THE NATURE, ORIGIN AND SIGNIFICANCE OF PIGMENT IN EMBRYOS OF AMBLYSTOMA

by

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Submitted to the Department of Anatomy and the Faculty of the Graduate School of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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May, 1923.

C O N T E N T S.

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I. INTRODUCTION.

Although various workers have observed the yellowish brown pigment scattered through amphibian embryos, the writer has been unable to find an adequate report in literature concerning it. The purpose of this work is to describe the origin, nature and significance of the pigment found in the myotomes and central nervous system of embryos of Amblystoma.

For valuable suggestions and criticisms the author is indebted to Professors G. E. Coghill and H. C. Tracy. Furthermore, through grants made to them by the Research Committee of the Graduate School, this work has indirectly profited in a considerable degree.

II. MATERIAL AND METHODS.

This study has been made chiefly on Amblystoma microstomium. The stages of development selected for observation have included those described by

Coghill ('16, p. 163) as: (1) embryos that move in response to an electric current, but not in response to tactile stimulation with a hair or other chemically inactive structure, designated the non-motile stage; (2) embryos taken very soon after there is a perceptible movement in response to tactile stimulation, designated early flexure stage; (3) embryos that move the trunk into a coiled condition, designated the coiled-reaction stage; (4) embryos that move to the right and left simultaneously in different parts of the body in a sinuous fashion, and have just acquired the power of locomotion, designated the early swimming stage. In addition observations were made on older embryos, the ages of which are indicated by the number of days from the time they began to swim.

Serial sections have been used extensively in these observations. The fixing fluids that have been employed are Bouin's corrosive sublimate and acetic acid, Van Gehocten's, Zenker's, without acetic acid, Orth's, Kleinenberg's, and formalin solutions. The first two are found to be especially suited to this purpose. Erythrosin and toluidin blue, alum-carmine and Lyon's blue, alum-carmine, haemetoxylin and orange G, neutral red, and neutral red followed by Janus green have been

used, but alum-carmine has proved to be best adapted for study of pigment in the central nervous system. Haematoxylin and orange G. show very satisfactorily the relations of the larger structures of the muscle cell, such as the nuclei, yolk globules, and the large masses of pigment which will be described further on in the paper. Neutral red and Janus green are superior for the demonstration of the finer structures of the cell, such as pigment granules, neurofibrils and myofibrils.

Teased preparations and frozen sections of fresh tissue have been used for the study of the nature and origin of the pigment. Preparations have been made by carefully dissecting various tissues such as post auditory myotomes, central nervous system, cutis, and undifferentiated mesenchyme. These tissues were placed in various stains for different lengths of time. After staining the larger tissues were teased out on a slide in a drop of the stain and then covered with a cover-glass. By gentle pressure on the cover-glass the tissue was spread thin enough to permit examination under oil immersion objectives. Thinner, or smaller pieces of tissue were, after staining, mounted in a hanging drop. Care was taken to use a small drop which would permit the use of oil immersion objectives. The stains used

in these preparations were neutral red, Janus green, Nile blue, methylene blue, Sharlach R and Sudan III. Unstained preparations were also studied.

III. O B S E R V A T I O N S.

A. PIGMENT IN MYOTOMES.

The relation of the pigment to other structures in the cell and it's distribution through the myotomes has been studied in corresponding myotomes of the different stages. Serial sections in the frontal plane have been used almost exclusively for these observations. Three embryos have been studied in the non-motile stage; four in the early flexure; seven in the coil, and six in the early swimming stage. Observations are given from type specimens of the different stages.

1. Non-motile Stage.

The cells in the dorsal portion of the third myotome are not formed into functional muscle fibers, but are radially arranged about the central part. Dorsoventrally there is a progressive change to the longitudinal arrangement of muscle cells containing two or three nuclei. Myofibrils are present in the ventral portion, although obscured by the yolk globules, which are very abundant. In the dorsal portion the

pigment occurs in the form of fine granules in the central part of the myotome. These granules are located about the central ends of the nuclei in the radially arranged cells, and around yolk globules. Farther ventrally, the pigment occurs in the middle third of the myotome longitudinally. The pigment granules occur about the nuclei, or the yolk globules, or in strands between these structures. It is more abundant in the ventral portion, and not only occurs as fine granules, but also in masses of closely adhering, or segregated, granules. These masses of pigment granules are here termed "pigment bodies." They occur in most instances at one pole of a nucleus. While the pigment bodies appear homogenous under low magnification, under oil immersion they are resolved into aggregations of granules. There are very few pigment bodies in the upper third of the myotome. The number increases dorso-ventrally through the ventral two-thirds of the myotome. This is illustrated in Fig. 1a, which is an optical projection of the third myotome, as viewed from the lateral side, showing the number and location of the pigment bodies. The total number of pigment bodies is sixty. The average diameter of thirty of the largest of the pigment bodies is 6.36 micra.

The tenth myotome is less differentiated than the third. The cells in the dorsal region retain their embryonic type and radial arrangement further ventrally than in the third myotome. The nuclei in this more dorsal region are nearly round. In the ventral portion, the cells become definitely oriented longitudinally, parallel to the long axis of the myotome. In this region the nuclei are slightly elongated. No myofibrils can be observed. The pigment, through the central portion, occurs in the same relative position as in the corresponding region of the third myotome. There are only eighteen pigment bodies in the entire myotome (Fig. 1b).

The eighteenth myotome is still less differentiated than the tenth. The radial arrangement of the cells about the central portion exists throughout the whole myotome. There are, apparently, no functional muscle cells. The structure of such a myotome is illustrated in Fig. VI. There are no pigment bodies present. The pigment granules are distributed through the central portion of the myotome about the yolk globules, and around the nuclei they are particularly abundant at the ends which are directed toward the center of the myotome.

The twenty-fourth myotome has not become differentiated.

2. Early Flexure Stage.

The third myotome in this stage is more differentiated than the corresponding myotome of the non-motile stage. The relatively undifferentiated dorsal portion is less in extent. The myofibrils can now be perceived in the middle part of the myotome dorsoventrally in regions where the yolk globules are not too numerous. The differentiation has increased over that of the non-motile embryo as shown by the elongation of the nuclei and muscle cells, and by the increase of myofibrils. There appears to be relatively less yolk. There is an increase in the amount of pigment. The pigment bodies number one hundred-fifty-two in the entire myotome (Fig. IIa). The majority are located near the ends of nuclei. The average mean diameter of thirty of the largest is 8.06 micra.

The tenth myotome has differentiated further than the corresponding myotome of the younger stage. It is less differentiated than the third myotome of the stage under consideration as described above. The undifferentiated cells in the dorsal region gradually change to the longitudinal arrangement in the ventral portion, where the outlines of the cells, the position of the nuclei, and the presence of myofibrils show that the muscle is functional in this region. The yolk globules are more

numerous at the ends of the myotomes than in the central part. The pigment granules and bodies have the same general arrangement as in the third myotome. Less pigment is present than in the third myotome, but more than in the corresponding myotome of the earlier stage. This myotome in the early flexure stage contains seventy-one pigment bodies (Fig. IIb). The average mean diameter of thirty of the largest is 7.65 micra.

The eighteenth myotome is more differentiated than the corresponding myotome of the non-motile stage. In the dorsal third, the cells occur in a relatively radial arrangement about the central part of the myotome. The cell boundaries are still indistinct, a condition which characterizes the whole of the corresponding myotome of the younger embryo. There is a gradual transition in this myotome from the radial arrangement of the cells in the dorsal third to the elongated longitudinally directed muscle cells which are characteristic of the ventral third of the myotome. These muscle cells are multinucleated, and each extends the length of the myotome. The pigment in the dorsal portion is distributed in the central part of the myotome as shown in Fig. VI. In the ventral, differentiated portion of the myotome, the pigment granules and bodies occur in the central third of the cells. There are only

twelve small pigment bodies in the entire myotome (Fig. IIc).

3. Coiled Reaction Stage.

The third myotome is further differentiated than the corresponding myotomes of the next younger stage, as shown by the increase in the amount of functional muscle in the dorsal portion, the increase in the number of myofibrils, and the elongation of the myotome. There appears to be relatively less yolk. The increase in the amount of pigment is slight. There are one hundred-sixty-one pigment bodies present. The average mean diameter of thirty of the largest bodies is 8.12 micra.

The tenth myotome is less differentiated than the third, but is more differentiated than the tenth myotome in the earlier stage. In the dorsal part, the nuclei are crowded to-gether in a transitional stage between the radial arrangement and the elongated cell types. The ventral half of the myotome contains approximately eighty-five per cent of the pigment bodies, which number one hundred-fifty (Fig. IIIb). Thirty of the largest of these bodies average 8.16 micra in diameter.

The eighteenth myotome shows a considerable increase in development over the corresponding myotome of the early flexure, and non-motile stages. The middle and

ventral portions are differentiated into fuctional muscle. The pigment granules and bodies have the same arrangement throughout the middle third of the myotome longitudinally, and through the myotome dorsoventrally, as described for the tenth myotome of this stage. There are thirty-five pigment bodies located in the ventral portion in the middle third of the myotome. The average mean diameter of thirty of the largest is 5.9 micra.

4. Early Swimming Stage.

The third myotome shows the greatest differentiation of any myotome studied. Cross striations are plainly visible. There is, apparently, a decrease in the amount of yolk as compared with the corresponding myotome of the coiled reaction stage. The yolk globules are smaller than in the corresponding myotomes of the younger stages. The number of pigment bodies has increased to two hundred-twenty-six as compared to one hundred-sixty-one in the coiled reaction stage, one hundred-fifty-two in the early flexure stage, and sixty in the non-motile stage. The average mean diameter of thirty of the largest is 8.65 micra. The bodies are typically arranged in the central portion of the myotome, at, or near the ends of the nuclei.

The tenth myotome is further differentiated than the

corresponding myotomes of the earlier stages. There is, apparently, less yolk with a decided increase in the number of pigment bodies. In this myotome there are three hundred-sixty-four pigment bodies as compared to one hundred-fifty in the coiled reaction stage, seventy-one in the early flexure stage, and eighteen in the non-motile stage. The average mean diameter of thirty of the largest is 8.24 micra. The dorsal part of the myotome has many granules, but few bodies. In the ventral part, the bodies are numerous with a relatively smaller number of granules; a similar arrangement prevails in the adjacent myotomes of that region. As the pigment bodies increase in number and size from the dorsal toward the ventral part of the myotome the granules appear to decrease in number until there are only a few in the ventral portion of the myotome where the pigment bodies become correspondingly more numerous.

A section through the middle of the myotome in frontal plane shows that the nuclei are arranged in three zones. In the outer zones the nuclei are arranged in a single definite row transversely, whereas, in the middle zone they are scattered throughout the middle third of the myotome, but separated from those of the outer zones by a characteristic space, void of nuclei (Fig. VII). The pigment bodies occur at, or near the

ends of the nuclei of the middle zone and at the inner ends of the nuclei of the outer zones.

The pigment granules are particularly abundant throughout the region between the two outer zones, while the end portions of the myotome contains few granules.

The eighteenth myotome is less differentiated than the tenth as the dorsal portion shows the radial arrangement for a short distance. There are one hundred-ninety-five pigment bodies in this myotome (Fig. IVc) as compared to thirty-five in the coiled reaction stage, twelve in the early flexure stage, and none in the non-motile stage. The average mean diameter of thirty of the largest is 6.23 micra. The distribution of the pigment granules and pigment bodies is the same as described in other myotomes.

The twenty-fourth myotome shows only a slight degree of differentiation. The pigment granules are distributed around nuclei and yolk globules, similar to the distribution described in the eighteenth myotome of the early flexure stage (Fig. V). In the entire myotome there are only five pigment bodies, which are located within a narrow zone in the middle third (Fig. IVd).

The positions of eighty-one pigment bodies were noted. Fifty-eight, or, 71.6% were at the ends of

nuclei; fifteen, or, 18.5% were at the sides of nuclei, and eight, or, 9.9% were not in the immediate vicinity of a nucleus. The reason 9.9% of the bodies were located some distance from nuclei may be due to their having been separated from nuclei by the sectioning of the tissues.

5. Comparative Summary.

The numerical relation of the pigment bodies in the third, tenth, eighteenth, and twenty-fourth myotomes is shown in Table I.

TABLE I.

Number of Pigment Bodies in Myotomes

Stages	3rd	10th	18th	24th
Non-motile	60	18		
Early Flexure	152	71	12	
Coiled Reaction	161	150	35	
Early Swimming	226	364	195	5

In the third myotome, the number of bodies increases from sixty in non-motile stage, to two hundred-twenty-six in the early swimming stage; in the tenth myotome, the increase is from eighteen to three hundred-sixty-five; in the eighteenth myotome, the number increases from zero to one hundred-ninety-five, while in the twenty-fourth myotome, the increase is from zero to five. The increase

in number appears to be slightly retarded in the third myotome after the early flexure stage. This is probably due to the growth, in this region, of the pronephros which prevents the full development of the third myotome, and is the probable explanation of the fact that the third myotome has fewer pigment bodies in the early swimming stage than the tenth myotome.

Table II shows the relative growth in size of the pigment in corresponding myotomes of the four stages.

TABLE II.

Third Myotome

	Dorsal	Middle	Ventral	Average
Non-motile	5.55	6.44	7.16	6.36
Early Flexure	6.09	8.79	9.4	8.06
Coiled Reaction	6.7	8.22	9.45	8.12
Early Swimming	6.96	8.06	10.95	8.65
Average	6.32	7.87	9.24	7.81

TABLE II
(Cont'd.)

Tenth Myotome

	Dorsal	Middle	Ventral	Average
Non-motile				
Early Flexure	6.29	8.19	8.48	7.65
Coiled Reaction	6.68	8.82	8.99	8.16
Early Swimming	6.7	8.83	9.2	8.24
Average	6.58	8.61	8.89	8.02

Eighteenth Myotome

	Dorsal	Middle	Ventral	Average
Non-motile				
Early Flexure				
Coiled Reaction	5.04	6.16	6.5	5.9
Early Swimming	5.2	6.49	7.02	6.23
Average	5.12	6.32	6.76	6.06

In computations for Table II, ten of the largest pigment bodies were selected for measurement in each of the following portions of the myotome: (1) dorsal, (2) middle, and (3) ventral. The greatest and least

diameters were measured in micra and the mean taken. This table shows that there is an increase in size of the pigment bodies from the youngest to the oldest stage and also from the dorsal to ventral portion of a given myotome. In the middle portion of the third myotome there appears to be a decrease in size from the early flexure to the early swimming stage. This may be due partly to error in selecting the bodies in the correct region. Also, the early flexure embryo may have been cut somewhat obliquely so that bodies that should be classified with those of the ventral part were included with those of the middle portion of the myotome.

The increase in size in the dorsal part of the third myotome from early flexure to early swimming stage is from 5.55 micra to 6.96 micra, an increase of 1.4 micra, ortwenty-five per cent. In the middle portion the increase was from 6.44 micra to 8.06, which amounts to 1.62 micra, or twenty-five per cent. In the ventral part the increase was 3.79 micra, or fifty per cent. The bodies increased in size dorso-ventrally. In the early flexure stage this increase is from 5.55 micra to 7.16, which amounts to approximately thirty per cent. In the early swimming stage the increase is 3.99 micra, or nearly sixty per cent.

6. Conclusions.

The results of the study of the serial sections show

that (1) the pigment is found chiefly in the central portion of the myotome, (2) the pigment granules are situated particularly about nuclei and yolk globules and extend in strands between these structures, (3) the pigment bodies increase both in number and size from the dorsal to the ventral part of the myotome, (4) the pigment bodies in corresponding myotomes increase both in number and size with the age of the embryo, at least up to the early swimming stage, (5) the amount of pigment increases as the yolk decreases, and (6) the amount of the pigment varies directly with the degree of differentiation.

B. PIGMENT IN THE CENTRAL NERVOUS SYSTEM.

Since there proved to be a distinct correlation between increase in pigment and the degree of differentiation in the myotomes, study was extended to the distribution of the pigment in the central nervous system as related to regions of differentiation and proliferation.

Altho observations have been made on a large number of embryos, type specimens were selected for a more exhaustive study. The embryos used were in my series designated, No. 304, non-motile; No. 309, early flexure; No. 308, coiled-reaction, and No. 302, early

swimming. They were all fixed in Bouin's fluid, sectioned transversely five micra, and stained with carmine. With a projecting apparatus, magnifying two hundred diameters, sketches were made of every fourth section of the nervous system, beginning at the anterior portion of the fore-brain. Outlines of the areas of abundant pigmentation were sketched in.

These drawings were continued caudad as far as the pigment could be seen through the projecting apparatus. The drawings were compared with reference to the general distribution of pigment and this comparison was supplemented and checked by direct study of the sections with the microscope. It should be noted that fine granules of pigment occur throughout the central nervous system, but that the regions in which the pigment was especially abundant were indicated by the projection apparatus. The visibility of the pigment through this apparatus thus became a standard for quantitative estimates.

The distribution of the pigment was noted particularly in the rhombencephalon and spinal cord. Different levels were selected for descriptive purposes in order to compare more easily the distribution of pigment in the different stages and to differentiating areas that have been previously described by Coghill ('14, '16).

Before consideration of these areas and their

relations to differentiation, it should be noted that the series of sketches of the sections mentioned above as well as Figure VIII were made before areas of differentiation had been taken into account by the author.

1. Rhombencephalon.

In the rhombencephalon, the pigment is located towards the surface in lateral and ventro-lateral areas. This occurs throughout the rhombencephalon and continues caudad in the spinal cord in lateral areas.

a. At level of the root of N. V.

In the non-motile stage the rhombencephalon still retains the upright position dorso-ventrally. The cells of the inner portion of rhombencephalon next to the ventricle are epithlial in appearance and arrangement. This extends nearly to the outside of the brain. Here in this region are a few cells that have already differentiated into nerve cells. Their relation to the trigeminal nerve has been described by Coghill ('16, Fig. 20). This lateral and ventro-lateral region is the area of abundant pigmentation shown by Figure VIII, row I, and column 1. There is a great abundance of pigment about

nuclei and yolk globules. It is most abundant towards the external surface of the brain. There is some pigment along the ventricular surface, which, probably, has been retained from the earlier ectodermal position. There is a large clump of pigment next to the ventricle in the floor plate cells.

In the early flexure stage, the rhombencephalon has differentiated further than in the non-motile stage, although there has been only a slight broadening. The area of differentiating neuroblasts is slightly wider in extent (See Coghill '16, Fig. 21). The pigment is distributed through this area as shown by Figure VIII, row II, and column 1. The pigment area has increased dorso-ventrally, and there appears to be a slight increase in amount of pigment. The largest and heaviest groups of pigment occur toward the outside. In the type specimen from which the drawings were made, there is more pigment immediately along the ventricular border of the rhombencephalon than in the other specimens. The pigment in the floor plate cells is massed to-gether in the central end of the cells adjacent to the ventricle.

In the coiled reaction stage, the rhombencephalon has broadened so that the areas of differentiation that

were lateral in the younger stages are now distinctly lateral and ventro-lateral. The cells adjacent to the ventricle are epithelial in appearance and arrangement as in the younger stages, but this area is less in extent. In this proliferating area there is no increase in the amount of pigment. In the lateral differentiating area there is an increase in the number of neuroblasts. (See Coghill '16, Fig. 35). As the area of differentiation has increased so has the area that contains the pigment in abundance. The pigment extends further toward the ventricle, although most abundant in the outer cells. This is shown in Figure VIII, row III, and column 1. There appears to be slightly more pigment than in the younger stages at the corresponding level. There is less pigment bordering the ventricle, except the large mass in the floor plate cells.

In the early swimming stage, there is a further differentiation in this stage over that of the coiled reaction stage. The fiber tracts and differentiating neuroblasts are easily perceived. The pigment is more abundant in the lateral and ventro-lateral areas as described in the younger stages. (See Coghill '16, Fig. 51-54). The pigment area, has increased in size, however, and is now wider and extends over half the distance to the ventricle. Outside of this

area, the pigment is not particularly abundant, except in the floor plate cells, where there is a large mass adjacent to the ventricle. In the lateral and ventro-lateral areas, the pigment occurs as fine granules. Sometimes under low magnification, some of the pigment appears to be in irregular stringy masses, but higher magnifications show that they consist of closely adhering granules.

b. At the level of the roots of NN. VII and IX.

As the differentiating areas are continuous in the rhombencephalon in the approximate location as described at the level of the root of the fifth nerve, a description of location of the areas at the levels of the roots of the seventh and ninth nerves would be a repetition. The areas of abundant pigment as described at the level of the root of the N. V are continuous in the lateral and ventro-lateral regions from the root of the N. V through the levels of the roots of the NN. VII and IX into the lateral areas of the spinal cord.

In the <u>non-motile</u> stage, there is apparently less pigment at the level of the seventh than the fifth nerve and approximately the same as at the ninth nerve. At the level of the ninth nerve the pigment is in a more lateral area.

In the early flexure stage, there is an apparent increase of pigment at the level of the seventh and ninth nerves as compared to that of the non-motile stage. This is most marked at the level of the ninth nerve which follows closely the increase of differentiation as shown by widening of the rhombencephalon at this level. The extent of the pigment increases correspondingly with the larger area of differentiation. The change in shape of the rhombencephalon at these levels and the increase in extent and amount of pigment is shown in Figure VIII, rows I and II, and columns 2 and 3.

In the coiled reaction stage, there is a further widening of the rhombencephalon with an increase in differentiating and likewise an increase in amount and extent of the pigment. In this stage, there seems to be approximately the same amount of pigment at the levels of the seventh and ninth nerves as at the level of the fifth nerve. No increase in amount of pigment could be noted in the proliferating areas next to the ventricle. The distribution of the pigment is shown by Figure VIII, row III, and columns 2 and 3.

In the early swimming stage, the differentiating areas are wider than in the younger stages, and in a

similar way, the area of pigmentation is wider and extends more toward the ventricle. The pigmentation is heavier and more occurs in clumps. The greatest amount occurs near the outer edge of the brain. There is approximately the same amount of pigment at these levels as at the level of the fifth nerve. There is no increase in pigment along the border of the ventricle.

2. Spinal Cord.

The distribution of the pigment in the spinal cord is shown by the projection apparatus to be in lateral areas. The pigment can be traced by this method to the level of the fifth myotome in the non-motile stage. In the early flexure stage, it extends to the level of the eighth myotome; in the coiled reaction stage, to the level of the ninth myotome, and in the early swimming stage, to the level of the eleventh myotome.

At the level of the third myotome in the non-motile stage, the pigment occure in the lateral areas as shown in Figure VIII, row 1, column 4. This corresponds to the differentiating area described by Coghill ('14, Fig. 28). In the early flexure stage, there is an increase in amount of pigment in the lateral areas. This is shown in

Figure VIII, row II, and column 4. There is no increase in pigment in the proliferating areas next to the centralcanal (See Coghill, '14, Fig. 29).

In the coiled reaction stage, the pigment has increased in extent as shown in Figure VIII, row III, and column 4. In the early swimming stage, there is additional increase in pigment in the differentiating areas as opposed to lack of increase in the proliferating areas. (Coghill '14, Fig. 30 and 31).

This increase in pigment at the level of the third myotome as the embryo developed is distinct. It is even more marked at the level of the eighth myotome. There was no pigment area visible at this level in the non-motile embryo. In the early flexure stage, there was a small amount visible in the lateral area. In the coiled reaction stage, this area is larger and contains more pigment. In the early swimming stage, the pigment has increased in amount and occurs in the area of most extensive differentiation. (See Coghill '14, Figs. 32 to 35).

3. Comparative Summary of Pigmented Regions in the Nervous System

The definite increase in the amount of pigment could not be measured.

The areas of abundant pigmentation increase in size at each of the levels of the central nervous system noted in the description. The comparative size of these areas is shown roughly in Figure VIII. There are no perceptible clumps in the youngest stages, and only occasionally do they occur in the older stages. The amount of pigment in the non-motile embryo decreases gradually through the rhombencephalon and early part of the spinal cord. In the early flexure and coiled reaction embryos, the pigmentation increases in the rhombencephalon at the level of the root of N. V, but seems to increase more rapidly at the levels of the roots of NN. VII and IX until in the early swimming embryo the amount is approximately the same in the lateral and ventro-lateral areas at the different levels under consideration.

The increase in amount of pigment and size of areas of abundant pigmentation is more marked in the spinal cord than in the rhombencephalon. Especially is this noticable at the level of the eighth myotome. The areas of pigmentation can be traced farther caudad as the embryo increases in age.

It is obvious that the areas of abundant pigmentation increase in size with the growth of the embryo, and that there is no increase in pigment in the prolifer-

ating areas adjacent to the ventricle. This is true in both the rhombencephalon and spinal cord.

It should be noted that in the youngest stages studied certain cells contain pigment. Coghill ('14) noted that the Rohon-Beard cells in the spinal cord contain masses of pigment. These cells to-gether with a few motor cells and certain cells in the floor plate, differentiate early and the pigment which is probably formed during their differentiation period, is retained in the cytoplasm during the four physiological stages studied.

4. Conclusions.

From the facts as observed one must conclude that (1) differentiating areas are areas of most abundant pigmentation, (2) the amount of pigment increases as differentiation proceeds, (3) proliferation is not accompanied by an increase of pigmentation, and (4) the amount of pigment is an index of the degree of differentiation in the central nervous system.

C. ORIGIN OF PIGMENT

The study of formation of pigment in amblystoma embryos has been performed primarily upon fresh preparations of living tissue. Observations have been made not only on the muscle and nervous systems, but also upon preparations from epidermis and undifferentiated mesenchyme. Dissected tissues, both stained and unstained, were mounted on slides and gently mashed under a cover-glass, or placed in hanging drops. Epithelial cells of the skin and undifferentiated mesenchyme cells were mostly used for this work because better preparations can be made from them than from the central nervous system and myotomes; the character of the pigment is the same in either case.

Preparations were kept under observation from one half to several hours. The longest observations were not continuous, but one field was observed at frequent intervals. The number of preparations were thus limited in the brief working time in the Spring. Of the number studied intensively, approximately thirty preparations were stained with neutral red, twenty, with Nile blue, twenty with Janus green, five with methylene blue, and ten unstained. Many more were stained to verify the staining reactions.

1. Unstained Preparations.

Preparations of undifferentiated entodermal cells are better adapted for study of relations of pigment and yolk. The yolk globules vary greatly in size from twenty to thirty micra in diameter to fine globules almost as small as pigment granules. The large globules are motionless. Very frequently the smaller globules exhibit movement which is probably Brownian movement. The movement is more evident in some fields than others. It is not appreciably increased by the use of a warming stage.

Pigment granules occur free in the preparations apart from yolk globules or appear on the surface of others. Few granules occur on large yolk globules. Medium sized globules frequently have on their surfaces two or more pigment granules. Occasionally a small globule is almost covered with pigment granules. The majority of yolk globules are free of pigment granules.

Pigment granules that are on yolk globules may or may not exhibit movement. Those on the larger globules either do not exhibit motion, or are seen to be gliding over the surface. The pigment granules on medium sized globules, move rapidly over the surface. Pigment granules on some of the smallest globules appear to be more agitated. This movement appears to be increased

because the yolk globule is itself in motion. In spite of the moving off the yolk globule and of the pigment granules over its surface, it is very seldom that a pigment granule is seen to detach itself from the yolk globule. The pigment granules that are free in the preparation exhibit movement, which is considered to be Brownian movement. Pigment granules are seen to move from place to place, but this may have been due to the streaming in the preparation. The movement of free granules in certain cells, for example young epithelial cells of the skin, have been seen to move not only by Brownian movement, but also by a jerky vibratory movement which is thought to be similar to the characteristic movement as described by Smith (1920) for pigment granules in embryo chick's eye.

There is no particular change in relation of the pigment to the yolk globules from non-motile to early swimming stage.

The pigment granules that are associated with yolk are yellow-brownish to brown in color. In the older specimen, preparations were made that contained pigment granules from chromatophores of the skin. These are larger in size and much darker in color than the pigment granules under consideration. There are no pigment granules in the nuclei.

In addition to the pigment granules found in the preparations, there are occasionally fine colorless granules situated on yolk globules. They do not occur in abundance and it is rare to find many in the same field.

2. Neutral Red Preparations.

Preparations were made of fresh tissue from amblystoma which had been grown in dilute solutions of neutral red, or of dissected portions of an embryo that were stained in dilute solutions of the dye. After killing the embryo, the tissue was dissected with fine needles, by aid of a binocular dissecting microscope, and placed in stain diluted by physiological salt solution. Different tissues, such as skin and subcutaneous tissue, muscle, and nervous system and of yolk were placed in solutions of neutral red. The dilution varied from 1:5000 dilution to 1:40,000. A dilution of approximately 1:20,000 was generally used. The tissues were stained from ten minutes to four hours. Tissues were either mounted on a slide in a solution of the dye, and covered with a cover-glass or mounted in a hanging drop. The cover-glass method is preferable for thick tissues such as myotomes, as the weight of the cover-glass spreads the tissue sufficientely to permit light

to penetrate the preparation. The hanging drops are preferable for younger stages, particularly for cells of the skin and subcutaneous tissue as the normal condition of the rounded form of the cells can be maintained and observed. The tissue fragment and the hanging drop in which it was suspended were both necessarily small in order to permit the use of oil immersion lens through all the preparation. Hanging drop preparations are particularly adapted for this work as air is kept out by sealing the edges of the cover-glass with a mixture of paraffin and vaseline, which permits observations for a number of hours.

Embryos of different ages were grown in solutions of neutral red of dilutions from 1:50,000 to 1:200,000. The more dilute solutions were generally used as the embryos take up the dye very readily. Tissue preparations were made from embryos that had lived two days or longer in the neutral red. The results of the intravitam staining were the same as staining fresh preparations of dissected portions.

Observations upon these intravitam preparations of the early swimming stage show the same general arrangement of pigment bodies in the myotomes as found in serial sections, namely, throughout the middle third of the myotome. The pigment granules

in muscle cells are located about nuclei and yolk globules and in strands between these structures or free in the cytoplasm. Although the pigment granules were visible in the central nervous system, it was impossible to determine their distribution in the fresh preparations.

In the youngest specimens studied, the epithelial cells of the skin contain much yolk in form of spherules or globules. In these cells the pigment occurs most abundant about the yolk globules in the periphery. The nucleus contains no pigment.

The differential staining of the yolk globules with neutral red and of the relations of the pigment to them is most striking. In preparations containing groups of yolk globules, the majority of the yolk globules do not stain. The larger globules do not react to the dye as readily as the medium sized and smaller ones. Yolk globules that stain are colored light pink to light red. Occasionally a portion of a stained globule is stained a deeper red. This deeper stained portion often has the appearance of an excrescence or of a ridge usually cresent shaped on the globule. Gradations in staining reactions of yolk globules also occur as well as differential staining of portions of the same globules.

There are, occasionally, granules on yolk globules that react to the dye. These are stained differentially from bright red to a deep red. These granules no doubt are identical with the 'beta' bodies as described by Coghill (1915). These granules glide over the surface of yolk globules. Some of the dark stained granules have been observed to leave a globule and when free in the preparation exhibit the Brownian movement, and they cannot be distinguished from the free pigment granules. The pigment granules whether free or in a group, appear to stain a very deep red.

While the majority of the observations were performed on preparations from undifferentiated entodermal cells, these observations were corroborated with evidences found in preparations of other tissue cells. Epithelial cells from skin of young embryos of non-motile stage show the same differential staining of yolk and of neutral red granules with reference to pigment. In such a cell, the structures are more compact. A larger percentage of yolk stains. There are more smaller globules that have neutral red granules or pigment granules exhibiting a vibratory gliding motion over the surface. The nucleus does not stain, or contain pigment granules.

The minute colorless granules observed in

unstained preparations were not seen in neutral red preparations. Melanin pigment granules from chromatophores of the older stages sometimes were included in the preparations. The melanin granules from chromatophores are larger and do not stain with neutral red. They are dark brown in color. Small yolk globules slightly larger than pigment granules occur in some fields and do not react with the neutral red.

3. Janus Green Preparations.

In preparations stained by Janus green, the yolk globules do not stain readily. The small number that react to the stain are light greenish blue in color. Upon the surface of some of these yolk globules are granules few in number that are stained by the Janus green. These vary in form and location. They may appear singly or as a row or ridge of fine granules. These granules, no doubt, are the 'alpha' granules described by Coghill ('15). Granules were observed to leave the globules, but not frequently. When they do leave they can not be distinguished from similarly stained granules in the preparations which are probably mitchondria, since Janus green is a specific stain for mitochondria. Mitochondria are not present in large numbers nor were they observed

to develop into pigment granules. The pigment granules are not stained by Janus green.

In some preparations there are occasionally globules that have a pinkish tinge in whole, or in part. This probably is due to reduction of the dye as noted by Coghill ('15). Cowdry ('14) also mentions that reduction of Janus green produces such a change. The change of granules from greenish blue to the pinkish purple color has not been observed. (Coghill '15).

4. Neutral Red and Janus Green Preparations.

The use of neutral red and Janus green as a double stain is in the main in agreement with Coghill ('15). The globules stained with green are the same as he called 'alpha' globules. In a preparation of yolk in which the 'beta' globules are most numerous I saw a group of four 'alpha' globules that were pink in part. Three of them had green colored granules on the surface which were, probably, 'alpha' granules. Further observation showed that the pink portion of one of them was increased. This may have been due to turning of the globules, but may have been caused by reduction of the dye. During the period under observation, none of the granules were seen to leave globules. However, the dark red 'beta' granules observed on the red colored

'beta' globules have been seen to leave the globules, and when this occurred they could not be distinguished from the pigment granules free in the preparation.

Sometimes preparations from skin of the younger stages would show very well the action of the two dyes. In a hanging drop, epithelial cells retain a rounded or oval shape; the nucleus is excentrically placed; the yolk globules very numerous, and pigment granules scattered through the cytoplasm. The nucleus does not stain. There are more 'beta' globlues. Some of the 'beta' globules have 'beta' granules on their surface. Excrescences occur on some 'beta' globules. The dark colored 'beta' granules can not be distinguished from the pigment granules free in the cytoplasm. The pigment granules stain a deep red. They are more numerous in the periphery of the cell. Brownian movement occurs to a limited degree. 'Alpha' granules were seen but are not numerous. In these cells a larger proportion of yolk globules stain than in a preparation from the entoderm. Also, there is more pigment in proportion to the amount of yolk. There is no pigment in the nuclei. Greenish 'alpha' granules have not been seen to change into pigment granules.

5. Methylene Blue Preparations.

Methylene blue was used very few times. It did not react uniformly through the preparations. In some fields the dye would stain a few of the yolk globules. In such a group the granules on their surfaces and the pigment

granules free in the preparation appeared to stain a deep blue. These yolk globules and granules, on their surface that reacted to the dye, may have been the 'beta' globules and 'beta' granules. In fields where the stain reacted, no colorless granules were seen.

6. Nile Blue.Preparations.

Nile blue was used as a stain for the dissected fresh material from embryos of different ages, and as an intra-vitam stain. Nile blue, even when used in very dilute solutions, was apparently too toxic for normal growth. In different groups placed in the dye, from one-fourth to all of the groups died in a few hours. Portions ofentoderm muscle and skin, dissected from stained specimens, were examined. The yolk globules stain differentially, some deeply, but most of them lightly. The muscles do not react readily to the stain. Granules, assumed to be 'beta' granules, reacted to the stain. Within some groups of granules there seems to be a gradation of color from a lighter blue to an intense blue. The pigment granules free in the preparation, stained a dark, intense blue. Melanin granules from chromatophores in the skin preparations did not react to the stain, or only slightly. By changing focus some of the melanin seemed to have a greenish tinge. This may have been due to diffraction or slight adhering of the dye to the granules.

Fresh preparations from living embryos react to Nile blue very readily. Dilutions from 1:50,000 to 1:60,000 were generally used. The nuclei do not stain. There are no pigment granules in the nuclei. Nearly all the yolk globules react to the dye, although some deeper than others. Pigment granules stain a very dark blue, so dark as to appear almost black. In some preparations there were free yolk globules that stained differentially. Deep blue granules were observed to be on the surfaces of some of the yolk globules and many of them exhibit gliding vibratory movement. This was most active on the smaller globules. Only one granule has been seen to leave a globule, and it then had the appearance of a free pigment granule. From the staining of the granules and yolk it seems that Nile blue stains the 'beta' globules and 'beta' granules. No colorless granules were observed.

7. Pancreatin Digestion Experiment.

The undifferentiated entoderm was removed from several embryos by careful dissection, with particular attention not to include an undue amount of pigment from the skin. Portions of the tissue were placed in a solution of pancreatin, and digested in an incubator for a number of hours. Other portions were incubated in physiological salt solution alone. Portions of the mixtures were

removed at intervals of from six to forty-eight hours and examined, both unstained and stained, for evidences of production of granules by the action of digestive enzymes.

The appearance of the unstained digested preparations is very much the same as the unstained preparations examined immediately after removal from the body of the embryo. The yolk globules vary in size. There were some colorless granules on yolk surfaces, but there was apparently no incfease in amount. Very few pigment granules were present and they were mostly free in the preparation. There was no apparent increase in number of pigment granules. The pigment granules were most numerous in fields containing many small yolk globules.

The use of neutral red did not show an increas in amount of pigment. However, it appears that more of the yolk stains than in living preparations. The smaller globules seem to take the stain more readily than the larger ones. The excrescences and 'beta' granules occur in very much the same way in incubated undigested as in digested preparations. In both preparations there was apparent differential staining of granules on the surfaces of globules from a lighter red to the dark red of the pigment granules.

Nile blue presents a similar picture. The majority of the yolk globules stain. The smaller globules stain deeper than the larger ones. More deeply stained granules occur on surfaces of the smaller deeply stained yolk globules. There was apparently no increase in number of granules. Sharloch R reacts in a similar way. The pigment granules stain slowly to a deep red. The yolk stains differentially. The smaller globules stain more deeply than the larger ones. The granules on the surfaces stain deep red.

The pancreatin digestion experiment did not show an increase in amount of 'alpha' or 'beta' granules. It did, however, seem to show that the action of the digestive enzymes on the yolk globules prepared more of them for staining. It appears that the action of pancreatin occurs most rapidly on the smallest globules.

8. Comparative Summary on the Origin of Pigment.

The various stains show similarity of reaction on the structures of the embryonic cells of the different tissues. The nuclei in the living tissue do not stain. The yolk globules in the various preparations show a differential staining with neutral red, Nile blue, methylene blue and Janus green. The first three react with many of the smaller yolk globules, and with progressively fewer with the increased size of the yolk globules. Certain granules

on surfaces of stained yolk globules react differentially to neutral red and Nile blue. Such granules when they detach themselves from the globules can not be distinguished from the pigment granules free in the preparation. The colorless granules observed in unstained preparations were not present in the stained preparations.

Janus green stains a comparatively smaller number of yolk globules and has fewer granules on their surfaces. These granules were not observed in the other preparations.

Pigment granules from chromatophores stain slightly with neutral red and Nile blue. These granules were larger and darker in color than the pigment under consideration.

The differential staining of yolk globules occurs in all tissues but a larger proportion of yolk globules stain in the epithelial cells of the skin than in theentodermal cells. In such cells the pigment occurs in the cytoplasm about yolk globules or free in the cytoplasm. There is no pigment in the nuclei.

9. Conclusions.

The work on the origin of the pigment shows the following: (1) presence of colorless granules in unstained preparations, (2) differential staining of yolk globules and of granules on their surfaces, (3) the pigment granules are formed in the cytoplasm, (4) the pigment does not come from

the nucleus, (5) the differential staining of granules on stained yolk globules indicates that the pigment may be formed from substances in the cytoplasm, part of which are liberated during the process of digestion of the yolk.

D. NATURE OF THE PIGMENT.

The pigment in the differentiating tissues of the embryos studied is not in sufficient quantity to permit separation from the tissues. For this reason a chemical analysis has not been made. Microchemical tests for the study of pigment in tissues are slowly being standardized. Many experiments were made to determine if possible the nature of the pigment.

1. Staining Reactions in Fresh Preparations.

Various stains have been used on fresh tissue to observe the reaction of the dyes on the pigment. Neutral red, methylene blue, Nile blue, Janus green and the fat stains, Sudan III and Sharlach R, were used.

The pigment unstained is yellowish brown to brown in color. The pigment granules stain with neutral red, Nile blue and methylene blue. The granules, after addition of the dye are darker in color, as a result, apparently, of actual staining rather than of merely a physical union of the dye to the pigment.

In preparations from the older specimens the pigment from the chromatophores were frequently included. This pigment is easily distinguished from the pigment in the differentiating tissues by difference in size and color of the granules. The pigment in the chromatophores stain very slightly with neutral red and Nile blue and not at all with Janus green. This pigment is melanin elaborated by specialized cells. Fat stains do not stain this pigment.

The fat stains, Sudan III and Sharlach R, were used primarily in an attempt to determine the relations of the pigments to fat. The results were the same in both cases, although Sharlach R was commonly used. Preparations were stained similarily as with the other stains. Yolk globules stain differentially; some a very light red; others a deep red. Portions of some globules stained deeper than the remainder of the globule. The majority of pigment granules occur about the deeper stained yolk globules. The granules on the surface of the deeper stained globules seem to stain with approximately the same intensity as the globules. The pigment granules free in the preparation appear either to have actually stained or the stain has saturated the surrounding tissue and thus causes the granules to appear darker. Biondi's stain containing osmic acid was used, with the result that the pigment granules are blackened by the osmic acid and retain the color through paraffin imbedding.

2. Staining Reactions in Sectioned Material

The observations on the distribution of the pigment have been on sectioned material stained with various histological stains. Such stains as eosin, erythrosin, orange G., and Lyon's blue do not react with the pigment. Other stains appear to act very weakly at times. This was observed with methylene blue, neutral red and Nile blue. Such reactions are very slight. They appear to be a physical combination only. The pigment does not stain with alum-carmine and Janus green. The pigment seems to be effected by imbedding in paraffin after alcohol-xylol treatment.

3. Solubilities.

The routine procedure has been to fix tissue in ordinary fixing solutions, carry it up through graded alcohols and clear in cedar oil or xylol and imbed in paraffin. The pigment does not disappear from the tissue. More powerful fat solvents were used than the alcohol, such as chloroform, acetone, ether and xylol. After prolonged treatment of twenty-four hours or longer the pigment did not disappear, or only very slightly. Dolley ('21) states that lipochrome or fat-holding pigment will disappear from tissue after treatment of one-half hour in absolute.

There was no appreciable disappearance of pigment after such treatment, even if tissues were kept in absolute alcohol from two to four hours.

4. Various Other Tests.

Hydrogen-peroxide and ferric chloride oxidized the pigment and changed it to a colorless state after a few hours. The pigment of the chromatophores also is bleached by the oxidizing agents.

The pigment gives a negative iron reaction th ferrocyanid of potassium followed by acid alcohol. This is true also of the melanin of the chromatophores. The pigment is insoluble in dilute acids, alkalies and ammonia water.

5. Summary and Conclusions.

Since the pigment reacts with fat stains, it seems to be necessary to class it with fat-holding pigments. The fat solvents, however, which dissolve fat-holding pigments, called lipochromes,do not remove this pigment appreciably, so that in tissues prepared with histological methods it presents characteristics of melanin.

E. DISAPPEARANCE OF PIGMENT.

The study of relation of pigment to differentiation has been confined to the early swimming and younger stages, because during this period blood vessels have not entered the myotomes and central nervous system. Soon after the

embryo swims the blood vessels are in close proximity to the brain and rapidly come in contact with it and later with the spinal cord. Before the feeding stage blood vessels can been seen between the myotomes. This added factor of blood vessels connected with the tissues to serve in nutrition and excretion necessitated a study of its effect on the amount of pigment.

In order to determine whether this pigment is distinctly of embryonic nature, tissues from several adult Amblystoma have been examined. It was found that the pigment had entirely disappeared from skeletal muscle. The same is true also of adult muscle of Rana pipiens, Necturus and Bufo. Serial sections have been studied of several embryos older than the early swimming stage to observe the time and method of the disappearance of the pigment.

In an embryo approximately two days older than the early swimming stage the pigment has the same general distribution as described for the early swimming stage. An exact count of the number of pigment bodies in muscle at this time has not been made. There is no apparent difference from the younger stage. Four embryos were observed that had just attained the feeding stage. At this time blood vessels can be seen between the anterior myotomes. In these myotomes the relations of the pigment in the cells have changed. Many of the pigment bodies are irregular in shape and appear to be breaking up. The pigment bodies are not confined to

the middle third of the myotome longitudinally. A few are now situated in the end region between the nuclei and the end of the myotome. Very frequently these bodies were situated close to the blood vessel. In a few instances pigment bodies were seen in a blood vessel. Although there appears to be a migration of pigment bodies or fragments of pigment bodies toward the blood vessels this occurs comparatively slowly.

In embryos two, seven and fifteen days older than the feeding stage there is progressively less pigment in the myotomes. The pigment begins to disappear first in the anterior myotomes and its disappearance proceeds caudad. In serial sections of portions of myotomes in amblystoma larva 38mm. in length, a few pigment bodies occur near the ends of some nuclei. Pigment granules are occasionally seen near the pigment bodies. The pigment is not abundant. Its complete disappearance occurs between this age, which is approximately six weeks after feeding, and metamorphosis. The pigment seems to be removed from the myotomes through the blood. This phase of the problem demands further study.

IV. SUMMARY OF CONTRIBUTIONS OF OTHER INVESTIGATORS

A strict chemical classification of animal pigments seems to be impossible because of the lack of knowledge of the exact chemical nature of the pigments. For purposes of this Summary, the following classification is adopted: (1) melanins, (2) lipochromes, (3) waste pigments, (4) blood pigments, (5) bile pigments.

A. MELANIN.

Melanin is an amorphous dark brown substance. It is characterized by insolubility in acids, fat solvents or dilute alkalies, bleaching by strong sunlight or H_2O_2 and non staining by fat stains. It is iron free. Some melanins are sulphur free. In others the sulphur content is as high as ten per cent in melano-sarcomas. Analyses of melanins show that the value of carbon varies from 48.95 to 60.02 per cent: for hydrogen from 3.05 to 7.57 per cent, and for nitrogen from 8.1 to 13.77 per cent. Melanins when decomposed by caustic potash yield skatol, indol and pyrrol derivatives. These probably are derived from the tyrosin and tryptophane in the protein molecule. Artificial melanin or melanoids can be produced by heating proteins with strong hydrochloric acid (Chittenden & Albro, '99).

Melanin is universally regarded as endogenous. There are three theories concerning the origin, namely, (a) from the nucleus, (b) from mitochondria, (c) from the cytoplasm.

A. Von Szily ('11) studied the production of melanin in the eye of different vertebrates and in melanotic tumors of the human eye. He describes the pigment granules as arising from colorless rod-like granules, derived from the chromatic material, which after being extruded from the nucleus wander through the cytoplasm and assume color, probably due to the action of cell ferments. The extruded granules are called "Pigmentträger". The extrusion of the "Pigmen tträger" and transformation into pigment can be followed through the different stages. The color appears first at one end of the granule and proceeds to the other. The nuclei from which "Pigmentträger" came may be productive or degenenerative. If productive there is no noticeable degeneration of the nucleus, but of degenerative the nucleus shows shrinkage.

Schultz (12) studied the formation of pigment by dermal chromatophores in a case of mycosis fungoides. He describes the differentiation of the nuclei, increase of chromatin content and extrusion of chromatin material into the cytoplasm. Pigment granules are formed from the chromatin material by specialized physiological activity either chemical or catalytic. The pigment thus derived from nuclear material is not a derivative of hemoglobin or produced by ordinary degeneration. After the active stage of production of chromatin material a portion of which is then thrown out into the cytoplasm, the cell goes into a resting stage.

Dyson (11) in a study of pigment formation in the skin believes that pigment is produced by the nucleus. Nuclear stain obscures the presence of pigment either in the nucleus or when it is deposited at the outer zone of the nucleus. The nucleus according to the author is composed of two classes of complex substances, fatty and proteid, which, owing to their complex nature and to the physiological activities occurring constantly in the nucleus, are continually changing their chemical relations. Thus during the metabolic processes of the cell the nucleus excretes certain substances containing both a fatty and proteid part. These substances are found in different degrees of transformation into pigment. This transformation was shown by different staining reactions and solubilities in alcohol, chloroform and hot and cold acetone. The melanin so formed is derived from the chromatic proteid portion of nuclear material which has been separated from complex lipoid granules. Such melanin has its origin from a lipochrome.

Furthermore Dyson holds that pigmentation may be increased in two ways (1) by stimulation of the epithelial cells by various agents such as, light, heat, irritants, etc. and such an increase is temporary, (2) by deficient drainage of tissue by obstruction of the lymphatics of the skin. In such cases a normal amount of pigment is formed but excess is not carried away. He thinks also that lack of nutrition caused by such obstruction might reduce vitality, and cause increase of pigment.

Wagner (10) studied the formation of the pigment that that first appears in the eggs of Rena temporaria and Rana esculenta. He describes the pigment to appear first next to the nucleus. The nucleus has several projections in which there are nucleoli. The pigment forms on the side of the nucleus next to the mass of chromatin material of the nucleoli and is pressed in columns between the protuberances. The pigment is within the cytoplasm through nuclear activity.

Champy('11) describes the formation of pigment from thread like mitochondria. The mitochondria break up into colorless fusiform granules, Luna ('13) also held to the theory of pigment formation from mitochondria. He did not observe directly the change from mitochondria into pigment in the cultures, although he still considered such a change possible in the living embryo.

Strong ('02) studied the formation of pigment infeathers ~~from remiges of~~ Starna hirundo, Linn, and advanced the theory that pigment is formed in the cytoplasm of pigment cells with the rods radially arranged about the nucleus. The nuclei do not contain pigment granules. Concerning the origin of the pigment he states, "The pigment arises in form of grayish or light yellowish corpuscles, of exceedingly small size, arranged along delicate protoplasmic strands, which radiate from the nucleus and sometimes anastomose more or less with one another."

Hooker('15) worked on plasma cultures of mesenchyme and epithelium from Rana pipiens embryos and came to the conclusion that the pigment is formed in the cytoplasm. The cells were at first clear and contained no pigment.

The first appearance of pigment was in the form of small brownish granules located immediately adjacent to the nucleus. As the number of granules increased they became scattered throughout the cytoplasm of the cell. Pigment did not appear inside of the nucleus. No colorless anlagen were observed and there was no extrusion of chromatin material from the nucleus. Briefly, he concludes that the pigment is formed in the cytoplasm, through the action of cell ferments upon substances in solution, withoutdegeneration of the nucleus or formation of colorless anlagen.

Smith ('20) studied the origin and development of melanin pigment in vivo and in vitro in the pigmented epithelium of the eye of the chick embryo. Mitochondria stained with Janus green, but there was no change of mitochondria into pigment. There was no extrusion of chromatin material from the nucleus. During the active production of pigment, there appeared on the nucleus small colorless granules. He considers that there may be two stages in the formation of the pigment, as follows: "(a) the formation of a colorlesschromatin, which is shown by the presence of colorless granules in the younger stages of development; (b) the production of color inthis chromogen, probably by action of cell ferments". The two processes may go on simultaneously, according to Smith, but the chromogen may be laid down more rapidly than is used in the production of color, and this excess of chromogen is formed into the colorless granules. He considers the colorless granules to be pigment, since they have the character-

istic size, shape and staining reactions of pigment granules. There were numerous fat droplets in his cultures but no relation was noted between them and the pigment granules. Smith holds to the theory of concentration of the pigment granules about the centriole. Between the centriole and periphery of the cell the pigment granules move in radiating paths.

In the last twenty years much of the work on origin of pigment has been performed in order to determine physicochemical reactions involved in its formation, and classification by microchemical tests. There have been many differences of opinions in the interpretaions of the chemical reactions.

Formerly melanin was considered to be a derivative of blood pigments as a product of disintegration. Ehrmann ('92) states as a result of his studies on pigment formation in amphibian eggs, while yet in the ovary, and in young embryos that he believes the pigment is deposited from pigment in the blood. He opposes the theory of formation of pigment by metabolic activity of the cells in which it occurs.

Since the time of Ehrman's work Bertrand and a host of other workers following him have demonstrated the presence of different substances in tissues that produce pigment by action of enzymes. It is now the generally accepted theory that melanin is formed in the cell by the action of an oxidase on an oxidizable chromogen.

Bertrand ('96) isolated an oxidase in plants (Russula and Dahlia) capable of producing a black pigment when it oxidized tyrosin. He called this oxidase tyrosinase.

Von Furth and Schneider ('01) found tyrosinase in haemolymph of Lepidopteran larva. Durham ('04) derived it from extracts of skins of fetal rabbits and guinea pigs. Gessard found free tyrosin in the ink sac of the cuttlefish and in melanotic tumors of the horse. These investigators and others have shown that tyrosin and tyrosinase are found in many different animals and in those parts of the body in which pigment occurs.

Bertrand ('08) by further investigation demonstrated the manner in which tyrosinase acts on tyrosin and other amimo-adids which are derivatives of tyrosin to form melamin. Thus he showed thatother compounds than tyrosin are capable of producing pigment. Thecolor varies with the different acids. Tyrosin becomes first grenadine-red then inky black, while p-hydroxy-phenyl-acetic acid becomes yellow then brown.

Block and Ryhiner ('17) found that certain cells of the skin produce an enzyme capable of oxidizing 3-4 dioxyphenylalanin to melanin. He calls this the 'dopa' reaction. The 3-4 dioxy-phenylalanin, which is a precursor of epinephrine, is a compound of orthodioxy-benzene (pyrocatechin) as a necleus with a-amin-propionic acid as a side chain.

Meirowsky according to Kissemeyer ('20) observes that the pigment is atochthonous in the epidermis and cutis. The cells produce an enzyme capable of oxidizing epinephrine to a pigment.

Kissmeyer ('20) obtained the 'dopa' reaction in cases of vitiligo in achromic patches. Whitfield ('20) reviewed the 'dopa' reaction and observes that hyperpigmentaion is due to excess of mother substance of normal pigment as a result of diseased suprarenal.

The present conception of formation of melanin pigment by many workers is that the mother substance ofpigment is produced by the chromaffin system and oxidized by cell ferments. Whitfield states, "Pigment and adrenalin are end products or intermediary products of the same original material, steps in the cycle of pyrocatechin metabolism."

Much work has been done by physiological chemists relative to the amino acids in blood and tissues. Folin ('14) has demonstrated that amino acids derived from protein digestion are absorbed unchanged by the blood stream and distributed unchanged to the tissues. Van Slyke and Meyer ('13) have also shown that "the disappearance of intravenously injected amino-acids from the circulation is the result of neither their destruction, synthesis nor chemical incorporation into cell proteins. The acids are merely absorbed from theblood by the tissues, without undergoing any immediate change." They have also shown that the amino-acids are absorbed by the tissues up to a certain point of concentration called the nutritive point of equilibrium. Osborne and Mendel ('12) found that nutri-

tive equilibrium is impossible if certain amino acids are lacking. Among these McLeod states that tryptophane is necessary for maintenance of body weight. They included tyrosin and tryptophane in this grouping. Thus different investigators have shown that amino acids, from which pigments may be formed or which are necessary for the formation of certain hormones, occur in sufficient quantities in the tissues to account for the formation of pigment. Other investigators have found that oxidases necessary for the formation of pigment occur also in different parts of the body.

Much has been done to demonstrate the formation of the oxidases and place of oxidation. Jaquet ('92) discovered that the oxidative property of the tissues is not dependent upon the life of the tissues nor the anatomical structure, but upon the presence of a soluble oxidase which could be extracted from both fresh and alcoholic tissue. J.Loeb ('99) held to the theory that the chief seat of oxidation in normal development and regeneration is the nucleus.

Lillie('02) clearly demonstrated that the nucleus is responsible for forming the oxidase of the oxidative processes in both fresh and alcoholic tissues. He concludes that his results "furnish ----------,conclusive evidence that in many tissues the nucleus is the chief agency in intra-cellular activation of oxygen,

and, further that the active or atomic oxygen is in general most abundantly freed at the surface of contact between nucleus and cytoplasm. "

B. LIPOCHROMES

Lipochromes are pigments found normally in the plant and animal tissues. They are the fat holding pigments and are characterized by staining readily with fat stains and by dissolving in fat solvents. "Melanins and pigments derived from hemoglobin do not stain with Sudan 111 and are not soluble in ether, etc., and hence can be readily distinguished from the fatty pigments ",(Von Furth, see Wells '20). Dolley ('21) has pointed out that this is some times difficult to do as some tissues contain both pigments, and the melanin may be overlooked as the fat stains invariably tinge the melanin and a small amount of lipochrome is difficult to distinguish.

Hueck ('12) observed that Nile blue is a reliable differential stain as it stains lipochrome a deep blue and melanin a green. He considers that Nile blue probably is an actual stain for lipochrome but probably combines physically with the melanin. Oxidation tests are not reliable as oxidizing agents such as hydrogen peroxide and ferric chloride as both bleach pigments

although the melanin, more slowly. Dolley ('21) states that solubility test in absolute alcohol is reliable for differentiation. Lipochrome disappears in about one half hour, while melanin is in soluble.

There have been many diverse opinions relative to the origin of the fat-holding pigments and as to whether the presence of the pigment is normal or abnormal. Rosin ('96), according to Dolley, described two kinds of pigment in the substantia nigra, one, a dark brown, and the other, a clear yellow. The latter he regards a lipochrome as it stains with fat stains. Lubarsch('02) and his pupil Sehrt ('04) worked on human material and found lipochrome in such tissue as liver cells, epithelium of the kidney,testes, seminal vesicles, in ganlion cells, in brown atrophy of the heart and in corpusluteum. Lubarsch advanced the theory that the lipochrome is a by product of metabolism. He called it"Abnutzungspigment" or "wear and tear" pigment. Sehrt ('04) observed that pigment in the tissues nearly always gave strong reactions to fat stains in fresh preparations and only weakly or not at all after treating with alcohol and imbedding in paraffin. He does not recognize the possibility of two kinds of pigment being present. He states that the pigment is bound more or less fast, mechanically or chemically, with fat and that a strong or weak combination produces a strong or weak reaction with fat stains.

Another theory was advanced that brown waste pigments are produced by metabolism of lipoids or fatty acids. Borst called these pigments lipofuscin. Hueck ('12) also calls the pigment lipofuscin and considers it to be a normal, physiological "wear and tear" product. He observes both lipofuscin and melanin in the same tissue and that it is difficult to distinguish between the two and that the two may be together in the same granule. Dolley concludes that the lipofuscin of Hueck's and the lipochrome of Lubarsh are one and the same.

Microchemistry at best has not been sufficient to determine the nature of the lipochromes and their relation to other pigments. Recently the biochemical and physiological studies of Palmer and Eckles (ϕ14) and Palmer ('19) have demonstrated that the plant pigments, carotin and xanthophyll, are widely distributed in animals and are intimately connected with animal metabolism. The carotinoids (carotin and xanthophyll) were found to be identical with the pigments in blood serum, skin, corpus luteum, milk and in the body fat of the cow, horse and hen. There is a remarkable species difference. Those that have colored fat such as cow, horse and hen carry the pigments in the blood serum while species with colorless fat such as sheep and goats do not have pigments in the blood stream. Palmar proved that chickens deprived of these pigments from time of hatching have no yellow pigment in their skin, egg yolk, fat and blood serum. Color is produced after feeding foods

containing carotinoids but it disappears if deprived of the pigments. This work by Palmar makes it improbable that any other fat-holding pigments than the carotinoids exist inman. Whipple ('21) states that "lipochrome is a peculiar pigment which is thought to have merely a passive function in the body with no relationship to the urobilin, urochrome or other pigments no relationship to the urbbilin, urochrome or other pigmentscontaining the pyrrol nucleus. At present we have no reason to suppose that the lipochromes have any direct relationship to the other body pigments."

C. WASTE PIGMENTS

Pigments found in various tissues of the body that ordinarily do not have pigment have often been noted by various worker. As the knowledge of pigment has increased and microchemistry developed the classification of these pigments has changed. Lubarsch ('02), Sehrt ('04), and others called the pigment, Abnutzung or 'wear and tear' pigment and classified it as lipochrome. A review of their work shows that there were really two kinds present, one a lipochrome which is now believed to be exogenous, and the other a waste pigment which is now classified as a malanin, by Dolley ('21) and Schmidtmonn ('20). Other workers named this pigment differently. Borst and Hueck noted the "wear and tear" pigments in the various tissues and called it lipofuscin. Lipofuscin has been shown by Dolley to be the same as lipochrome.

Hemofuscin, as has already been cited, has been considered a waste product. It gives the reactions of melanin. Dolley ('21) classified it as a melanin. Straeter ('14) considers the pigmentation of involuntary muscles to be a waste product.

Imhofer ('13) says that pigment in brown atrophy of the heart is a waste product.

Brahn and Schmidtmann ('20) in their recent work "Zur Kenntnis des Melanins und des braunen Abuntzungspigments" sought by microchemical methods to determine the nature of the 'wear and tear' pigments. They came to the conclusion that the fat staining of pigment granules is a mixture of a more or less accidental nature and that the pigment cannot be considered a fatty acid. Schmidtmann considers that the pigment in brown atrophy of the heart is a waste product and that it is melanin, as shown by its physical and chemical reactions. The exact method of formation was not given.

Dolley ('21) finds that the pigmentation of brown atrophy of the heart consists of not only the lipochrome usually ascribed to it but also a melanin which is a waste product. He believes that the lipochrome is derived from plant carotinoids and carried to the heart by the blood, while the melanin is "an endogenous pigment derived from disturbed metabolic processes".

Only one author has to the writer's knowledge described granular pigmentation in skeletal muscle. Imhofer ('13)

working on muscles of human vocal cord finds the pigment occurring morphologically in two forms, namely, in large bodies with wide variation is size and in small dot-like granules. Pigment occurs generally at one pole of a nucleus. If two nuclei are close together the pigment forms bridges or strings between them. In fresh preparations the pigment reacted postively with Sudan, Sharlach R. and osmic acid. The pigment reacts with neutral red and becomes a deep red. Fat solvents such as alcohol, ether, benzene and acetone dissolve part of the pigment. More of the pigment is dissolved by the solvents in the younger individuals than in the older. Solubility, therefor, decreases with age. Imhofer concludes that the pigment has two components, a lipoid and a pure pigment component. The lipoid component is a Sudanofil and stains with Sudan while the pure pigment component does not react positively with Sudan. These two components cannot be separated completely, although their staining reactions differ with Sudan III. The pigment is negative to iron reaction. Paraffin imbedding removes part of the pigment.

In the first few years of a child's life he found the muscles of the larynx to be free of pigment, which later is always present and the amount increases with age. Imhofer does not consider the pigment in the vocal cords to be a pathological product, but he believes it to be a result of a physiolgical action incident to the wasting of the muscles of

the vocal cords.

A review of Imhofer's work shows that he is in reality dealing with two pigments, one a lipochrome and the other a melanin. This is apparent from the solubilties and staining reactions.

Dolley and Guthrie (18') studied the pigmentation of nerve cells. In their review of the literature they cite that many workers such as Lubarsch, Sehrt, Obersteiner, Calligaris, Hueck, and Marinesco note the presence of two pigments, one a fat-holding and the other negative to the fat stains, while Mühlmann opposes the duality of the pigment. The etiology of these pigments presents many different opinions. The majority believe the pigment is a normal 'wear and tear' product, while others consider it to be a senile or pathological product. The whole situation is very confusing.

Dolley and Guthrie in a series of experiments on rabbits and dogs produced depression and found that pigment is formed in the Gasserian, spinal, vagus, and sympathetic ganglia. According to Dolley('21) "by depression is meant simply any interference or blocking of function by stimulation from without or by the deficiency of conditions essential to life, such as food or oxygen."..The depressants used were morphine and heat. The pigment found was melanin, as shown by microchemical methods and it presists after paraffin imbedding following alcohol-xylol treatment. They state

"it is our more fortunate lot to be able to place the melanotic pigment as an end result of an orderly process and to make the process universally account for the pigment whether it occors in youth or age or disease. The pigment thus has its place in a logical generalization which includes repose, the functional cycle and the pathology of the nerve cell."

D. BLOOD PIGMENTS.

Blood pigments are derived directly or indirectly from the hemoglobin of the blood such a hematin, hematoidin, hemosiderin. Hemoglobin, the normal coloring matter of blood, is a compound protein, having a large, complex molecule, which consist of a protein group (globin) and a coloring matter (hematin). It is elaborated by the erythroblasts and remains in the red blood corpuscles during their life. Hemoglobin contains iron which is not detected by iron reactions owing to the close chemical union of the iron to the remainder of the molecule.

Addis('14) has advanced a theory concerning the normal metabolism of hemoglobin in the body. Splenic phagocytes acting on red blood corpuscles free the hemoglobin which is carried by other phagocytes to the Kupffer cells of the liver which pass it on to the liver cells. The pigment matter, hematin, is there separated from the globin and converted by removal of its iron

into the bile pigment, bilirubin. The bilirubin is excreted into the intestine, reduced to urobilinogen, a part of which is reabsorbed and polymerized into urobilin which is then polymerize into a larger complex. In the liver this complex has the original side chains restored to the pyrrol nuclei which is then used to from new hemoglobin. This Addis presents as only a "working hypothesis" that can be used in the study of pigment metabolism, a problem that is as yet far from being understood. Recently Whipple ('21) has suggested another hypothesis concerning pigment metabolism which will be considered later.

Pathologically hematin is found in extravasations as an amorphous dark brown blackish substance. It is seldom found in small hemorrhages. Some workers believe that hematin by chemical changes gives rise to two pigments, hematoidin which is iron free and hemosiderin which gives the iron reaction. Brown ('11) does not consider hematin to be an intermediary product in hemoglobin disintegration as it persists a long time in the malarial spleen. When once formed it disentegrates very slowly. He believes hematin to be an intermediary product in formation of bile pigments from hemoglobin.

The question of metabolism of blood pigments is unsettled. Hematoidin, whether it arises by chemical change directly from hemoglobin or from hematin, is found

in extravasations as orange colored or red rhombic plates or ~~as~~ in yellowish granular masses. It is isomeric with bilirubin. Hematoidin is formed in places where blood is hemolized and only the edges of such places are in contact with living cells. According to Schmidt,(Wells '20) Nencki and Sieber give the following equation for the formation of hematoidin:

$$\underset{\text{(hematin)}}{C_{32}H_{32}N_4O_4Fe} \;\&\; 2H_2O = 2\underset{\text{(hematoidin)}}{C_{16}H_{18}N_2O_3} \;\&\; Fe$$

Hemosiderin gives the iron reaction, is relatively insoluble and is formed after laking of blood. When deposited in such tissues as liver, spleen, heart or pancreas, it is distinguished from lipochrome by solubilities and iron reaction. It usually occurs as yellowish brown, to brown granules. Although the exact fate of hemosiderin is not known it is supposed to be utilized in the manufacture of new hemoglobin.

Hemofuscin is a brownish black pigment found in involuntary muscle fibers. The name hemofuscin according to Schmidt (Wells '20) was given by Von Recklinghausen as he considered it a derivative of hemoglobin. The iron reaction is negative and it is insoluble in fat solvents. Some have considered it a lipochrome, but this conclusion is questionable because of the insolubility of the substance in alcohol, ether and chloroform. Others have considered hemofuscin to be melanin. According to Von Furth (Wells), Taranoukhine believes the pigment in brown atrophy of the

heart is neither a derivative of melanin nor a lipochrome, but a waste product. Straeter ('14(confirms the view that it is a waste product in involuntary muscles and cannot be considered a lipochrome. Dolley ('21) classifies hemofuscin as a melanin.

E. BILE PIGMENTS.

Bile pigments are generally considered to be derivatives of hemoglobin formed principally by the liver cells. Bilirubin, an isomer of hematoidin, is the chief one of the bile pigments. Bilirubin occurs usually in solution and only occasionally may be found in granular form. From it biliverdin is formed by oxidation. By further oxidation bilicyanin is formed from biliverdin. In the intestines bilirubin and biliverdin are reduced to urobilin or stercobilin, part of which is absorbed into the blood and excreted in the urine. Part of it is excreted as a chromogen called urobilinogen.

Whipple and Hooper ('16) and more especially Whipple ('21) have worked extensively on the subject of production of bile pigment and its relation to destruction of hemoglobin. In a recent article Whipple ('21) has reviewed the experimental evidence showing the relations of the various factors of pigment metabolism in the body. The usual conception of metabolism of hemoglobin as given in textbooks and by Wilbur, Addis and others is not according to Whipple,

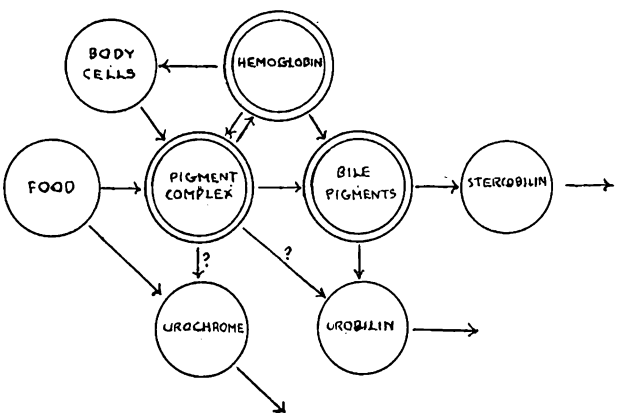
substantiated by experimental evidence. It has been generally believed that bile pigments are formed from destruction of blood cells, that they are excreted into the intestine, that stercobilin is reabsorber and that part of this is used in the production of new hemoglobin, and part, excreted by the kidneys as urobilinogen and urobilin. Whipple states that there is no evidence to prove that stercobilin is absorbed, and considers as strong argument against this view the fact that bile fistula dogs observed for over two years show no anemia or lack of pigment and no reaction to feeding of bile pigments. Whipple and Hooper showed evidence that production of bile pigment is not directly related to hemoglobin destruction. Further, with injections of hemoglobin into bile fistula dogs the pigment output does not parallel the amount of hemoglobin injected.

Whipple has shown that the food factors are important in bile pigment production. Feeding bile fistula animals fresh bile pigments or fresh or cooked blood or digested portions of blood does not increase the output of bile pigments. That change of diet changes bile pigment elimination is shown by the increase in bile pigment after a sudden change to a diet of carbohydrateexcess. A sudden change from carbohydrate excess to meat diet decreases the bile pigment output. Hemoglobin regeneration following anemia is increased by various food stuffs. Red meat, cooked liver, hemoglobin and butter fat give an increase of hemoglobin. Body protein is also an important factor in production of hemoglobin and bile pigments. This was shown

by starvation experiments. Bile pigment excretion continues during fasting periods. Further, hemoglobin will be formed during these periods and actually increases in amount. This shows that disintegration products of body cells may be used in formation of new hemoglobin.

Whipple asserts that bile pigment normally is a product of functional activity of liver cells. In addition, Whipple and Hooper ('16) showed that bile pigments can be formed by other cells than liver cells. This was demonstrated by the appearance of bile pigments in the blood stream of the head and neck in two hours, after exclusion of the liver from the circulation. Also they showed that hemoglobin can be transformed into bile pigment in twelve hours in the serous cavities.

Whipple, after a review of these various facts, has evolved a new working hypothesis as an explanation of the metabolism of pigment. This is shown in the following diagram:



The essential feature of this hypothesis is the "pigment complex." He described the "pigment complex" as a

"group of substances which are essential parts of the mature body pigments." Whipple believes that experimental evidences show that the "pigment complex" is formed from substances obtained from (a) food, (b) body cells and (c) hemoglobin. Formed from the "pigment complex" are hemoglobin, bile pigments and, possibly, urobilin and urochrome. Whipple holds that the pyrrol group seems to be the essential factor in chemical composition of the substances in the "pigment complex."

F. SUMMARY OF THE LITERATURE.

Altho the review of the literature has been brief, the writer has endeavored to give a comprehensive view of the development of the conception of pigment metabolism.

The pigments that are involved in body metabolism appear to be resolved into two main classes: exogenous and endogenous. Lipochromes are entirely exogenous, as they are carried to the tissues by the blood plasma. their presence can be demonstrated by staining reactions with fat stains and by solubilities in fat solvents.

Pigments of endogenous origin are melanin, waste pigments, blood pigments and bile pigments.

Melanin, as a class name, includes a large number of closely related pigments. While it is generally accepted that melanin is produced by the action of cell ferments

upon an oxidizable chromogen there is much work to do to complete the knowledge of the substances that enter into the formation of melanin and the chemical reactions involved.

Waste pigments have received various names and have been considered both pathological and physiological products. Recent workers classify the waste pigments as melanin since they give the characteristic reactions. This opposes the theory that melanin is produced by specialized cells since waste pigments are formed in many different tissues.

Blood pigments and bile pigments are so closely related that many text books classify them in one group as hemoglobinogenous pigments. Whipple's work suggests that while the pigments are closely related and one group (blood pigments) may, in body metabolism, disintegrate and aid in the formation of the other (bile pigments), in reality both groups are elaborated from a common group of pigment forming substances. Diet factors influence pigment metabolism.

V. D I S C U S S I O N

In this discussion of the writer's results in the light of the work of other investigators, the pigment, with which this paper is concerned, will be considered with reference to (1) its origin (2) its nature, and (3) its significance.

A. ORIGIN OF THE PIGMENT.

The pigment described in differentiating tissues of Amblystoma occurs in cells that are particularily adapted for study of pigment formation. Not only is this true for study of the origin of pigment, but also, for study of intracellular metabolism, since each cell from the beginning has food, in form of yolk, sufficient for growth, differentiation and embryonic function. During the period under consideration there are no blood vessels in the tissues concerned to serve in nutrition or excretion. During this period of development, before the blood vessels enter the tissues, the cells of the tissues must produce all necessary enzymes for absorption of the yolk and for the metabolism of differentiation and proliferation. Some enzymes or hormones must prepare the yolk for assimilation similarly to the process of digestion; other enzymes must aid in the physico-chemical reactions of metabolism.

Since these cells are thus adapted for study of intracellular digestion, assimulation and metabolism, they have been particularly examined for evidences concerning the

place and mode of formation of the pigment that occurs within them.

The writer finds no evidence that the pigment is produced by the functional activity of the nucleus. No pigment has been observed either in the nucleus or imbedded in the nuclear membrane. Observations in the younger cells, especially in the muscle sections, show that pigment granules occur on the surface of the nuclei, but there is no evidence that ~~it~~ they came from nuclear material. No extrusion of granules from the nucleus, pigmented or colorless, have been observed. In no phase of the work have nuclei in pigmented cells, appeared shrunken or degenerative.

According to Child ('15) " undifferentiated protoplasm is protoplasm reduced morphologically to its lowest terms". In the embryonic cells under consideration that are morphologically simple the observations have been primarily made. The most conspicious structures in the cytoplasm are the yolk globules, which are so numerous that the hinder the observations of the relatively permanent structures that are formed in the cells. Observations have been made on both unstained and stained preparations to observe the possible presence of chromogen and it's transformation into pigment. The two possible sources for the chromogen are : (1) mitochondria, and (2) substances in the cytoplasm.

Mitochondria are not present in large numbers.

This was noted by Coghill ('15) and verified in the present series of experiments by the use of Janus green. This dye, which is a specific stain for mitochondria, stained certain granules on the surfaces of yolk globules, as was noted in the observations. These granules may develop into mitochondria, but this has not been verified. At no time has the staining reactions of these granules and of mitochondria suggested that mitochondria develop into pigment.

The chromogen of the pigment seems to arise in the cytoplasm as colorless granules on surfaces of yolk globules. These colorless granules are observed in the unstained preparations but are not observed in preparations stained by various dyes. The use of neutral red, especially, shows a differential staining of granules on yolk globules from a light to dark red. The darker granules when they detach themselves from the yolk cannot be distinguished from pigment granules free in the preparation. The size, position and staining reactions of the granules on the surface indicate that the colorless granules are the chromogen of the pigment granules and that they arise on the surfaces of yolk globules. This does not exclude the possibility of the formation of the chromogen in the cytoplasm apart from the yolk globules. In fact this is suggested in the serial sections of the youngest embryos when the pigment was observed particularly in the vicinity of the nuclei. In those cells the physiological activities

naturally occur near the nucleus and tha action of the cell ferments converts the chromogen into pigment. Later the chromogen probably is formed faster than it becomes pigmented as suggested by Smith ('20) thus explaining the presence of colorless granules. Smith notes that the pigment in the embryonic chick's eye develops first as a colorless chromogen and later becomes colored.

The nature of the chromogen is debateable. As has been cited in the literature, pigment may come from a number of substances. In the production of melanin different workers such as Bertrand, Gessard, Durham, and Bloch have shown that such compounds as tyrosin, tryptopha nm, 3-4 dioxyphenylalanin, epinephrine occur in tissues in sufficient quantities for the production of the pigment. The cell ferments are formed by the nucleus. Whipple ('21) concludes that substances containing the pyrrol group are essential for formation of hemoglobin and bile pigments. Melanin on decomposition yields compounds containing pyrrol group. It is probable that these substances, such as tyrosin and pyrrol derivatives, arise during the differentiation of the cells in the embryo of Amblystoma.

As the young cells contain a nucleus, relatively undifferentiated cytoplasm and food in form of yolk, the substances that enter into the formation of the pigment must be produced through the digestion of the yolk or as a result of metabolism. Or they may be formed by a combination of

both these processes. As has been noted in the observation, the pigment is formed in the differentiating tissue. So that whenever in the process of differentiation substances capable of forming pigment are produced in excess chromogen might be the result.

Some of these substances no doubt are formed from the yolk during its digestion. The differential staining reactions of the yolk globules by neutral red and Nile blue show that physiological processes occurring on the surface of the yolk produce or liberate certain substances that stain more readily than the yolk globule as a whole. The digestive enzymes present may break up the yolk into the amino-acids which in turn are used in building up the protoplasm of the cell. As the yolk is the same for all cells, then those cells which are differentiating the most rapidly, may not have use for all theconstituents of the yolk and it would be these substances that are produced in excess that may enter into the formation of chromogen. Since during the early embryonic period in Amblystoma there are no blood vessels i n the muscles and nervous system these substances must remain ~~remain~~ within the cells that produce them.

The writer has made no attempt to determine the chemical composition of the yolk. McClendon ('09), working on centrifuged frog's eggs, was the first to attempt the chemical analysis of yolk. He concludes that it consists of

some fat, considerable lecithin and a large quantity of protein. He considered the protein to be a nucleo protein as it was rich in phosphorus. Jenkinson ('14) reports on the composition of yolk of frog's eggs and considers the protein to be a globulin or nucleo protein and that a purine base, which probably is xanthine, can be obtained. He points out the difficulties of chemical analysis even in quantities of the yolk and the impossibillity of such analysis of a single egg. These investigators have shown the presence of proteins that can be broken up into amino-acids and synthesized into body protein, so for the Amblystoma embryo it is assumed, from the similarity of appearance of yolk and other cellular structures of the two amphibian species, that the yolk is of such composition that pigment forming substances can be formed from it during its digestion.

The writer's observations upon the origin of this pigment have been generally performed on the epithelial cells of the skin rather than upon those of the entoderm, since the ectodermal cells show a greater degree of differentiation. The metabolic rate is presumably higher in cells that are differentiating more rapidly and it is in these rapidly differentiating cells that the pigment increases at the most rapid rate. Whipple ('21) has suggested a "pigment complex", which is composed of substances derived from the food, body cells and hemoglobin. In these cells

under consideration, it has already been suggested above the pigment may come from substances derived from the food. It is also probable that other substances are formed during metabolism which enters into the formation of this pigment. These substances formed by metabolic processes could, if there were vascular and excretory systems, be carried out of the tissue and either utilixed by different organs of the body or excreted, but in the absence of circulatory systems the substances that are probably comparable to Whipple's'pigment complex' are necessarily retained within the cell.

The waste products from metabolism may be formed in either the anabolic or catabolic phase of metabolism. The anabolic phase involves the building up of complex elements out of more simple molecules. McLeod states that chemical transformations that occur in the cell are difficult to obtain in the chemical laboratory. He says "for in the cell these chemical transformations are capable of being guided to a very remarkable degree of nicety so as to produce intermediate products that are used for some special purpose, either by the cell that produced them, or after transformation by the blood, etc., by cells in other parts of the organism". Such products, which McLeod calls intermediate products, the writer considers, enter into the formation of pigment, In this inert condition they are retained within the cell until the tissues are penetrated by the vascular system.

Many workers have shown that waste products of many tissues have been formed into pigment and recently Whipple ('21) has shown that substances from the breaking down of the body protein enter into the "pigment complex", from which bile pigments and hemoglobin are formed. Thus in the differentiating tissues of Amblystoma embryos substances formed during the catabolic phase of metabolism probable enter into the formations of the pigment. As soon as the tissue begins to differentiate the building up and tearingdown of the protoplasm begins. The substances from the wear and tear of tissue cannot be eliminated, and thus enter into the formation of the pigment.

The group of substances mentioned above that are formed during the differentiation and embryonic function of the tissues under consideration might well be grouped into a "pigment complex". By "pigment complex" is meant in this connection a group of substances that are formed in the differentiating tissue from three probable sources as follows: (1) excess of substances formed during digestion of the yolk that are not needed by the tissue cells, (2) intermediate products of building up of the protein during the anabolic phase of metabolism and (3) products of breaking down of the cell protein. It is considered that as these substances accumulate they are converted by activity of the cell into pigment. The chromogen as a precursor of this pigment probably is laid down faster than the color develops in it. This would account for

the presence of the colorless granules which appear to be changed into pigment.

B. NATURE OF THE PIGMENT.

The pigment in differentiating tissues of embryonic Amblystoma does not occur in sufficient amounts to allow isolation from the tissues. A chemical analysis of it, therefore, could not be made. In order to determine the nature and classification of the pigment it was necessary to depend upon micro-chemical methods.

This pigment is a waste product of normal metabolism and has many characteristics in common with the waste pigments found in the human body. As has already been cited, Lubarch ('02) and Sehrt ('04) found that the 'wear and tear' pigment reacted to fat stains stronger before sectioning and was partly soluble in fat solvents. These investigators did not continue the work farther but called the pigment lipochrome. Other workers have noticed the fat staining characteristics and partial solubilities of waste pigments. Hueck ('12) found that 'wear and tear' pigment reacts to SudanIII, Sharlach R and Nile blue. He believes the reaction of Nile blue is characteristic for fatty acids. Hueck suggests the possibility that 'wear and tear' pigments are products of fat metabolism. Ciaccio ('15) working on auto-oxidation experiments of lipiods found that unsaturated fatty acids are oxidizable andundergo important changes of color, consistency, solubility properties, and reactions

towards alkalies and acids, while they retain their natural characteristics to react toward fat stains. He distinguishes two types, one derived from phosphatids which react weakly toward fat stains, and the other derived from fatty acids which react intensely with Sudan III and Sharlach R. Schmidtmann ('20) after extensive study of sectioned material from brown atrophy of the heart concludes that the pigment which is present in sectioned material is melanin, because it does not differ in staining reactions and solubilities from melanin. Schmidtmann does not believe that it is a metabolic product of fat but that the staining of the pigment granules in fresh tissues indicates that there is a mixture of fat with the pigment of more or less accidental nature and that there is a connection between nutrition and fat content of pigment.

Dyson ('11) working on melanin of the skin noticed that there was less pigment after treating the material with fat solvents and partial staining with fat stains. He concludes that the chromogen of melanin has two parts, one a protein portion which presists as the pigment, and the other a lipoid portion which separates from the protein portion. Dolley ('21) from his work on the pigment in brown atrophy of the heart finds that there are two pigments present, one a lipochrome, derived from the food, and the other a melanin which is a product of metabolic

activity of muscle cells.

Concerning the nature of waste pigments there are many differences of opinions. Formerly it was held that waste pigments are lipochromes of animal origin, later there were suggertions that they are a mixture of pigment derived from protein with fat, or from fat metabolism, and the recent workers conclude that there are two waste pigments, a melanin and a fat holding pigment. The latter is a plant pigment derived from the food.

The reactions of the pigment in the differentiating tissues of Amblystoma in sectioned material agree with the characteristics of melanin. It stains only slightly with fat stains or basic dyes. It is iron free. It presists in sectioned material after imbedding in paraffin. A very small portion, if any, of the pigment is dissolved by fat solvents. The objections to classifying the pigment as melanin are two, namely, (1) the staining reactions in fresh preparations, and (2) the presence of the pigment in tissues which do not ordinarily elaborate melanin.

Various stains were used in fresh preparations to determine the nature of the pigment. It has been already noted that the colorless granules, which are believed to be the chromogen, and the pigment granules, react positively with Sudan III, Sharbach R., Nile blue and Neutral red. This indicated that the granules, chromogen and pigment, are either related to fat metabolism or intimately mixed with the fat or fatty acids in the cytoplasm.

It is plausible that metabolic products of the fats and fatty acids form colored compounds, according to the work of Ciaccio. The yolk contains fat which undoubtedly is used in the reactions of the metabolism. If intermediary products of metabolism of fat or end products formed that are not needed in the cell they may contribute to the chromogen of the pigment. That pigments in animals are formed directly from fats has so far not been proven.

The writer is of the opinion that the mixing of the fats and fatty acids of the cell in a physical manner with the pigment accounts for the positive staining reactions with fat stains in fresh preparation. That this might be possible has been suggested by various authors such as Dyson, Hueck and Schimedtmann. The writer's study of the relation of fats to the pigment was not carried further than the use of the fat stains. The same pigment granules that stain with fat stains in fresh preparations react only very slightly to them or to basic dyes in sectioned material. This could easily occur as the alcohol used in dehydration of the tissue would extract the fat.

The writer does not believe that the waste pigments should be classified as melanin without further study. Schmidtmann ('20) and Dolley('21) state that the pigment in brown atrophy of the heart is melanin because it gives many of the micro-chemical reactions of melanin. If this

pigment is considered melanin it destroys the general conception of majority of workers that melanin is a product of specialized cells produced for a particular purpose. It seems more reasonable that the pigment formed in the heart from metabolic products by the activity of the cells would not be found there if the interchange of products of the cell and substances in the blood plasma were normal. If these same substances that form pigment where the circulation is impaired were normally carried from the tissues, then they might be regarded as substances that occur in the "pigment complex" of Whipple. The writer believes that in the differentiating tissues of Amblystoma the substances, which form the pigment, are comparable to those substances in Whipple's "pigment complex".

It may be shown in the future that the substances that are used in the formation of melanin are at least a part of the substances in the "pigment complex". Further work is needed on pigment metabolism to show the relation of the substances from which melanin is formed with those substances from which hemoglobin and bile pigments are derived.

The waste embryonic pigment in Amblystoma is derived primarily from metabolic products of protein metabolism and food, and is intimately mixed with fats, probably physically and not chemically. This pigment is comparable to bile pigments, since it is a waste product. In certain other features it resembles melanin but it cannot be

unconditionally classified either as melanin or lipochrome. In certain specialized cells it may be retained and further differentiated into melanin in definitive organs.

C. SIGNIFICANCE OF PIGMENT.

The pigment in different tissues of embryonic Amblystoma might be classed as melanin or bile pigment, but this classification would mean very little if the relation of the pigment to the tissue were not considered in terms of the life of the whole organism. In other words, the significance of the pigment can be understood only as it is considered in the light of the principles underlying the development of the embryo as a whole.

A few investigators of different Amphibian eggs and embryos have offered suggestions concerning the probable significance of the pigment . Bellamy ('19), from his studies on frog eggs, said "It may be noted here also that pigment appears more densely in the most active regions of the egg where other evidences indicate that oxigations are proceeding more rapidly than elsewhere. In other words, the density of pigmentation seems to be an expression of the rate of at least certain oxidations occurring in that region." He suggests that increased pigmentation is related to regions where cell division is most rapid. Jordan ('93), from observation on the newt, suggested"that the pigment marks physiological activity, and that the less heavily

pigmented cells of the ventral wall of the archenteron owe their relative lack of pigment to ~~their~~ more sluggish metabolism attendant upon less rapid cell division." King ('02), working on the egg of Bufo lentiginosis, doubts the probability that pigment increases with raoid cell division, for, she says "It is certainly true that the large yolk cells in the egg of Bufo divide less frequently than the cellsin the upper hemisphere, but there ≠ is no evidence that the deeply pigmented cells of the outer surface of the upper hemisphere or of the dorsal wall of the archenteron divide more rapidly than the cells that are found between them."

While the observations of the above mentioned investigators were incidental to their work on other major problems, in the writer's studies it is found that pigment makes its appearance as a result of "oxidation processes" or "physiological activity" in early embryos of Amblystoma. It is obvious, however, that there are more remote factors in pigment formation than simply "oxidation" or "physiological activity" as conceived by Bellamy, Jordan and King. These factors have to do with the difference between differentiation-metabolism and proliferation-metabolism, for these observations demonstrate that pigment accumulates in regions of differentiation but does not accumulate in regions of proliferation. While both these processes

involve"oxidation" and "physiological activity" the accumulation of pigment in connection with the one and not with the other shows that the physiological processes must be fundamentally different in the two cases.

In differentiation there is a building up of structures that render the protoplasm more complex. Accompanying the building up of these relatively permanent structures of the cell, by which differentiation is determined, is a certain amount of wear and tear in the protoplasm itself, and this must involve both oxidation and reduction processes. Among the products of these processes of "wear and tear" would be the mother substances of pigment. The metabolism of proliferating cells , however, does not result in the building up of complex,relatively permanent structures in the protoplasm. On the contrary, according to Child ('15) mitosis involves dedifferentiation in the cytoplasm. Proliferation,therefore, must lack the"wear and tear" processes that are characteristic of differentiation, and for that reason lacks the function of pigment production.

Bearing upon the question of the relation between differentiation and dedifferentiation Child("15) says"cells which divide rapidly do not undergo any great degree of differentiation, and the cells which resume division after undergoing differentiation first undergo a greater or less degree of dedifferentiation." Therefore, in embryonic cells which are undergoing rapid proliferation, such

as those of the ventricular region of the central nervous system of the embryo, the slight differentiation metabolism which may be taking place must be counterbalanced by the predominating processes of dedifferentiation. In such relations differentiation and dedifferentiation must be conceived as antagonistic processes, and the antagonism between these processes must be conceived as involving all that has to do with the substances of the "pigment complex." Any substances of the "pigment complex" that may arise during the resting phase of the cell might become dedifferentiated in the following mitosis. This view receives support from Child's work on flat worms, in which he shows conclusively that in dedifferentiation highly differentiated tissue structures actually disappear.

The application of this principle of antagonism to the development of Amblystoma during the period under consideration explains why mitosis does not occur in the myotomes which are undergoing rapid differentiation and rarely if ever occurs in the rapidly differentiationg regions of the central nervous system. It renders intelligible, also, the polarity of the amphibian egg with reference to pigmentation. Bellamy ('19) considers that the polarity of the frog's egg is determined by the relation of the egg to the blood supply. He says "this polarity is marked by the localization of most of the protoplasm in

the pigmented hemisphere, by the eccentricity of the nucleus nucleus, and by the distribution of the pigment." He holds,also, that "Local increase of activity results in the formation or the increase of pigment and local decrease in activity results in diminution of pigment." However, the results of the writer's studies show conclusively that the term "activity" is wholly inadequate to indicate the real nature of the processes under consideration. The conception of polarity as the localization of differentiation-metabolism renders the problem of polarity of the amphibian egg intelligible from the point of view of the writer, for yolk is produced in the cytoplasm through differentiation-metabolism and it is necessary that this process take place in the region of oxygen and food supply. But this differentiation-metabolism involves the production of the substances of the pigment complex, and in the presence of abundant oxygen they readily become transformed into pigment. The localization of pigment in the egg is,therefore, determined by external conditions as Bellamy proposed, but its origin is specifically involved in the process of differentiation.

VI. C O N C L U S I O N S.

1. The pigment that is characteristic of amphibian embryos arises in the cytoplasm from colorless granules which are considered to be chromogen and which represent a pigment complex made up of (1) substances that arise in excess from the digestion of the yolk, (2) intermediary products of anabolic phases of metabolism, and (3) products of catabolic phases of metabolism.

2. This pigment can not be unconditionally classified as either melanin or lipochrome but is regarded as a waste pigment comparable to the bile pigments.

3. This embryonic pigment is an indicator of metabolism that is characteristic of tissue differentiation as opposed to metabolism that is characteristic of cell proliferation.

4. Differentiation-metabolism and proliferation-metabolism are regarded as fundamentally antagonistic processes in development.

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DESCRIPTION OF FIGURES

Reference letters:

PB - Pigment body

PG - Pigment granules

N - Nucleus

YG - Yolk globule

S - Strands of pigment granules

Figures:

Figure I. - An optical projection of the pigment bodies in a non-motile embryo as viewed from the side of the (a) third myotome, (b) tenth myotome.

Figure II.- Projection of an early flexure embryo similar to figure I except in addition it contains the projection of (c) the eighteenth myotome.

Figure III.- Projection of a coiled-reaction stage embryo similar to figure II.

Figure IV.- Projection of an early swimming stage embryo similar to figure II except that (d) represents the twenty fourth myotome.

Figure V. - This is a microphotograph (X440) of a frontal section of the third myotome taken through the middle dorso-ventrally of an embryo of

the early flexure stage. The fixing fluid used was van Gehuchten and stained with Erythrosin-toluidin blue. The nuclei (N) stained bluish purple but they appear faintly in the microphotograph. The numerous yolk globules (YG), although stained red in the section , appear black and are nearly as dense as the pigment bodies (PB). The pigment granules are not visible at this magnification. The pigment bodies, which are composed of pigment granules , appear irregular in shape. The numerous yolk globules obscure the other structures in the cells.

Figure VI.- This figure is a microphotograph (X 850) of a section taken from the dorsal portion of the tenth myotome of a non-motile stage embryo. The cells are radially arranged about the center of the myotome. The pigment granules occur in the central part of the myotome. S represents strands of granules extending from ends of nuclei toward the center of the myotome. Yolk globules (YG) are faintly visible and some have pigment granules on their surface. The nuclei appear black. The

embryo was fixed in Bouin's fluid and stained with neutral red and Janus green.

Figure VII.- This is a microphotograph (X 440) of a section in frontal plane of the tenth myotome of an embryo of the early swimming stage. The section was taken from the dorsal portion of the myotome. The fixative was corrosive-sublimate and acetic acid and the stain was alum-carmine and Lyons blue. No pigment granules are visible at this magnification. Arow of nuclei can be observed at either end of the myotome. The pigment bodies (PB) are mostly located near ends of nuclei.

Figure VIII.- Figure VIII is a reproduction of drawings of sections taken at different levels of the central nervous system of the various stages noting the location of the areas of abundant pigmentation on one side only. Row I was made from a non-motile stage embryo; row II from an early flexure stage embryo; row III from a coiled-reaction stage embryo; row IV from an early swimming stage embryo. Column 1 was taken at the level of the V root; column 2, at the level of the VII root; column 3, at the level of the IX root; column 4, from the spinal cord at the level of the

third myotome; and column 5, from the spinal cord at the level of the eighth myotome.

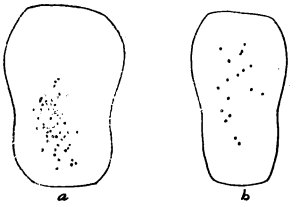

Figure I

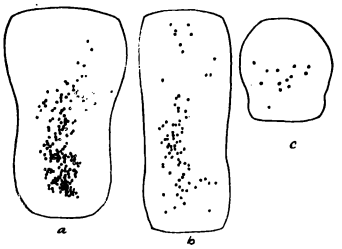

Figure II

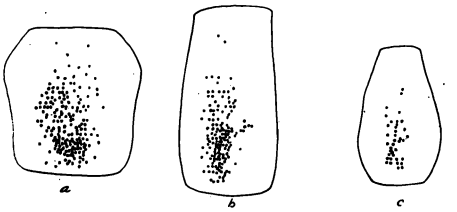

Figure III

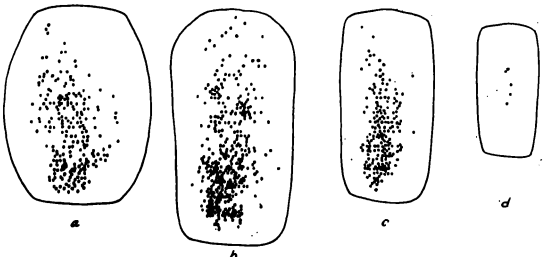

Figure IV

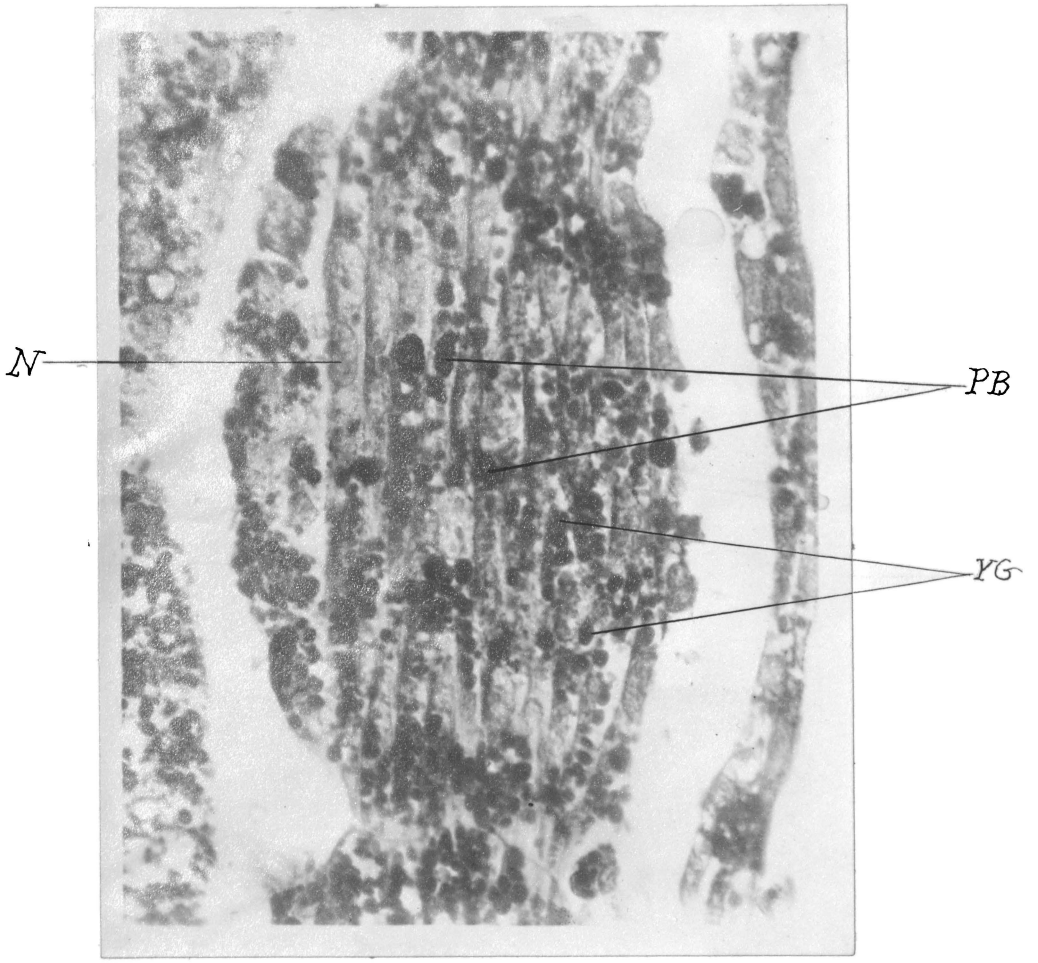

Fig. V

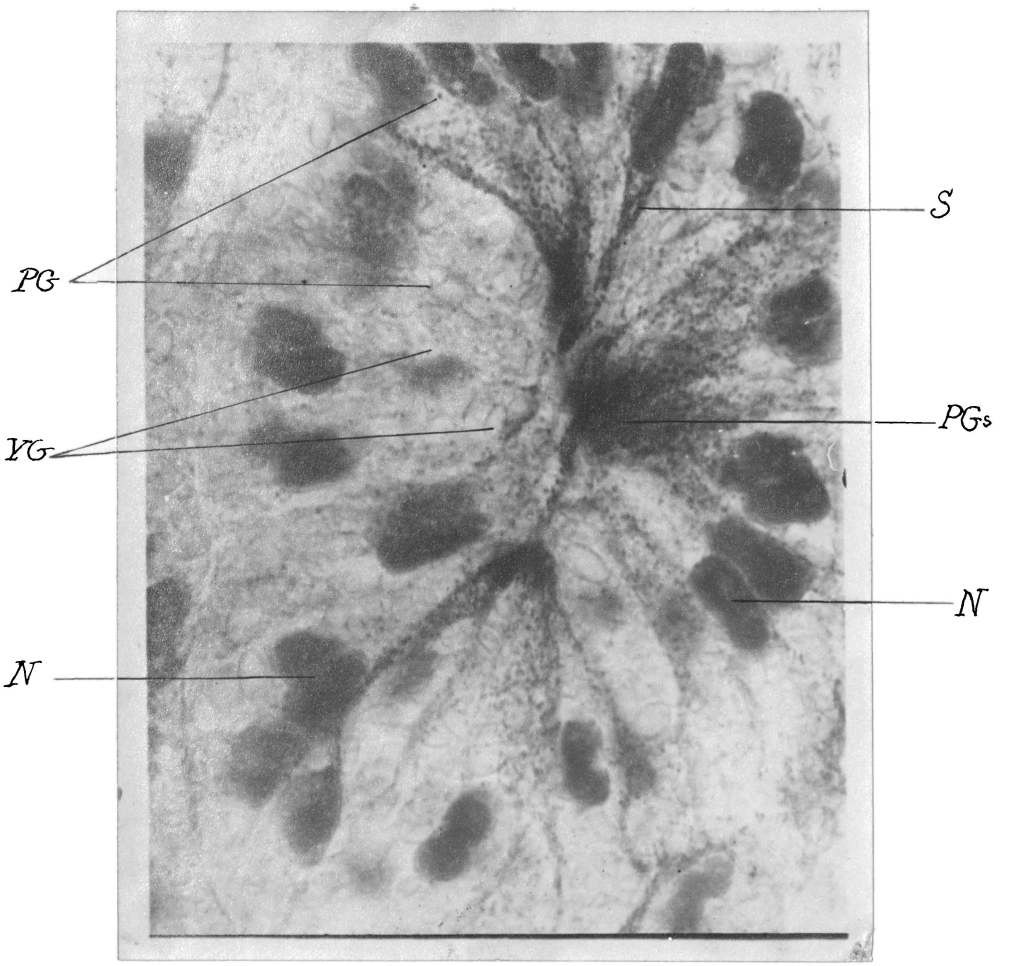

Fig. VI

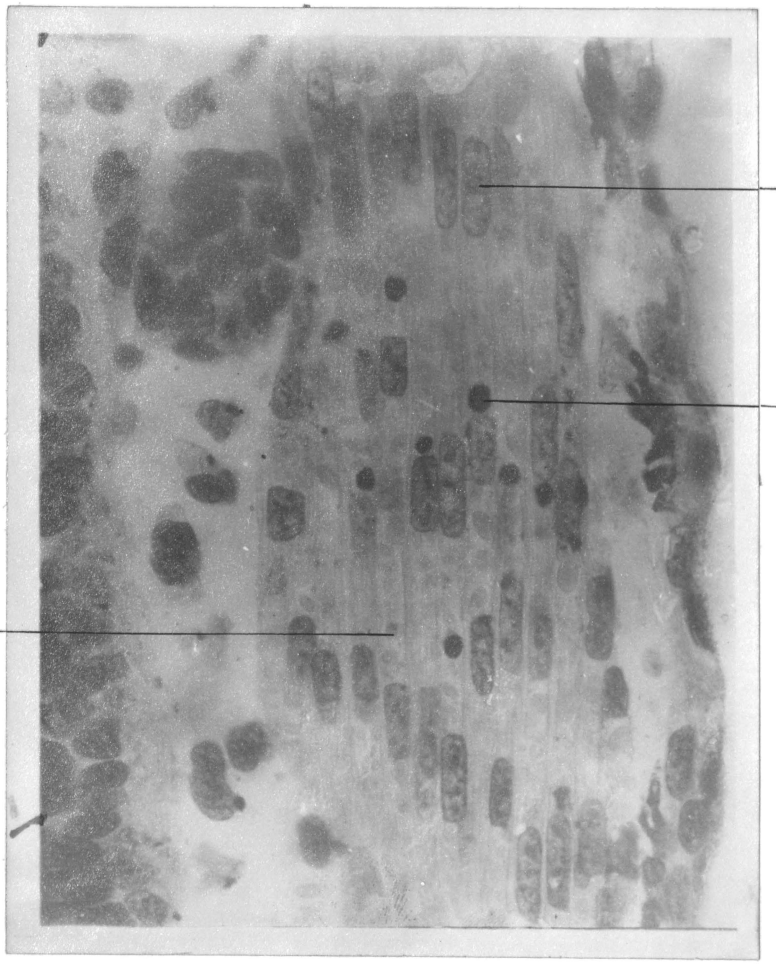

Fig. VII

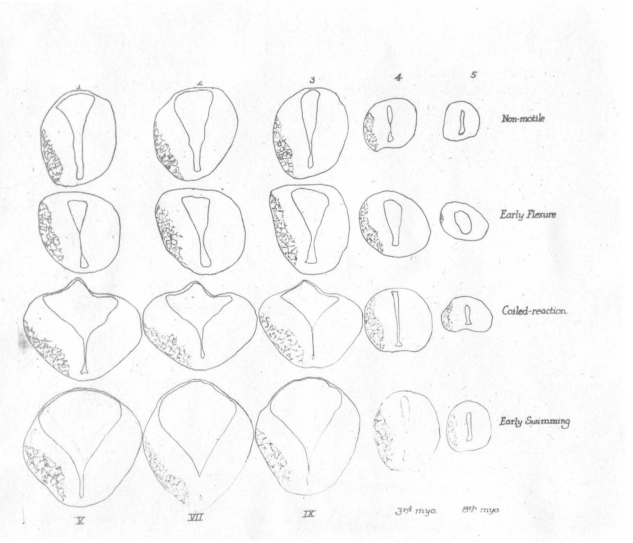
3
4
5
Non-motile
Early Flexure
Coiled-reaction.
Early Swimming
V
VII
IX
3rd myo.
6th mys

Fig. VIII