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INDUCED MODIFICATIONS IN PIGMENT DEVELOPMENT IN SPELERPES LARVAE.*

(Preliminary Paper)

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INTRODUCTION.

We present here a brief account of a series of experiments having as their aim the inhibition, or the modification of pigment development.

We believe that it is a fairly well established fact that the black melanic pigment results from the interaction of an oxidizing enzyme of the tyrosinase type and some oxidizable chromogen, the exact nature of which has never been elucidated†. One of us, (Gortner 1911, b,) has shown that certain organic phenols inhibit the action of tyrosinase in the test tube and the suggestion was made that perhaps certain types of colorless animals owe their lack of pigment to the presence of inhibitory compounds. The present series of experiments was carried out in order to test the inhibitory powers of the *m.* di-hydroxy phenols *in vivo* as contrasted with their action *in vitro.*

The material upon which the experiments were carried out, consisted of eggs and embryos of the salamander, *Spelerpes bilineatus*, Green. This material is unusually suitable for such work inasmuch as the eggs contain no pigment when deposited, and the early stages of pigmentation in the embryo can thus be followed from day to day.

* Presented at the annual meeting of the Ohio Academy of Science, Columbus, Nov. 30, 1912.

† For literature see Kastle (1910), Riddle (1909), and Gortner (1911, a).

By macerating larvæ which were about to begin pigmentation and adding tyrosin to the aqueous extract of the crushed larvæ, we observed the color changes which are characteristic of tyrosinase. We have also satisfied ourselves that the onset of pigmentation in the Spelerpes larvæ is due to the beginning of chromogen secretion, the tyrosinase having been already present for some time.

Experimental.

Our experimental data groups itself under four heads: (1), Experiments with Tyrosin; (2), Experiments with Orcinol, (3. 5. di-hydroxy toluene); (3), Experiments with Resorcinol, (*m.* di-hydroxy benzene) and (4), Experiments with Phloroglucinol, (*sym.* tri-hydroxy benzene).

Experiments with Tyrosin.

This series comprised 41 experiments (not including an equal number of checks) and a total of 428 individuals. The checks in every case came from the same bunch of eggs and were kept under the same conditions as the tyrosin-treated lot with the exception that no drugs were used. What is true of the tyrosin checks is also true in the checks of all the subsequent experiments. Owing to the slight solubility of tyrosin (one part in 2454 parts of water at 20°) it was impossible to test the effect of high concentration. Twenty experiments, comprising 208 individuals showed no marked effect of the tyrosin, *i. e.* they were usually indistinguishable from the corresponding checks. We find however that in 11 of these experiments the tyrosin was of a lower concentration than 0.008% and below this concentration we have succeeded in but one case (0.006%) in producing an effect and in this one case the larvæ "reverted" to normal after 28 days. Six of the remaining nine experiments which showed no effect are shown by our records to have been "poisoned", either by confinement in too limited quarters or by bacterial infection. The checks of those which were confined in too small dishes (small stender dishes) showed the same abnormal traits that were observed in the treated material. Of the remaining three experiments which failed to show a marked effect, two were in tyrosin of 0.025% concentration and the remaining lot in 0.010% tyrosin. The former showed some influence for a time but later "reverted." The other showed no influence.

Twenty-one experiments, comprising 220 individuals were profoundly influenced by the tyrosin treatment and became "good" or "typical" tyrosin types. The tyrosin influence is shown by; (1), The more rapid appearance of pigment in the treated lot as contrasted with their checks; (2), The extremely small size and later the entire absence of pigmentless spots in the larvæ, the spaces where spots are normally visible being filled

with dense black pigment; and (3), the dense dull-black color of the larvæ compared with which the check often appears very light.

There is no mistaking the "tyrosin type", for an inexperienced person will always pick them out as the darkest individuals in a series.

Of the 21 experiments which showed an effect, 15 had a tyrosin concentration of 0.010%, 1 of 0.0125%, 2 of 0.020%, 1 of 0.040%, and 1 of 0.006% (this last being the only one of the entire 41 experiments which showed an effect at this concentration, and which, as noted above, "reverted" after the 28th day).

The time of treatment averages about 60 days, and in three experiments (Nos. 560, 595, 609) which are still running (Dec. 6) the larvæ were in tyrosin for 72 days and have since been in pure water only (no tyrosin) for 123 days. They are still appreciably darker than the corresponding checks, and show enough of the characteristics of the "tyrosin type" to be readily classified as such. During the later period the larvæ have at least doubled their previous length, but it is impossible to say whether their continued darker color is due to a continued more active pigment formation or merely to a distribution over a larger area of the dense black mass of pigment already present†.

Experiments with Orcinol.

Orcinol, as noted above, inhibits the action of tyrosinase upon tyrosin in the test tube, and we hoped to be able to inhibit, or at least to modify, the course of pigment development by rearing the larvæ in solutions of orcinol. We found the drug to be quite toxic, not so much so of itself as the oxidation products which are formed by the action of light upon a solution of orcinol. However, by changing the solutions every day, or every second day, and keeping the dishes, together with the controls, in a dimly lighted room, we were able, in part, to prevent the toxic action. In this manner we have been able to keep larvæ in a solution of 0.020% concentration for 50 days.

Altogether 35 experiments were run, including 513 individuals (not including checks). Later it seemed advisable to subdivide some of the experiments so as to accurately test the effect of varying length of immersion in the drug solution. A total of 115 such removals were made, each one in reality being a separate experiment in itself, thus making a grand total of 150 experiments. Concentrations of orcinol ranging from 0.0125% to 0.025% were employed.

† As the larvae become older the characteristic spots of the checks become less conspicuous and are later lost so that the types become less differentiated, and the depth of color is about the only criterion available at this stage of development.

To briefly summarize the effect; we obtained, *in every instance*, a retardation of growth accompanied by a much greater retardation in pigment development than would correspond to the retardation in growth. In some experiments where the concentration of the orcinol was very low and where the length of the immersion was short we did not obtain permanent after-effects and the later course of development resembled that in the checks. When, however, the strength of the orcinol was sufficiently high (0.020% to 0.025%) and the period of treatment sufficiently long, varying from one day to a week or more depending upon the initial age of the embryo, we have apparantly obtained permanent modifications. The nature of these effects depends to some extent upon the initial age of the egg or embryo. When eggs at a stage of development between the early blastula and late neural groove are kept in the solution less than six days they rarely show as abnormal types as those which have been exposed to the action of the drug for from 6 to 20 days. They do show, however, the typical retardation of pigment development, and various other characteristics (see below) sufficient to classify them as "orciny."

Where these early embryos are kept in the solution more than six days, the course of development is decidedly different. The larva developes in many cases apparently normally though somewhat slowly, until within a short time before hatching, or in some cases for several days after hatching, when huge swellings appear, sometimes filling the entire body with great serous cavities, through the walls of which may be seen the alimentary canal and blood vessels, stretched almost to breaking. In this condition they may live for days, but eventually die without further development.

If, however, the embryos are older when treated—*i. e.* with the head strongly differentiated or at any later stage to the beginning of pigmentation (which occurs shortly before hatching)—the effect is widely different. In no instance do we obtain the blistered larvæ, but instead, short heavy individuals, about one-third shorter and twice as broad as the checks. These animals we class as the true "orcinol type". They are distinguished from the checks by their shorter length, greater girth, absence of any conspicuous spots, the development of heavy awkward "flippers" in the place of delicate limbs and toes, the coarse reticulation of the pigment pattern, their sluggish movements, and, what is most disappointing, their inability, or at least their disinclination, to take food. This last trait prevents our knowing how permanent the type may be, the better orcinol examples (which were numbered by the dozens) having, without exception, grown smaller and at last died, apparently of starvation, in an average of eight to nine weeks after hatching. A few of the less extreme types are still alive (Dec. 6) 161 days after removal from the solutions, and in almost every instance the coarse reticulations and the heavier body form still persist.

Experiments with Resorcinol.

A total of 150 experiments, including 103 which had as their aim the test of the effect of varying length of immersion in the drug, were conducted using 636 larvæ, not including checks in each series. We find that resorcinol is more potent than orcinol, not alone in being more toxic, but the type produced by it is, if possible, more definite. The same swellings of the serous cavities are produced if the eggs are treated before reaching the late neural groove. When treated before reaching the blastula, no larvæ were hatched.

When larvæ which had the head strongly differentiated or were in any stage between this and a day or two after the beginning of pigmentation, were treated with resorcinol in sufficient concentration (0.020% to 0.025% and in one instance 0.05%) and for a sufficient length of time (4 to 10 or more days) they were highly modified and produced one of two types. Both types begin with a retardation of development and a great retardation of pigmentation. The first pigment appears in the eye and in a day or two a narrow V appears on the shoulders, followed a little later by a narrow line down the spine. *This condition persists as long as the larvæ remain in the resorcinol*, but unfortunately the drug is so toxic that 15 to 18 days immersion invariably causes death. We have had many instances where the larvæ which were treated with resorcinol appeared almost entirely devoid of pigment except for the dark eyes, when the corresponding checks were completely pigmented and the spots were fully developed.

When the larvæ are removed from the resorcinol solution after varying lengths of time we obtain the same two types referred to above. The more extreme type (See Fig. No. 1) resembles the "orcinol type" but is heavier, the "flippers" are more enlarged, and the pigment reticulation is very fine as contrasted with the coarse reticulations of the orcin type. This type persists for 60 to 70 days when death by starvation ensues.

The second type probably represents those individuals which have not been so profoundly modified. The body form is almost normal, the limbs and toes are well developed, but the spots are absent and the pigment pattern is very fine and dull in color. The majority of this type also die of starvation, and on Dec. 6—about 161 days from the beginning—we have only a very few individuals remaining. None of these have been "typical" but have been classed as "fair resorcin" or "somewhat modified" and all but two of these larvæ still show modification. At this period of development, however, the checks have lost their characteristic markings so that a closer analysis is impossible. In nearly every instance in both the orcinol and resorcinol series, the surviving individuals are lighter than the checks.

Experiments with Phloroglucinol.

From the position of the hydroxyl groups we expected to find that phloroglucinol caused greater effects than orcinol. In a series of 20 experiments comprising 174 individuals we find that no retardation occurs, providing that oxidation by light is prevented. On the contrary, a slight *acceleration* of pigmentation takes place and the spots are almost invariably larger and more distinct throughout the entire course of development. Beyond this, and an apparent slight stimulation in growth, no effects have been noted. The drug was employed in a strength of 0.025%.

Summary.

By subjecting the eggs and larvæ of *Spelerpes bilineatus* to the action of dilute solutions of tyrosin, orcinol, resorcinol and phloroglucinol, we have observed the following effects on the general development, and in particular on the development of the pigment pattern:

(1). Tyrosin causes an acceleration of pigment development and later produces larvæ which differ from the check by the absence of spots, and the presence of a much more dense deposition of pigment.

(2). Orcinol, when applied for six or more days to embryos younger than the late neural groove causes monstrosities. When used with embryos at a later period of development it causes the body to become short and thick, the spots to become irregular or wholly absent, the entire color pattern to be blurred, the general character of the pigment pattern to be a coarse reticulation, the limbs to become "flippers", and the larvæ to be unable, or disinclined, to take food.

(3). Resorcinol causes much the same modifications as orcinol, with the exception that the pigment reticulation is very much finer. A second resorcinol type does not show the abnormal body form.

(4). Phloroglucinol causes no abnormalities, and when any result is to be noted it is the more distinct markings of the color pattern and a slight acceleration of pigment development.

(5). All of these modifications are persistent for weeks after removal from contact with the drugs, and to all appearances the orcinol and resorcinol types would be permanent were it possible for the larvæ to take food.

The work is being continued.

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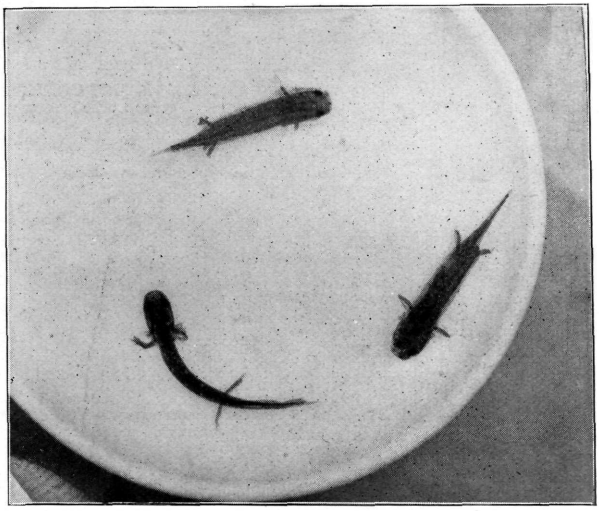

FIGURE 1.

Photo from life (x 2.3) of two Spelerpes larvae which were kept in 0.05% resorcinol for seven days, beginning just before pigmentation started. Their heavy form and the peculiar pigmentation readily distinguish them from the accompanying check. The photograph was taken thirty days after the larvae were removed from the resorcinol solution.