EVIDENCES ASSOCIATING PINEAL GLAND FUNCTION WITH ALTERATIONS IN PIGMENTATION

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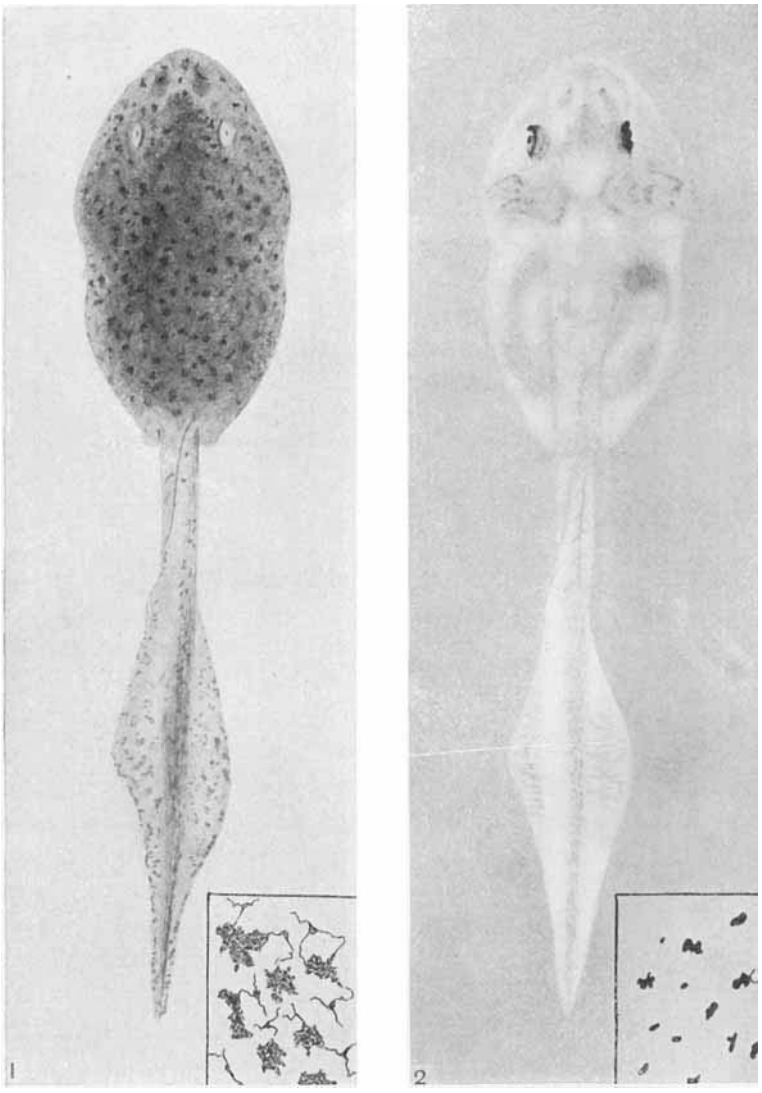
SEVEN FIGURES

INTRODUCTION

In the spring of 1915 we undertook to establish the influence of pineal gland substances upon growth and differentiation processes in tadpoles. The tadpoles employed were hatched in the laboratory and immediately placed upon a diet of pineal gland. On the tenth day of larval life it was readily observable that in the pineal fed groups the coloration was uniformly lighter than in the control, muscle-fed groups. This alteration was at first attributed to some unknown difference in environmental conditions—light, background, etc., as the color of these organisms was known to vary considerably in response to such stimuli. These changes when first noticed were trivial in degree, but as development progressed the alterations in pigmentation became correspondingly greater. Thirty minutes after feeding pineal tissue, the tadpoles which prior to the feeding had been uniformly dark, became so translucent that all the larger viscera were plainly visible through the dorsal body wall (figs. 1 and 2). This translucency appeared with such regularity and so punctually after pineal feedings and was so markedly absent in the control groups that the phenomenon was made the subject of special study. Out from this work have come many acceptable evidences of a pineal gland influence upon pigmentation, upon the phases of colloidal state, and upon the vegetative nervous system.

The following report is the record of the unfolding of this further work.



Figs. 1 and 2 Drawing of the same tadpole just prior to (fig. 1) and forty-five minutes after (fig. 2) pineal feeding.

NATURE OF PIGMENTATION

The color phenomena observable in many animal life forms are due to the absorption or reflection of light rays by chemical substances in the integument of the animal. These materials are usually found as granules of pigment lying in specialized cells, the chromatophores. Chromatophores are divisible into several types, but of these only two are found in frog skin. First, the melanophores, lying in the skin, peritoneum, etc., contain granules of dark-brown or black melanin and are often contractile. Second, the xanthophores, which are found only in the adult skin, contain granules of light yellow xanthin and are never contractile.

The relationships between pigmentation and environment are perfectly obvious but little understood. The color changes of the common tree toad (Hyla arborea) have been accounted for in various ways. One writer holds that the pale condition is the result of the stimulative effect of light upon the chromatophores and that the dark phase is due to the absence of that stimulant (1). Another observer (2) claims that light alone has very little effect, but that changes of temperature control the coloration. The problem is complicated by the fact that the same individual may not react in the same way under exactly similar conditions. Certain it is, however, that animals do respond to environmental changes by alterations in their coloration. It has been shown by numerous experimenters that these changes are to a great extent, under the control of the eyes. A change of environmental light is caught by the eye and the resulting stimulus transmitted to the chromatophores by the central nervous system. That this is the usual method of procedure is indicated by the atypical reactions of blinded individuals.

The mechanism of these changes is entirely dependent upon the contraction or expansion of the melanophores. Both xanthophores and melanophores enter into the color effects but the former play a passive role and always present the same appearance. The melanophores of frog tadpoles are of two distinct

types. The simpler and less conspicuous form is limited to the epidermis. It consists of a cell-body with two or more simple processes (*Ep. M.*, figs. 5 and 6). They may lie singly in the epidermis or, in such abundance as to form a definite reticulum. These melanophores are not contractile (3).

The second type of melanophore is found in greatest abundance in the sub-epidermal connective tissue. During late metamorphosis these cells migrate to the corium. In the expanded condition the sub-epidermal melanophores present a very typical 'mossy' appearance. The cells lie so closely approximated that they form a nearly continuous sheet (*Sub. M.*, fig. 6). There is a lighter, central space in each cell probably representing the nucleus. In the contracted condition (*Sub. M.*, fig. 7) the melanophores appear as irregular dots in which no structure is visible.

It has been fairly well established that the contractile melanophores are innervated by motor fibres proceeding along both sympathetic and spinal nerve paths (4). Hooker has shown physiologically that the reactions of the melanophores of the frog are synchronized by the action of the central nervous system. By histological methods, Ballowitz (5) has demonstrated motor nerve endings in melanophores of bony fishes. Laurens (6) working with Amblystoma larvae has shown that the melanophores may contract as the result of direct stimulation. Often, however, this primary reaction is overcome by an opposite, secondary reaction initiated by the central nervous system.

There are three principal theories to account for the mechanics of melanophore contraction. Ballowitz (1) claims that the contraction is an intra-cellular migration of the pigment granules within fixed cells. The protoplasm of a chromatophore is filled with numerous, extremely fine, radially arranged, anastomosing canals within which the pigment is forced back and forth by the alternate contraction and relaxation of the protoplasmic canal-walls.

Spaeth (7) believes that the chromatophores of fishes are fixed stellate cells, within which the pigment granules, carried in a rather fluid cytoplasm, stream into and out of the processes

during expansion and contraction. He explains this migration as a strictly colloidal phenomenon, the contracted and expanded conditions representing respectively the aggregate and disperse phases of a colloidal suspension. This is perhaps the most widely accepted and tenable view.

Hooker (8) has advanced a third explanation. He believes that the melanophores of frog larvae lie in preformed spaces and that the cells expand and contract as a whole within the spaces which enclose them. The acts of expansion and contraction, according to this theory, are brought about by pseudopodia, the pigment granules being carried in the cell cytoplasm. On this premise the pigment cells are to be considered amoeboid.

These expansions and contractions are commonly brought about by changes in the intensity of light or heat, but many other agencies will cause a specific reaction. Spaeth (7) has made a detailed study of the reactions produced by a great variety of stimuli upon the melanophores of Fundulus. He notes that the reactions are in every way comparable to those obtained by the same agents upon smooth muscle. He raises the very pertinent question as to whether melanophores may not be considered as modified smooth muscle cells.

In 1910 Babak (9) noted the reversal of the normal reaction to light when Axolotl larvae were blinded. In diffuse light the melanophores of normal seeing larvae contract. After painting the eyes with an opaque substance the melanophores expand in light. In the same way the melanophores of normal larvae expand in darkness, while those of blinded larvae contract. Fuchs (10) explained the phenomenon as due to the intervention of the parietal organ (the pineal gland of higher organisms). He reached this conclusion from a consideration of the phylogeny of this organ. The embryology of the parietal organ in some of the lower reptiles indicates very clearly that this body is a remnant of a third eye. Fuchs assumed that it had retained some of its controlling power over the melanophores. In the normal larvae its influence is completely over-shadowed by the superior power of the functioning eyes. In the blinded

larvae the parietal organ again assumes control. Laurens has completely disproven this hypothesis (11).

It is noteworthy from the present study that although the pineal gland does not exert a controlling influence upon pigmentation comparable to that arising from environmental stimulation of the retina, nevertheless it contains an active substance capable of directly inducing pigmentation changes irrespective of environmental conditions.

MATERIAL AND METHODS

Eggs of the species Rana pipiens, Rana cantabrigiensis and Bufo Americana were collected in the vicinity of Detroit, and hatched in the laboratory. Immediately after hatching and before the oral orifice had opened they were grouped in trays into colonies of 200 and food placed in the trays. The food was weighed, each colony receiving the same amount triweekly. All foods were taken voraciously by the tadpoles. By means of a water dropping and disposal system the tadpoles were at all times in fresh, aerated tap water. Moreover the trays were frequently shifted to average environmental conditions of light and temperature. In the observations on pigmentation we used some 12,000 tadpoles.

The food consisted of desiccated glandular material and fresh plant food. Of the glands we tested the effect of pineal (adult and preadult) thyroid, parathyroid, and suprarenal. Brain tissue and beef muscle were used as controls. Different lots of tadpoles were fed upon Spirogyra, bread crumbs and hemp seed as a further check. A single lot of tadpoles was fed on desiccated retinae from beef eyes as a particular experiment. Of these tissues the pineal gland alone produced the phenomenon we have called the pineal-pigment cycle.

EXPERIMENTAL EVIDENCE

Effect of whole pineal tissue on pigmentation

Certain endocrinous tissues are known to alter pigmentation in tadpoles. This was noted by Gudernatsch (12), in his feed-

ing experiments on these animals. Concerning the pigment altering tissues he states:

> After five weeks feeding, those (Tadpoles) fed on adrenal cortex became much lighter than those fed on adrenal medulla or any other food. This difference in color became more evident as the experiment proceeded, until the cortex-fed tadpoles had an extremely light, greenish yellow tint. The spleen and thymus fed tadpoles became extremely dark during the course of the feedings. Those fed on liver developed a dark greenish color, those on ovary a yellowish color.

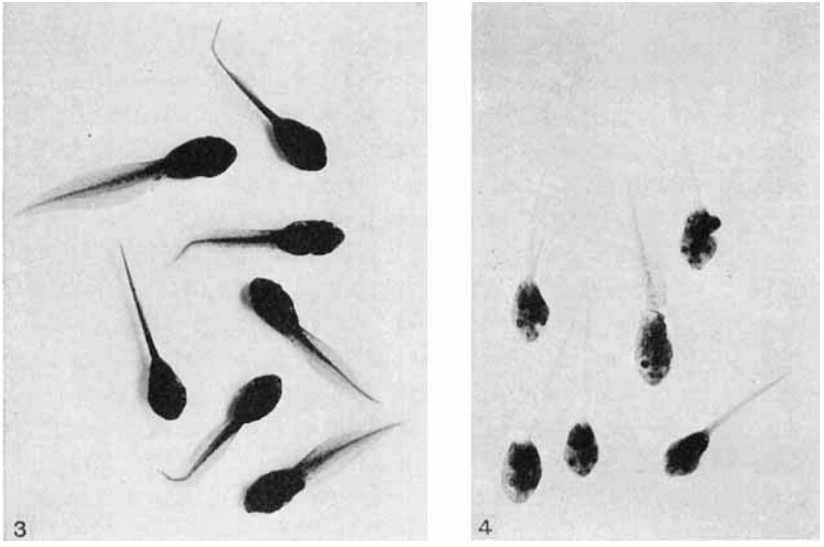
The type of pigment variation observed by Gudernatsch is obviously distinct from that observed by us. In his animals the pigment alteration was slow in appearing and persistent. In ours the change appeared early in life, occurred sharply in relation to feeding, was cyclic and transient.

After the tenth day of larval life pigment changes were always evident after every feeding of the pineal tissues and the animals continued to react until their forelegs protruded. Sufficient blanching of the bodies occurred within thirty minutes after pineal feedings to differentiate these colonies from their controls. A maximum condition of translucency was attained in about forty-five minutes, and three to six hours later restoration to the original color was complete. The difference was first noticeable in the region about the eyes due to the absence of larger viscera. It can be demonstrated, however, that the reaction occurs simultaneously over the whole body. At the height of the reaction the integument was so transparent that the brain, the olfactory tracts, the kidneys, the beating heart and the intestines were all clearly visible through the dorsal body wall.

Figures 1 and 2 are drawings of a single tadpole just prior to and forty five minutes after feeding pineal material. The darker portions in 2 are due to the denser viscera, the pigment conditions being the same over the entire animal.

Photography fails to give a true picture of this phenomenon but since actual photographs are more valuable as exact evidence than drawings figures 3 and 4 are here included. These are respectively photographs of the same group of tadpoles

just prior to and thirty minutes after feeding 5 mgm. fresh pineal gland. A true evaluation of the relative pigmentation may be had by comparison of the tadpoles of figure 3 with the periphery of those in figure 4. The dark color in the center of the bodies in figure 4 is due to the opacity of the denser viscera and intestinal contents and not to a difference in the pigmentation of the skin.



Figs. 3 and 4 Photographs made by reflected light.
Fig. 3 Normal tadpoles—just prior to pineal feeding.
Fig. 4 Same tadpoles 30 minutes after feeding acetone extract of pineal gland. The darker portions of these tadpoles are due to denser viscera—heart, gills, intestinal contents, etc. The degree of translucency is identical in all parts of the skin.

If a portion of the skin from a light and from a dark tadpole be dissected loose and examined under a microscope the reason for the difference in shade will be readily apparent, (figs. 6 and 7). The two types of melanophores are present. In the normal (dark) piece of skin the sub-epidermal melanophores (fig. 6, *Sub. M.*) are expanded to such an extent that they form an opaque sheet in which there are left a few scattered openings. In the

light piece of skin these melanophores are contracted to rough spheres of pigment (fig. 7, *Sub. M.*). The epidermal melanophores exhibit an unchanged appearance in both drawings. A sagittal section of normal skin (fig. 5) shows the relation between the two types of melanophores and the various layers of the integument.

These described alterations in pigmentation are invariably induced in tadpoles upon the administration of pineal materials, be they the fresh minced glands, simple desiccation preparations, or simple aqueous extracts. In an effort to associate these changes with certain constituents of the pineal gland, various fractions of the pineal were prepared and employed and are now about to be described.

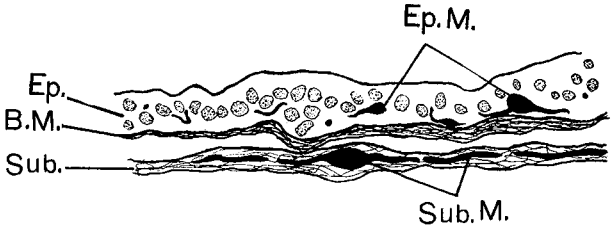


Fig. 5 Sagittal section of integument taken from the eye region of a normal tadpole. Section illustrates the two types of melanophores and their position relative to the tissue layers.

ABBREVIATIONS

Ep., epidermis
Int., integument, including epidermis and sub-epidermal tissue
Ep.M., epidermal melanophores
Sub.M., sub-epidermal melanophores
B.M., basement membrane
Sub., sub-epidermal connective tissue

Effect of pineal fractions on pigmentation

In the preparation of these split materials the fresh glands were either ground up and immediately extracted or desiccated and subsequently extracted. From the results of a wide variation in fractionation methods, chief interest centers around the acetone and alcohol extractives and their residues. In the case of acetone the process was carried out in a Soxhlet apparatus. On freeing the extractives from acetone there resulted

a brownish-black fatty mass with an odor suggestive of crude fish oil. Portions of these extractives and the residue were preserved intact for experimentation. Other portions of both were reextracted with alcohol. Likewise fresh pineal material

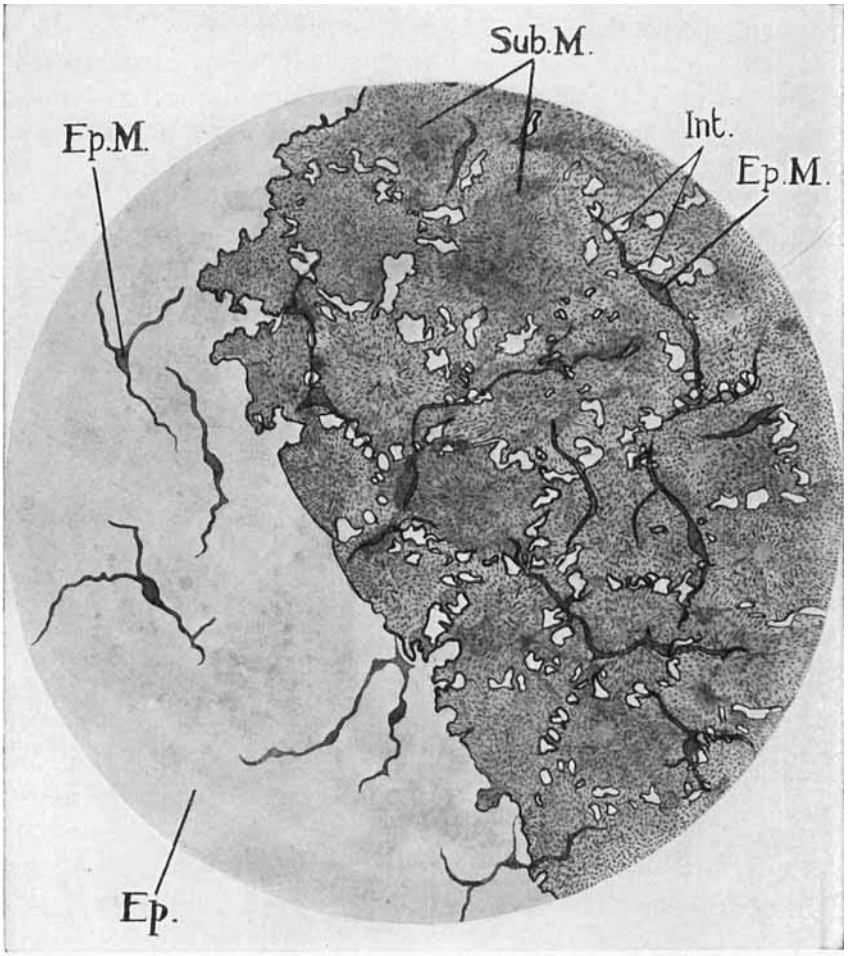


Fig. 6 Surface view of integument from normal tadpole. On the left side of the drawing the sub-epidermal connective tissue has been torn away, leaving the epidermis alone. Thus on the right side both types of melanophores are visible, on the left the epidermal type alone. The pigment granules in the sub-epidermal melanophores are evenly distributed throughout the cytoplasm, illustrating the disperse phase.

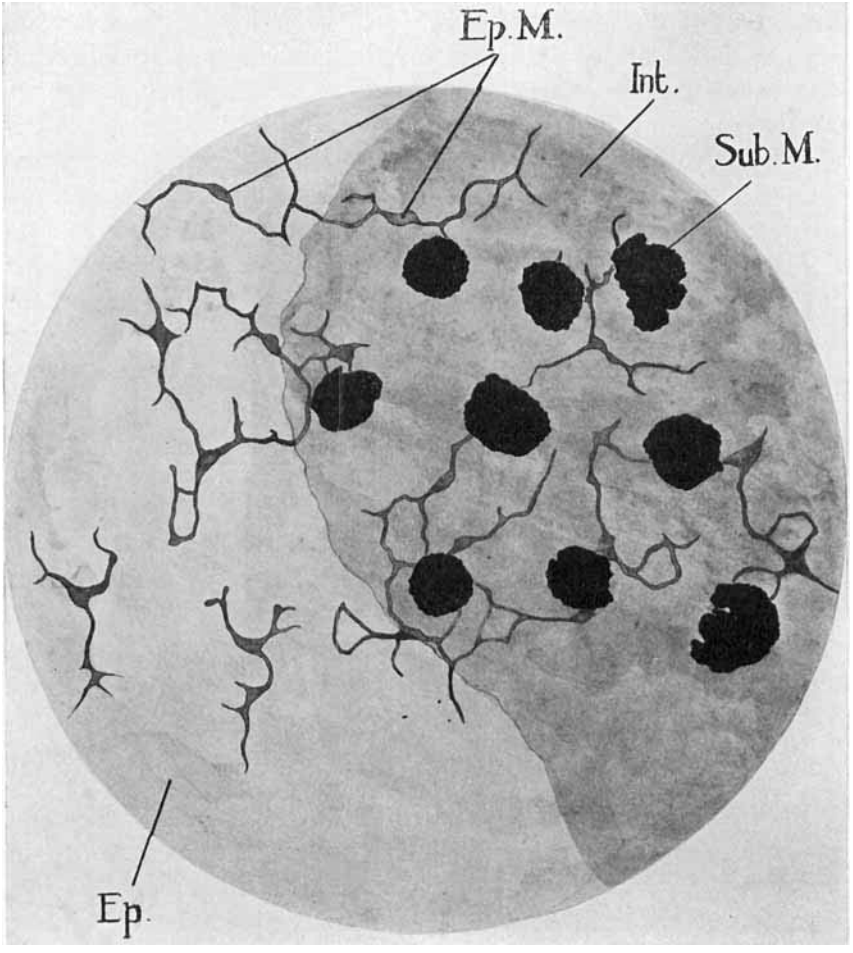


Fig. 7 Similar view of integument from tadpole fixed 30 minutes after feeding acetone extract of pineal gland. In this case the pigment granules in the subepidermal melanophores are collected into a mass in the centre of the cell—the aggregate phase.

The material was fixed in Bouin's fluid, dehydrated, cleared in xylol and mounted without staining in balsam. The drawings were made with the aid of an Abbe camera lucida, with Leitz objective 6 and ocular 4, giving an approximate magnification of 590 diameters, but have been reduced $\frac{1}{3}$ for publication. All material was taken from the region between the eyes.

was extracted with alcohol and the residue and extractives respectively extracted subsequently with acetone. These several preparations were tested as to their influence upon pigmentation on several hundred tadpoles from the same hatchings. At once it was apparent that the pigment altering principle was completely dissolved in acetone. The typical pigment cycle was induced by this extract while the residue and all acetone extracts of muscle tissues induced no pigment changes. The residue from acetone extraction was, however, capable of inducing the growth stimulating action that McCord has described for the pineal gland, while the acetone extracts which were exquisitely active in inducing pigment alterations were only slightly active in stimulating rapid growth. The inference is that at least two separate distinct principles exist in the pineal, the one producing the pigment phenomena, the other stimulating rapid growth. In the case of the alcoholic extraction the active substances were not readily soluble, for the alcoholic extractives, the alcoholic residue, and the acetone reextractives, all yielded positive pigment results. The acetone extractives yielded quite readily to aqueous emulsifying and this form proved to be the most convenient mode of employing this material.

Quantitative relations in time and amount of contraction

In the feeding of pineal materials to the tadpoles the time interval necessary to establish maximum contraction of the pigment cells increased as the concentration decreased. Tadpoles placed in a 1:500 pineal emulsion were noticeably lighter in five minutes and required but thirty minutes to arrive at maximum translucency. In higher dilutions the maximum translucency was attained only after a longer interval and in very high dilutions producing only qualitative changes, the maximum was not attained. The dilution of 1:100,000 was the highest that produced a macroscopically discernible qualitative action. These reasonably constant quantitative relations have afforded us a means for the evaluing of the strength of our several preparations and may on extended study prove to be

a trustworthy method for the standardization of pineal products for at the present time no method exists for testing the strength of such preparations.

The quantitative relations between concentration and time will be evident from the following table.

DILUTION OF ACETONE EXTRACT	MAXIMUM REACTION ATTAINED IN
	minutes
1 : 500	30
1 : 1000	45
1 : 2000	60
1 : 5000	105
1 : 10,000	Qualitative change but maximum not attained.
1 : 100,000	Maximum not attained.

The maximum reaction was determined by comparison with a standard consisting of several tadpoles which had been placed in a 1 : 500 pineal emulsion thirty minutes prior to the beginning of the experiment. The translucency thus obtained was found to be the greatest possible. It served as the criterion for comparison as to the degree of depigmentation induced by pineal preparations of unknown activity. In the practical standardization of pineal preparations, the end reaction of greatest feasibility was the comparative time intervals necessary to attain to maximum translucency. Such a method is obviously open to the criticism that frog larvae are only obtainable for a short period in the year. With Bufo Americana pigment changes were found to be too trivial to be of value in standardization. Rana pipiens were exquisitely responsive and amirably suited for standardization purposes except in that only during the spring months are they obtainable. We are now experimenting with certain amphibian larval forms that may be obtained throughout the year.

We have determined that the growth stimulating principle in the pineal is distinct from the principle concerned in pigment changes and this on further investigation may militate against this proposed means of standardization.

Effect of pineal gland upon melanophores of other forms[1]

Pineal gland extracts have no demonstrable effect on either type of chromatophore of Loligo (squid) or upon the melanophores of adult Fundulus. The extracts however determine distinct contraction of the melanophores of young Fundulus as may be noted in accompanying table.

No. 1 ONE WEEK-OLD FUNDULI IN BOILED SEA-WATER		No. 2 ONE-WEEK-OLD FUNDULI IN EMULSION OF ACETONE EXTRACT OF BEEF MUSCLE IN SEA-WATER		No. 3 ONE-WEEK-OLD FUNDULI IN EMULSION OF ACETONE EXTRACT OF PINEAL GLANDS IN SEA-WATER	
Time		*Time*		*Time*	
9.15	Complete expansion of melanophores	9.15	Complete expansion	9.15	Complete expansion
9.38	Complete expansion of melanophores	9.40	Complete expansion	9.37	Beginning contraction
9.46	Complete expansion of melanophores	9.47	Complete expansion	9.45	Contraction increasing
9.59	Complete expansion of melanophores	10.00	Complete expansion	9.58	Contraction almost complete
10.31	Complete expansion of melanophores	10.32	Complete expansion	10.30	Complete contraction
11.51	Complete expansion of melanophores	11.52	Complete expansion	11.50	Complete contraction
11.55	All transferred to fresh sea water				
1.55	p.m. Complete expansion	1.56	Complete expansion	1.54	Partial expansion
3.01	p.m. Complete expansion	3.02	Complete expansion	3.00	Complete expansion of some of the melanophores, especially in tail; partial expansion of others

Mode of absorption of the pigmentation altering principle

Several experiments were tried with the object of showing whether the pineal tissue must be ingested or not in order to produce the reaction.

[1] Experiment conducted by A. Noble at Woods Hole, Mass.

1. Pineal emulsion on anesthetized tadpoles. Several tadpoles of equal depth of pigmentation were completely anesthetized with of ether after which half were placed in an emulsion of acetone extract of beef pineal, the others in a like emulsion of muscle tissue. Five minutes later those in the pineal emulsion were preceptibly lighter, later acquiring a marked translucency. The latter remained unchanged. The tadpoles recovered from the anesthetic and gradually regained their original appearance.

2. Hypodermatic injection of pineal emulsion. Several tadpoles of equal depth of pigmentation were divided into two groups. One received 0.01 cc. of an emulsion (1–500) of acetone extract of pineal gland, injected hypodermatically into the peritoneal cavity; the other received the same amount of normal saline solution injected in a similar way. Shortly after injection the pineal treated animals became lighter, eventually reacting to the maximum degree of translucency. The other tadpoles remained practically unchanged in appearance. As a further control a third group was immersed in the pineal emulsion for the length of time consumed in making the injection and the tadpoles were then washed in tap water. They remained unchanged. This control showed that the effect produced was due to the injected pineal material and not to any accidental absorption through the skin.

3. Effect of pineal emulsion on eviscerated tadpoles. A number of tadpoles were completely eviscerated. These tadpoles live and swim about as freely as their fellows. Following this procedure part of the animals were removed to an emulsion of the acetone extractive of beef pineal gland and shortly passed through the same pigment changes as normal tadpoles placed in this emulsion at the same time. The eviscerated and normal tadpoles remaining in tap water did not change in color.

These observations prove conclusively that the principle involved is directly absorbable through the gills or skin. The effect is produced without the intervention of the processes of digestion. There is no indication, however, as to whether the principle acts directly upon the melanophores, or indirectly through the medium of the central nervous system.

Effect of pineal extract upon unstriated muscle

Aqueous extracts of fresh pineal glands were tested according to the method of Dale and Laidlaw (13) upon isolated strips of guinea-pig uterus. The extract produced a typical though feeble contraction of the uterine muscle. Three cc. of a 20 per cent pineal extract was roughly equivalent in activity to 0.004 cc. of a 20 per cent pituitary extract. Thus pineal extracts stimulate certain smooth muscle cells as well as pigment cells to contraction. This similarity of action goes far to confirm Spaeth's hypothesis that the melanophore is a type of smooth muscle cell.

COMMENT

Many acceptable evidences associate the pineal gland with an earlier optical function. In the reptilian stage of evolution this parietal eye probably attained to its highest development. In the embryos of certain lizards (Lacerta agilis) the typical eye structure is still evident, but in no form living at the present time does the pineal gland retain an ocular function, of high order (14).

The color changes in forms are obviously in adjustment to environmental conditions. The eye is the essential controlling factor in this adjustment. When in blinded animals certain definite changes in pigmentation still occur, on theoretical ground it is tenable to assume that the pineal body retains sufficient ocular mechanism to exert its influence upon the pigment cells. This is the hypothesis suggested by Fuchs (10). Laurens has established experimentally that such an activity on the part of the pineal is highly improbable (11). Accepting the contentions of Laurens, it is the gist of our work that while the pineal does not act in the rôle of its ancient ocular function, it contains within itself an active principle capable of inducing pigment changes independent of and wholly apart from environmental conditions. The pineal pigment changes dominate and appear despite environmental conditions tending toward the

opposite phase. The salient observations that we have recorded in detail on the foregoing pages are:

1. Up to near tenth day of larval life in tadpoles, pigmentation is not influenced by pineal feeding. Evidences relative to this are not precise, but suggest that this is due to incomplete development of the nervous mechanism involved.

2. Beginning at this time and continuing until near the termination of metamorphosis, the addition of traces as small as 1 part acetone extract in 100,000 parts water determine distinct cyclic pigment changes peculiar to these preparations. Prior to feeding, both controls and experimental animals are uniformly dark colored. Shortly after feeding the pineal fed groups begin to lose color until within thirty minutes, all macroscopic pigment is lost so that all the larger viscera are clearly visible (figs. 1 and 2). The condition is transient and the cycle is complete with full restoration of color within from three to six hours, unless further pineal food is added to the trays. As metamorphosis is completed the pigment is no longer altered by pineal materials, due to rearrangements of chromatophore types and sites in the adult animal.

3. The response in pigment change is quantitative. A method is described for the standardization of pineal preparations.

4. The pineal substance responsible for the pigment changes is wholly extracted by acetone. The residue after acetone extraction is an inert substance as to pigment influence. However this residue has an influence on growth and differentiation. The inference is that the gland contains more than one active substance.

5. The reactions produced by pineal extracts add some evidence to Spaeth's contention that the melanophores are modified smooth muscle cells. The similarity of contraction of certain smooth muscle organs under the influence of pineal extracts and the contraction of melanophores is in keeping with Spaeth's hypothesis.

The very nature of this pineal-pigment cycle affords an excellent method of approach to the mechanics of melanophore function and from this the larger problems of colloidal state.

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