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PHYSIOLOGICAL OBSERVATIONS ON ALKALOIDS, LATEX AND OXIDASES IN PAPAVER SOMNIFERUM*

RODNEY H. TRUE AND W. W. STOCKBERGER

In the summer seasons of 1902–4, while carrying on a series of investigations on the opium poppy, the writers made a number of observations on the occurrence and behavior of certain oxidizing enzymes present in this plant. These results, although verified by a number of repetitions, were not published at that time, it being then intended by the writers to broaden somewhat the scope of the investigation before bringing forward their results. However, the continuance of the work was subject to a number of contingencies and was not developed in the manner anticipated. Although much has happened since then that might concern the interpretation of the data then gathered, the facts themselves seem to point to certain broad conclusions which now as then are likely to interest the plant physiologist. Accordingly, it has seemed still in order to present these results with this explanation.

The plant material here used was grown by Mr. S. C. Hood, scientific assistant, from authentic seed at Burlington, Vermont, in the experimental grounds of the Vermont Agricultural Experiment Station in connection with co-operative work being carried on between that station and the Bureau of Plant Industry.

DISTRIBUTION OF OXIDASES AND OF LATEX IN THE PLANT

The oxidase was detected chiefly by means of the guaiac tincture test for oxidases of the laccase type used by Schönbein[1] and many

* Published by permission of the Secretary of Agriculture.

[1] Schönbein, C. F. Ueber das Vorkommen des thätigen Sauerstoffs in organischen Materien. Journ. Prakt. Chem. **105**: 203–308. 1868.

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others. The peroxidase reaction was studied chiefly by means of the guaiac tincture followed by hydrogen peroxide. The guaiac test was supplemented by the test with pyrogallol and sometimes with gallic acid.

The presence of an oxidase of the general type represented by the laccase of Bertrand and by the tobacco oxidase of Loew was easily demonstrated. This oxidase as found in the freshly expressed juice gave a reaction with guaiac tincture, pyrogallol and gallic acid. After precipitation with an excess of strong alcohol, the solution obtained on redissolving the precipitate in distilled water gave an intense reaction. Rough tests showed that in such solutions both the oxidase and the peroxidase reactions were inhibited by an exposure for four minutes to a temperature of 70° C. It appeared that this limit varied with the concentration and age of the enzyme solution. This inhibition of the reaction made it clear that the causes of the color changes lay in something easily modified by heat, not in any of the more stable substances shown to be capable of bringing about like color changes in the guaiac tincture. It having been seen in preliminary tests that the oxidase and peroxidase reactions in the different organs of the plant differed widely in intensity, systematic data were sought on this point.

In order to get evidence on the question of distribution from fresh materials, the reagents were taken to the field and applied to freshly cut surfaces of the growing plants. Here the order of intensity shown by the guaiac reaction agreed with that seen in the discoloration of the pulp. The most marked oxidase reaction was always seen in the more active younger parts of the plant. The fresh roots showed an almost complete lack of oxidase while the buds and petals were heavily loaded with it. The conclusion seemed justified that in this plant the intensity of the oxidase reaction increases from the base toward the summit of the plant. Similar tests for the peroxidase reaction showed clearly the presence in all parts of the plant of substances causing this reaction and no marked difference in intensity seemed to characterize any special part of the plant unless a greater activity was seen in the buds and flower parts.

The question immediately presented itself as to what particular tissues or substances contained the oxidases. When the guaiac tincture was applied to the cut surface of the growing plant, the drops of latex which instantly appear first gave the oxidase color reaction

and remained much more intensely colored than the surrounding parts. When the latex was gathered by allowing the exuding drops to fall into a little distilled water an intense oxidase reaction was likewise seen. A study of the reaction on cut surfaces of leaves, stems and roots showed that the reaction was most intense where the latex was most abundant and that, indeed, as far as could be judged by this crude method, the latex content and oxidase reaction ran roughly parallel. The petals seemed to form a possible exception in giving an intense oxidase reaction while yielding little latex on wounding. However, the mass of tissue here is small and the very numerous small branches of the latex system may offer obstacles to the quick and abundant outflow of the latex such as would be strikingly seen on the cut surfaces of the more massive structures.

Further interesting light on the relation of the latex to the oxidase reaction came from a study of young poppy plants. Plants from 30 to 45 centimeters tall on which no flower buds had as yet appeared gave no clear oxidase reaction on cut surfaces, and the plant juices were watery rather than milky. As the plants developed the suspended matter giving to the latex its characteristic milky appearance increased and the oxidase reaction also appeared as already described. Whether this coincidence between the degree of milkiness of the latex and the intensity of the oxidase reaction has any special physiological significance can not now be stated. However, a number of wild plants having a milky juice were tested in the same way and a strong oxidase reaction appeared whenever the juice was treated with the guaiac tincture. The addition of H_2O_2 in these cases gave a very strong reaction for peroxidase. The following plants were tested: *Euphorbia maculata*, *Sonchus asper* and *Hieracium aurantiacum*.

The study of these reactions on fresh plants was supplemented by a laboratory examination of extracts prepared from different parts of the poppy plant. Normal poppy plants approaching maturity were carefully dug up, promptly and thoroughly cleaned and quickly cut into the following portions: Roots, lower stems, leaves, upper stems, capsules (immature), and flower buds. Each portion was quickly reduced to a fine pulp by use of a meat grinder, placed in a clean beaker similar in size and shape to the others used in the series and macerated over night in a volume of water proportional to the weight of the fresh pulp and sufficient to cover it. On the following morning the various macerations were found to have undergone a

change of color in the surface layer. The root material showed but a slight change of color, a grayish tint being seen rather than the reddish-brown color characteristic of the others. The material from the stem portions was slightly reddish brown, with a distinctly more intense color toward the upper part of the plant. This intensity further increased in the leaf material. The reaction still more intense in the capsules was exceeded by the flower buds which gave a most intense color. The petals and stamens were also shown by separate tests to be most active. Tests with litmus paper showed that the extracts from the petals, lower stem and roots were neutral. All others showed a trace of acidity. In so far as this evidence went it seemed to indicate that the oxidase reaction was most abundant in the younger, growing parts of the plant.

The solutions of the different portions of the plant after being expressed from the pulp were treated with three volumes of commercial alcohol and briskly shaken. Precipitation was complete after several hours when the precipitate was filtered. This precipitate was dissolved in water as far as possible but a considerable insoluble residue always remained. The resulting solutions when tested for the oxidase reaction gave distinct though not strong oxidase reactions which in order of intensity was nearly the reverse of the order seen in macerated materials after standing over night in the beakers.

This interesting result seemed to indicate several possibilities. If it be assumed that the browning of the surface layer of the watery extract was caused by the precipitated substances responsible for the blue color in the guaiac tincture an apparent contradiction in the evidence seemed to exist here. That it may, however, be apparent only seemed to follow from further experiments.

In the course of the preparation of enzyme-containing precipitates the ground pulp was macerated in water over night in loosely covered beakers. In the morning a more or less deeply colored brownish layer was seen at the top of the material. Portions of the solution which were carefully drawn off by means of a pipette from this colored layer and from the uncolored portion near the bottom showed a marked difference in their activity toward the guaiac solution. Although the color of the superficial layer seemed to indicate that marked oxidative activities had taken place in the region of contact with the air, but a very faint oxidase reaction was seen when the reagents were employed. On the other hand, the uncolored portion from the

bottom of the beaker gave a strong oxidase reaction. The cause of the disappearance of the oxidase reaction from the brown surface solution was next sought.

A very active oxidase solution was prepared by quickly grinding up buds in distilled water and filtering off the solid portions. This solution which immediately began to take on a brownish tinge on exposure to the air was quickly divided into two portions. One was put into a bottle which was completely filled, tightly stoppered and placed near the bottle through which air was being drawn. After about 20 hours the aerated solution was found to have taken on a dark-reddish brown color suggesting a coffee infusion indicating that an intense action had taken place under the influence of the greatly increased air contact. This solution showed an almost complete loss of the oxidase reaction when tested with guaiac tincture. The second portion preserved out of contact with the air and which showed no clear deepening of color during the interval gave a very strong oxidase reaction. Both solutions were neutral to litmus. It seemed to be indicated that either the oxidase had been exhausted during the reaction if still present or had been in some way inactivated.

It was thought possible that products of oxidation that without doubt had accumulated in the solution during aeration might in some way have inhibited the oxidase reaction. Accordingly an attempt was made to free at least partially the supposed enzyme from these products. The dark colored solution was treated with three volumes of commercial alcohol and the resulting bulky flocculent pale-colored precipitate filtered off after about two or three hours. The filtrate retained the brown color almost completely. The washed precipitate was thrown into a volume of distilled water equal to about half the volume of the original solution. As is usual with such precipitates a considerable part remained undissolved. The solution obtained carried a trace of color but not sufficient to obscure a definite oxidase reaction. Test with guaiac tincture, however, failed to give even minimum traces of such a reaction. Since by the same method active oxidases were regularly prepared from fresh material, it was clear that the process of isolation had not destroyed the enzymes. Although a considerable part of the products of enzyme action had without doubt been removed no return of oxidase activity was seen, a fact strengthening the suggestion that the enzyme was exhausted or inactivated through use. It is of course not clear to what degree the process of

precipitation and re-solution separated the oxidase from the oxidation products. In so far as color may be accepted as an index, it seems probable that a very considerable degree of separation was effected.

Taking all evidence into account, the conclusion was strongly indicated that the enzyme was used up or inactivated during the course of the reaction. It is interesting to note in this connection the similar conclusions arrived at by Bunzel[2] in his recent and more exact studies.

In order to get further evidence on this point, a series of experiments was made with the juice of potato tubers. Freshly prepared aqueous extracts made in the same way as the poppy extracts gave active oxidase and peroxidase reactions. The solutions darkened very rapidly on standing and when tested after four hours gave no oxidase reaction. This solution was then treated with two volumes of strong alcohol, filtered after about an hour and the precipitate dissolved in distilled water. The resulting solution gave no oxidase reaction. The conclusion drawn from the poppy experiments seemed to be strengthened by the evidence gained from the work done on potatoes. This conclusion is hardly compatible with a catalytic explanation of oxidase action.

It is recalled that in the making of opium, the crude material from which morphine and several other alkaloids are obtained, the essential process consists in so scarring the full-grown but still green capsules as to cause the latex to run out onto the surface where in contact with the air it dries down from a thinly fluid milky juice to the dark brown, gummy substance known as opium.

Distribution of Alkaloids in the Plant

In view of the relations just discussed, it seemed desirable to ascertain in how far the distribution of the alkaloid, morphine, might show a relation to the distribution of latex and of the oxidase reaction. Accordingly, a number of full-grown plants of the black-seeded form of the opium poppy a meter or more high were brought in to the laboratory where they were cut up as quickly as possible into the following parts: Roots, lower stem, midstem, upper stem, leaves, flower buds, and capsules. The capsules were approximately full-

[2] Bunzel, H. H. The measurement of the oxidase content of plant juices. Bulletin 238, Bureau of Plant Industry, U. S. Dept. Agric. 1912.

grown but were still green and full of sap. Each of these portions was separately ground to a pulp and twice extracted with a hydro-alcoholic menstruum. The green weight was taken just before grinding and the pulp after extraction was expressed and again weighed. The amount of crude morphine was then determined for each extract and calculated with reference to the quantity of the plant material which had yielded it.

The results of the morphine determination are expressed in a ratio of morphine present to the same unit weight of plant material calculated for each of the plant samples. The relative yields of morphine were as follows:

Root..... 29.0	Lower stem.... 2.6	Midstem.... 1.3
Upper stem.... 2.2	Leaves..... 6.1	Buds..... 102.0
Capsules..... 42.0		

Some morphine was found in all parts of the plant. The roots in which there seemed to be but little latex yielded morphine in fair quantity, while in all parts of the stem the amount was almost negligible. However, the highest yield of morphine by far was found in the buds and capsules, both actively developing structures. Disregarding the root, the distribution of morphine in the plant seemed to conform fairly well to the distribution of oxidases and latex as previously noted.

The suggestion of a direct relation between the oxidases and morphine formation led to a series of observations on the latex itself. It was found that when the latex flowing from the freshly incised capsules was allowed to fall directly into strong alcohol the material failed to respond to the usual qualitative tests for alkaloids, while similar samples collected at the same time either in water or in a petri dish where it was allowed to dry gave all of the usual reactions with the common alkaloidal reagents. Fresh latex from the same plant was repeatedly collected both in alcohol and in water and tested with the result just described.

Conditions of Alkaloid Formation in the Plant

The results would seem to be explained on the assumption that morphine does not exist preformed either in the living plant or in the latex but develops in the latter when it is exposed to the action of the air or in the plant through oxidation changes as the tissues mature and

die. Apparently the oxidase which occurs so abundantly in the latex may be credited with playing an important part in these oxidations. Morphine was absent in the solutions of the free latex only when oxygen was excluded or when the action of the oxidases was inhibited.

Further evidence on the part played by the air in the production of morphine was added as a result of an experiment in which fresh capsules of the poppy were dried in an atmosphere from which air (oxygen) was excluded.

Three uniform lots of fresh poppy capsules of normal growth were collected on the tenth day after the fall of the petals.

Lot I was spread out on a bench at a north window of the laboratory and allowed to dry by simple exposure to the air.

Lot II was dried out in an air bath oven at a temperature varying between 90 and 100° C.

Lot III was dried out in an atmosphere of CO_2. A Remington copper still filled with CO_2 was taken to the poppy field where selected capsules were cut off and immediately put into the still through a suitable opening. This opening which was near the top of the container was tightly stoppered except when opened to receive the capsules. When the desired quantity of capsules had been collected, the still was brought back to the laboratory and in large part submerged in a water bath in which from 8 o'clock a.m. to 5 p.m. the water had a temperature close to the boiling point. No heat was applied during the night. As soon as the still with its load of capsules was in position in this water bath connection was made with a cylinder of liquid CO_2. The gas passed first through a wash bottle containing sulphuric acid, thence through a glass tube dipping into a vessel of boiling water in which the gas was heated. From here it was carried to the bottom of the still where it diffused among the capsules. The excess CO_2 and the vapor from the drying capsules escaped through a small opening in the top of the still. A continual flow of gas was maintained day and night until the capsules were dry. When dry, the capsules were collapsed, brown in color, and very brittle.

The three lots of material were analyzed by Mr. W. O. Richtmann, at that time pharmacognostical expert in this bureau. Those dried in the open air at room temperature and in the air-bath oven were found to contain normal amounts of total alkaloids. The lot dried in the atmosphere of CO_2 contained no aklaloids at all.

An attempt was made the following year to repeat this experiment.

Mr. Hood collected and dried two lots of capsules, one in contact with the air, the other in an atmosphere of CO_2. Unfortunately, however, the experiment was hardly a repetition. Some of the capsules had begun to desiccate before the experiment was set up. An accident occurred to the container holding the lot drying in CO_2 with the result that the material was exposed for some time to the air. The experiment, however, was carried through. On analysis by Mr. Richtmann the air-dried material was found to contain 0.064 percent crude morphine, those dried in CO_2, 0.032 percent crude morphine calculated on dry weight at about 60° C. When the modifying conditions just described are taken into account, the results obtained seem to confirm those of the first experiment.

From the evidence at hand, we believe ourselves justified in tentatively advancing the conclusion that morphine as such does not exist in the poppy but is formed from a mother substance present in the latex through the action of oxidases using the oxygen of the air. It seems quite probable that the mother substance consists of a complex molecule which, under the action of atmospheric oxygen wielded by oxidases, is split along a fairly well determined cleavage line with the result that a rather constant N-containing product having the constitution of the alkaloid morphine arises. Should the reaction occur under somewhat different conditions, it seems possible that the lines of cleavage might shift somewhat, giving a different proportional quantity among the many alkaloids obtained from the poppy. When oxygen is absent and presumably oxidase action also, cleavage, if it takes place at all, may take place along quite different lines with the result that no morphine appears. That other alkaloids are affected as well as morphine is shown by the entire absence of an alkaloidal reaction in the material dried in CO_2. A certain kind of analogy between this situation and that seen in glucosides which are split up through the action of enyzmes is strongly suggested.

Inasmuch as physiological opinion concerning the significance of alkaloids to the plants producing them has tended strongly toward the view that they are waste products of plant metabolism, it seemed desirable to carry the investigation further. Obviously morphine itself can hardly represent to the poppy plant an accumulation of N-containing waste products.

It could hardly be taken for granted, however, that all alkaloids stand in a like relation to the plant. Accordingly, belladonna plants

grown on the experimental farm of the Department at Arlington Farm, Va., were used as material for experimental study. This plant was chosen because of the absence of latex and because it is a fair representative of a large and much studied group of alkaloid-bearing plants. The roots, known to be rich in alkaloids, chiefly atropine, were dug in November, 1905, quickly and carefully washed, cut into transverse slices from 3 to 6 mm. thick and divided into two lots. It has been shown by Sievers[3] that there is a wide range of individual variation in alkaloidal content in belladonna. The chance for error from this source was reduced by dividing the slices of each root equally between the two lots.

One lot of 450 grams fresh weight was placed in a glass jar the mouth of which was closed except for holes to permit the escape of excess gas and water vapor. CO_2 was led from a tank through sulphuric acid, thence through a copper coil immersed in boiling water to give the gas a greater water-absorbing capacity and thence into the bottom of the jar containing the sliced roots. The jar itself stood in a water bath kept at boiling temperature from 9 a.m. to 4:30 p.m. while the gas flow was maintained throughout the day. The material was dry and hard on December 2.

A second lot was placed in a similar jar likewise heated in a water bath, but provided with a supply of air instead of CO_2. Drying was finished on November 29 when the material was hard and dry and had a pronounced odor of brown sugar.

Duplicate analyses of the powdered material made by Mr. W. O. Richtmann showed the following result:

TOTAL ALKALOIDS IN BELLADONNA ROOTS

Treatment	Sample	Total Alkaloids, Percent
Dried in CO_2 current	*A*	0.665
	B	0.665
Dried in air current	*A*	0.642
	B	0.625

These data show clearly that the alkaloidal yield of belladonna root is not dependent on the action of oxygen and, therefore, is governed by physiological conditions quite different from those govern-

[3] Sievers, A. F. Individual variation in the alkaloidal content of belladonna plants. Journ. Agr. Research 1: 129–146. 1913.

ing the formation of morphine. The application of guaiac solution to the freshly cut root produced a very strong oxidase reaction, apparently most intense in the cortical regions. The addition of hydrogen peroxide did not markedly intensify the color.

As far as the evidence here submitted goes, the belladonna alkaloids exist ready formed in the plant perhaps as accumulated waste products.

Summary

In conclusion, the results here reported may be briefly summarized:

1. It appears from work done on the opium poppy, *Papaver somniferum*, that the oxidase reaction is most active in the upper parts of the plant, especially in the floral structures, capsules and actively growing parts. The peroxidase reaction shows less variation in its intensity in the different parts of the plant.

2. The intensity of the oxidase reaction roughly parallels the distribution of the latex which in itself is most active.

3. The oxidase seems to be either "used up" or otherwise inactivated during the course of its action. A like exhaustion or inactivation of the supposed enzyme was seen in the case of the oxidase of the Irish potato. These observations would indicate that perhaps the oxidase reaction is not due to a catalyzing agent, therefore is not due to an enzyme.

4. With exception of the root, the intensity of the oxidase reaction runs roughly parallel with the alkaloidal content. In the root, the alkaloid content is relatively higher than the intensity of the oxidase reaction.

5. Alkaloids seem not to exist as such in the poppy plant but to appear as products of the action of the oxidases on constituents present in the latex reacting in the presence of oxygen.

6. The alkaloids of *Atropa belladonna* differ from those of the poppy in that they are found to exist in structures dried without contact with free oxygen and seem to exist ready formed in the plant.

Office of Drug Plant, Poisonous Plant,
Physiological and Fermentation Investigations,
Bureau of Plant Industry, U. S. Department of Agriculture.