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Inhibition of Tyrosinase Melanin Formation by Sodium Benzenone-Indophenol.

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It was discovered that the indophenol dyes produced a pallor in amphibian larvae raised in solutions of these dyes.[1] This suggested the possibility of solving many of the fundamental problems of pigment formation through a study of the mechanism of action of these dyes. The original work was repeated and confirmed, but extreme variation was found with regard to the effectiveness of the dyes in different species.[2] It was also proved conclusively that the dyes do not exert an influence on pigment via the hypophysis.

The present paper gives the results of experiments devised to test the hypothesis that the dyes acted directly on the pigment granules by destroying them after they were formed or by inhibiting their formation.

In preliminary experiments it was found that phenol indophenol (sodium benzenone indophenol) does not destroy pigment from the following sources: 1. Artificial pigment produced by the oxidation of either dihydroxyphenyl alanine or tyrosine. 2. Natural pigment in colloidal solution produced by brushing pigment out of the choroid coat of frozen ox eyes. 3. Natural pigment contained in melanophores in pieces of dead amphibian skin.

It was concluded from these experiments that the dye does not destroy or dissolve pigment granules after they are formed. It was thought that the dye might be inducing the pallor by influencing the enzymes responsible for pigment formation. Since tyrosinase has not been conclusively demonstrated in vertebrate forms, while dopa oxidase has, it has been assumed by many[3, 4, 5] that dopa oxidase is the enzyme responsible for pigment formation. Contrary to expectations, it was found that the dye did not inhibit the dopa reaction, but accelerated and intensified it. In addition, it accelerated the auto oxidation of dihydroxyphenyl alanine to dopa melanin.

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1 Lewis, M. R., *J. Exp. Zool.*, 1932, **64**, 57.

2 Figge, F. H. J., *Ibid.*, 1938, **78**, 471.

3 Bloch, Br., and Schaaf, F., *Biochem. Z.*, 1925, **162**, 181.

4 Laidlaw, G. F., *Anat. Rec.*, 1932, **53**, 399.

5 Miescher, G., *Arch. f. Mikro. Anat.*, 1923, **97**, 326.

The effect of the dye was then tested on tyrosinase. It was found that the production of melanin by the action of tyrosinase on tyrosine could be either completely or partially inhibited by the dye.

Tyrosinase was obtained from mealworms *(Tenebrio mollitor)* or potatoes. The mealworm enzyme preparation was made by grinding the larvae to a fine pulp in a mortar. Ten cc of 0.25% sodium carbonate per gram of mealworm was added. This was then centrifuged in a hand centrifuge to separate the coarse particles. The gray supernatant fluid and the fine black and gray precipitates were removed from the centrifuge tubes, shaken vigorously with a few drops of chloroform, and used in this form as the active tyrosinase preparation. Part of this was then boiled to inactivate it for use in the control bottles. The potato tyrosinase was plain potato juice prepared by grating potatoes and filtering the juice through a medium coarse filter paper. The attempts to prepare concentrated enzyme preparations according to the methods of Raper,[6, 7] Gortner,[8] or Przibram[9] were not very satisfactory because the enzymes deteriorated so rapidly. The enzyme was prepared just before each experiment. The substrate was a 0.25% solution of tyrosine buffered with Sörenson's phosphate buffer mixture to pH 7.5.

It was found in the early experiments[2] on amphibian larvae that old dyes were not as effective as fresh dyes. The dye used in these experiments was manufactured a week before the initial experiment of this series. A new dye solution was made up for each individual experiment.

Fifty cc wide-mouthed bottles were used for reaction vessels. The rate of melanin formation was determined by calculating the percentage change in light absorption in any given reaction mixture. Light absorption was determined by means of a very simple photoelectric colorimeter, which made use of the same principles and resembled slightly, the Evelyn[10] photoelectric colorimeter.

The reaction bottles are listed in the table in pairs according to the concentration of the enzyme. One of the bottles of each pair contains, in addition to the substrate and enzyme, the quantity of dye indicated in the table. The degree of inhibition for any dye/enzyme ratio may be estimated by comparing the percentage change in light absorption in any given pair of reaction bottles. At the end of the first hour the bottles containing enzyme and substrate only showed

6 Raper, H. S., *Biochem. J.*, 1926, **20**, 725.

7 Raper, H. S., and Wormall, A., *Ibid.*, 1923, **17**, 454.

8 Gortner, R. A., *J. Biol. Chem.*, 1911-12, **10**, 89.

9 Przibram, H., and Brecher, L., *Arch. f. Entw-mech.*, 1919, **45**, 83.

10 Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, 63.

a change in percentage light absorption ranging from 9.5% to 45%. None of the "d" bottles containing dye showed any change. It is, therefore, assumed that all concentrations of dye completely inhibited pigment formation during this period. The figures on percentage change in light absorption after longer time intervals showed that the enzyme tyrosinase was completely inhibited in the bottles containing small amounts of enzyme and large amounts of dye. The smaller quantities of dye with larger amounts of enzyme only partially inhibited the enzyme.

TABLE I.

Bottle No.	15 cc substrate		Rate of melanin formation as indicated by percentage light absorption at end of		
	cc enzyme	mg dye	1 hr	5 hr	14 hr
1	.1		9.5	68	92
1 d	.1	.5	0	0	0
2	.2		10	78	94
2 d	.2	.4	0	0	0
3	.3		20	84	97
3 d	.3	.3	0	0	36
4	.4		39	91	98
4 d	.4	.2	0	0	50
5	.5		45	94	98
5 d	.5	.1	0	32	77

This experiment was repeated 5 times, using mealworm tyrosinase and once with potato tyrosinase. Readings were made at one or 2 hour intervals. The plotted curves for all experiments were essentially uniform and the results were in such close agreement with the quantitative variations in the 5 different concentration ratios that further repetition was deemed unnecessary.

The conclusion to be drawn from this is that sodium benzenone indophenol inhibits the enzyme tyrosinase and thus prevents the formation of pigment granules. The pallor which this and similar dyes induce in young amphibian larvae may be explained on this basis. This indicates that even though it has been impossible as yet to demonstrate tyrosinase in these forms, this enzyme, and not dopa oxidase, must be responsible for most of the melanin formation in young larval amphibians.