FORMATION OF DIHYDROXYPHENYLALANINE FROM TYROSINE BY COUPLED OXIDATION WITH ASCORBIC ACID*

C. G. VAN ARMAN†, M.S., AND K. K. JONES, PH.D.

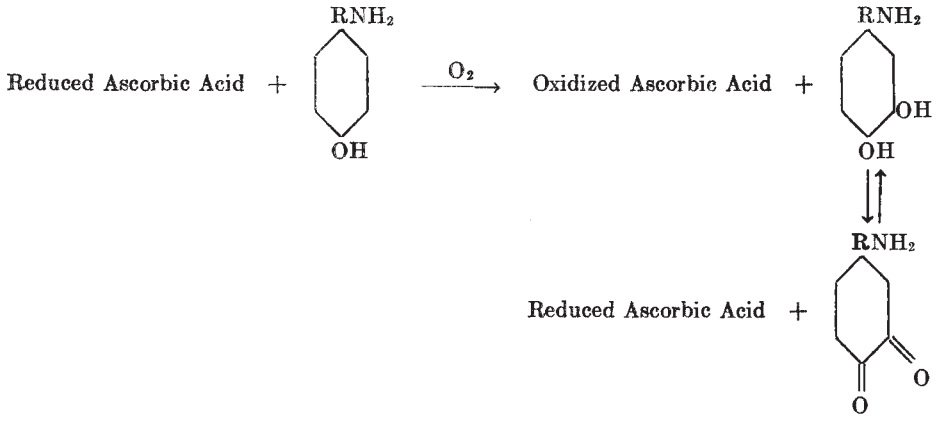
Dihydroxyphenylalanine (dopa) has been thought to be intermediate in the formation of many important biological compounds, such as the melanin pigments and various pressor agents. This compound has been obtained from plants and from lower forms of animal life, but its isolation from normal mammalian tissues is open to question. Its presence in mammalian tissue has been inferred from several facts. There is, first of all, an enzyme in the pigment cells of the skin which is a specific oxidase for 1-dopa (1). This enzyme has been shown in melanotic tumors of the mouse (2, 3). Small amounts of dopa are formed by irradiation of tyrosine with ultraviolet light (4, 5, 6). It is well known, also, that dopa quickly turns into melanin-like pigments upon oxidation; Raper (7) has proposed a mechanism for the reaction. Some workers believe that pigment in the skin is formed in this manner. Normal human urine contains hydroxytyramine which probably arises from dopa by enzymic decarboxylation. A specific decarboxylase has been demonstrated in kidney tissue of several mammalian species (8, 9, 10). The presence of this decarboxylase implies the presence of dopa in the tissues.

There is good evidence (11) that the parent substance of epinephrine is phenylalanine which could become transformed to tyrosine first, and then to dopa. Bloch and Löffler (12) propose that the bronzing of the skin in Addison's disease may be due to the fact that this compound can no longer be converted into epinephrine and stored in the adrenal glands but becomes converted to pigments instead. Large amounts of 1-dopa have been found in the urine of a patient suffering from the rare disease, tyrosinosis (13). Thus far, however, its presence in normal tissue is not proved.

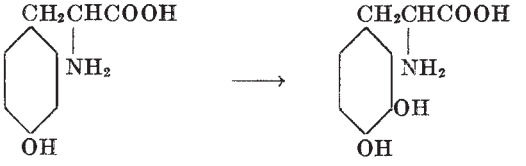
Nevertheless, further indirect evidence may be obtained by finding whether or not dopa may be formed in vitro by some chemical system known to exist in the body, acting upon some precursor, also known. The most logical choice for the precursor is, of course, tyrosine, because simple introduction of a phenolic hydroxyl group would yield dopa. One oxidizing system in the body is ascorbic acid plus oxygen—and this system occurs notably in both the skin and the adrenal glands (14, 15, 16). Coupled oxidation with ascorbic acid has been invoked to account for a number of biological reactions. The relation of ascorbic acid and skin pigment has been discussed by Cornbleet (17). The mechanism postulated by Beyer (18) for introduction of second phenolic hydroxyl group is as follows:

* From the Department of Pharmacology, Northwestern University Medical School.
† Roche Fellow in Pharmacology.

Read before the Ninth Annual Meeting of the Society for Investigative Dermatology, Chicago, June 20, 1948.



The over-all result in this particular case would be the reaction:



Tyrosine Dihydroxyphenylalanine (Dopa)

EXPERIMENTAL

Twenty-five mg. of l-tyrosine were dissolved in 35 ml. of M/15 phosphate buffer at pH 7.0 together with 100 mg. of ascorbic acid and the solution aerated for 70 min. at 38.5°C. in a 50 ml. test tube by introducing air at the rate of one small bubble per second. A 6 ml. aliquot was then removed to a centrifuge tube, 2 ml. of a suspension of aluminum added as an adsorbing agent, and the suspension made just basic to phenolphthalein by means of a few drops of 4N NaOH. The aluminum hydroxide had been prepared according to Richter (19). After being shaken, the tube was centrifuged and the supernatant liquid discarded. Three-tenths ml. of a 25 per cent solution of sodium dihydrogen-phosphate and 2 ml. water were then added, the tube shaken to elute the dopa off the solid particles, and then centrifuged again. Approximately 0.06 ml. of the supernatant liquid was applied to a strip of Whatman #1 filter paper and a paper chromatogram was prepared in a manner similar to that of Consden, Gordon and Martin (20).

The principle of paper chromatography is simply that a mixture of compounds, especially amino acids, may be separated along the length of a narrow long strip of paper and detected by various sensitive color reactions. The position of any colored band thus developed is characteristic for a given compound, since it depends upon the partition coefficient for this compound between the phase of solvent (in this case phenol) saturated with water and the phase of cellulose saturated with water. The terminology introduced by Consden, Gordon and Martin for the position of the band is "R_f," which denotes the ratio of the dis-

tance which the given compound has traveled by the solvent. The use of various solvents and proper choice of a color reaction may further increase the specificity. The equipment is extremely simple, (fig. 1) but precautions are necessary to obtain good results.

Strips were run in an inert atmosphere because if run in air no dopa could be found on control tests. Nitrous oxide was chosen for the inert gas because it is heavy and displaces air in the experimental jar by being run in from the top.

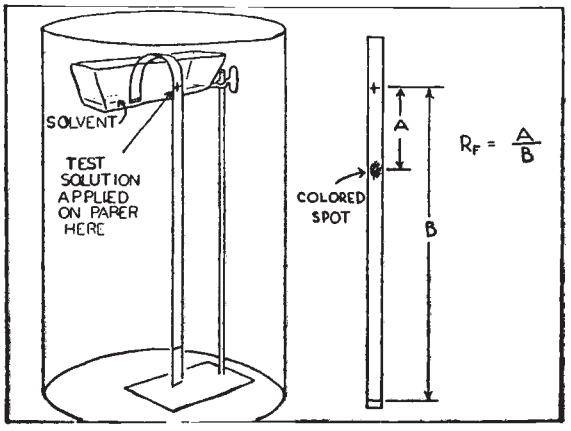


FIG. 1

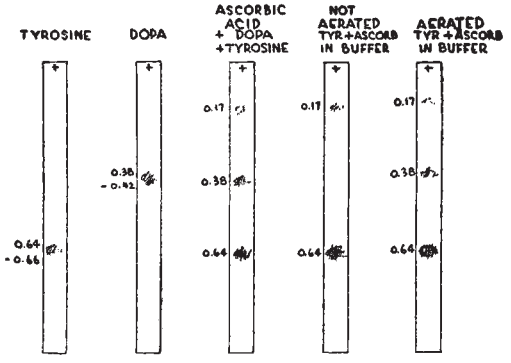


FIG. 2

Runs were made at 24–25°C. using 90 per cent phenol as the solvent. Colored bands were developed with 0.1 per cent ninhydrin (triketohydrindene hydrate) in butanol by immersing the strip briefly, and then drying and heating at 110°C. for 10 minutes.

RESULTS

A definite purplish-pink band was found having an R_f value of 0.38. This agrees with the value of 0.38 found in control runs under the same conditions with known preparations of l-dopa (fig. 2). A supporting test upon the solution from the second centrifugation is given by the transient rose-red color developed

after iodine and potassium iodide (Richter's test). This latter reaction is said to be specific for the catechol nucleus and an ethylamine or isopropylamine side-chain; however, it works well with l-dopa, the color fading rapidly.

DISCUSSION

The R_f value found on the paper chromatogram makes it practically certain, as explained above, that dopa is the compound producing the colored bands. This, together with the positive Richter's test, leaves little doubt that ascorbic acid and oxygen can cause the formation of dopa from tyrosine. Little can be said about the quantities produced except for a rough estimate based upon the depth of color in the colorimetric tests and chromatograms. From these it was estimated that some 3 to 4 per cent of the tyrosine was converted under the conditions described. The fact that the skin and adrenal glands both contain relatively large amounts of ascorbic acid makes this mode of formation of dopa appear feasible in the animal body.

SUMMARY

Dihydroxyphenylalanine was formed in vitro by the action of ascorbic acid and oxygen upon l-tyrosine. Its identity was established by paper chromatography. The synthesis of dopa in vivo by this mechanism appears feasible, but has yet to be established.

REFERENCES

1. Bloch, B.: Chemische Untersuchungen über das spezifische pigmentbildende Ferment der Haut, die Dopadecarboxylase. Ztschr. f. physiol. Chem., **98:** 226, 1916–1917.
2. Bloch, B.: Das Problem der Pigmentbildung in der Haut. Arch. f. Dermat. u. Syph., **124:** 129, 1917.
3. Hogeboom, G. H. and Adams, M. H.: Mammalian tyrosinase and dopa oxidase. J. Biol. Chem., **145:** 273, 1942.
4. Lerner, Fitzpatrick, Calkins and Summerson: Enzymatic oxidation of tyrosine and dihydroxyphenylalanine by melanoma extracts. Fed. Proc., **7:** 167, 1948.
5. Arnow, L. E.: The formation of dopa by the exposure of tyrosine solutions to ultraviolet radiation. J. Biol. Chem., **120:** 151, 1937.
6. Arnow, L. E.: The preparation of dopa-melanin. Science, **87:** 208, 1938.
7. Rothman, S.: Oxidation of tyrosine by ultraviolet light in its relation to human pigmentation. Proc. Soc. Exper. Biol. & Med., **44:** 485, 1940.
8. Raper, H. S.: The aerobic oxidases. Physiol. Rev., **8:** 245, 1928.
9. Holtz, P. and Credner, K.: Über das Vorkommen der Dopadecarboxylase in Pancreas. Arch. f. exper. Path. u. Pharmakol., **199:** 145, 1942.
10. Holtz, P. and Credner, K.: Die enzymatische Entstehung von Oxytyramin im Organismus und die physiologische Bedentung der Dopadecarboxylase. Arch. f. exper. Path. u. Pharmakol., **200:** 356, 1942–1943.
11. Holtz, P.: Über die Bildung einer blutdrucksteigernden Substanz aus Dioxyphenylalanin durch tierisches Gewebe. Angewandte Chem., **51:** 383, 1938.
12. Gurin, S. and Delluva, A. M.: The biological conversion of radioactive phenylalanine to adrenaline. J. Biol. Chem., **170:** 545, 1947.
13. Bloch, B. and Löffler, W.: Untersuchungen über die Bronzefärbung der Haut bei der Addison'schen Krankheit. Dtsch. Arch. klin. Med., **121:** 262, 1916.
14. Medes, G.: A new error of tyrosine metabolism: tyrosinosis. The intermediary metabolism of tyrosine and phenylalanine. Biochem. J., **26:** 917, 1932.

15. GIROUD, A., LEBLOND, C. P., RATSIMAMANGA, R. AND RABINOWICZ, M.: L'acide ascorbique au vitamine C au niveau du tégument (derme, épiderme, pigmentaires). Bull. Soc. franc. de dermat. et syph., **3:** 482, 1935.
16. SZENT-GYORGYI, A.: L'acide ascorbique (Vitamine C). Bull. Soc. chim. biol., **15:** 694, 1933.
17. GLICK, D. AND BISKIND, G. R.: The histochemistry of the adrenal gland. I. The quantitative distribution of vitamin C. J. Biol. Chem., **110:** 1, 1935.
18. CORNBLEET, T.: Vitamin C and pigment. Arch. Dermat. & Syph., **35:** 471, 1937.
19. BEYER, K. H.: The ascorbic acid-dehydroascorbic acid system in the synthesis and inactivation of sympathomimetic amines. J. Pharmacol. & Exper. Therap., **76:** 149, 1942.
20. RICHTER, D.: Inactivation of adrenaline in vivo in man. J. Physiol., **98:** 368, 1940.
21. CONSDEN, R., GORDON, A. H. AND MARTIN, A. J. P.: Qualitative analysis of proteins: a partition chromatographic method using paper. Biochem. J., **38:** 224, 1944.

DISCUSSION

Dr. Stephen Rothman: The presenters are to be congratulated on the ingenious application of their method and the successful isolation of dopa from tyrosine. Recent studies indicate that mammalian epidermis does contain a partially inhibited tyrosinase. In this year's Federation Proceedings, Fitzpatrick et al reported that in the presence of traces of dopa the oxidation of tyrosine is enormously accelerated.

Concerning the nature of inhibitory substances in human epidermis, data are accumulating that these are sulfhydryl compounds. I wonder if the concentration of ascorbic acid used by Drs. Van Arman and Jones can be regarded as physiological. The ascorbic acid content of the human epidermis seem to be extremely low.

It has been known for a long time that ascorbic acid inhibits melanin formation in the quinone stage. This is the reason that in the presence of ascorbic acid dopa accumulates when tyrosine is being oxidized (J. Invest. Dermat. Vol. 3, No. 2).

Dr. Theodore Cornbleet: The method exhibited is an aerobic one; whereas, that in vivo is probably anaerobic. Nevertheless, all the evidence points up the significance of the reaction described by the essayists in the formation of melanin. We have found ascorbic acid constantly present with pigment. Figge has shown that the redox potential of ascorbic acid is optimum for tyrosinase activity. Roper's classical scheme for the formation of pigment from tyrosine oxidizes the latter as a first step to dihydroxyphenylalanine. On the other hand, according to Lea's work the presence of ascorbic acid inhibits the enzymic oxidation of tyrosine. The clinical evidence in Addison's disease supports the latter view. We have shown that the administration of vitamin C materially reduces the urinary pigmentary substances in patients with malignant melanoma.

Rothman has postulated the presence of sulfhydryl substances in the skin that inhibit melanogenesis. It seems to me that there is much to be said for this view, except that I believe that ascorbic acid instead of sulfhydryl substances is the inhibitor. As previously said, the presence of ascorbic acid inhibits the enzymic oxidation of tyrosine. Sunlight, heat and other agents can oxidize or

otherwise dispose of the blocking agent, vitamin C, whereupon, the enzymic reaction remains free to proceed to the final production of melanin.

Maurice Oppenheim, M.D.: In 1902, 46 years ago, I published a preliminary report from the University Clinic, Professor Neumann in Vienna, under the title, "To The Question of Pigment Formation Out of Tyrosine." I followed a publication of my friend von Fürth, Professor of Physiological Chemistry at the University of Vienna, who found that certain mushrooms like russula turn blue and black after being exposed to the air. He concluded that under the influence of tyrosinases the tyrosine forms melanin, and he found such conditions in animals too. I concluded after certain experiments I made that the human epidermis contains tyrosine, which under influence of oxydases form pigment. I could not continue my experiments because my teacher Professor Ehrmann was convinced that the melanin is a product of hemoglobin and that no other source of pigment exists in the human skin. I would like to add that the pictures that Dr. Zimmerman has shown us, in the development of pigmentation in the Negro fetus are almost identical to the pictures that Ehrmann published of the skin in Tritone and Salamander embryos. I appreciate the excellent paper of Van Arman and co-workers and the other discussers. This is only a brief historic remark on this subject.

Mr. C. G. Van Arman: I am sorry that we do not recall the paper in the Federation Proceedings cited by Dr. Rothman. However, we have known that tyrosinase (and/or dopa oxidase, if they are different) is found in skin and in melanotic tumors. We did not mean to say that the enzyme had not been demonstrated, but only that dopa itself—the diphenolic compound—had not been proved beyond all question. Of course the presence of the enzyme does very strongly imply, almost proves, the presence of dopa.

The question whether our experimental conditions can be considered physiological is difficult. The pH was 7.0. The concentration of ascorbic acid was approximately 2 milligrams per milliliter, which is considerably greater than that found in the skin, but not much greater than that in the fascicular layer of the adrenal cortex.

We agree with Dr. Cornbleet's comments that the oxidation-reduction potential of a substance seems related to its ability to introduce a second hydroxyl group. This seems quite in line with what we have read in the literature. Apparently a low potential is necessary for a compound to function in coupled oxidations. Sulfhydryl groups of course do have such a potential.

Dr. K. K. Jones: The formation of dihydroxyphenylalanine in the body is assumed as a step in the formation of melanin. It is difficult to demonstrate a way in which it may be formed that is physiological. Most of the methods by which it is formed from tyrosine or phenylalanine are not compatible with cell life.

The formation by ascorbic acid oxidation is physiological in that it uses compounds present in the cell and occurs at pH levels and redox potentials found in living tissue.

There is however a peculiar condition in that a strong reducing agent facilitates oxidation. The explanation is that the energy of the oxygen atom does the oxidizing. The energy of the oxygen molecule is about 117,000 calories. Ascorbic acid by accepting one atom of the molecule makes it posible for the other atom to attack the benzene or phenyl ring and therefore insert the one or two hydroxyl groups necessary to form dopa.

Ascorbic acid does not block the tyrosinase enzyme action. It prevents further oxidation of the end products of enzyme action such as dopa quinone. The mechanism is yet to be explained.