STUDIES ON THE DIFFERENTIATION OF HEMOGLOBIN IN *RANA PIPIENS*.

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Jimmy B. Throneberry

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This is to certify that the

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Major professor

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STUDIES ON THE DIFFERENTIATION OF HEMOGLOBIN IN *RANA PIPIENS*

By

JIMMY B. THRONEBERRY

AN ABSTRACT OF A THESIS

Submitted to
Michigan State University
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ABSTRACT

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by Jimmy B. Throneberry

The objectives of this investigation were to determine when and where hemoglobin appears in the development of Rana pipiens, and whether treatment of early embryos with this protein, or with antibodies against it, have effects on the hematopoetic process. A number of rabbits were immunized against hemoglobin, isolated according to a modification of Drabkin's technique, from adult R. pipiens. The agar-diffusion technique of Ouchterlony was employed to determine the specificity of antisera, and also to detect the time of appearance of the hemoglobin molecule in development. Various modifications of the benzidene-peroxidase test were employed to localize the hemoglobin molecule, the most successful being a method employing 3, 3'-dimethoxybenzidene. In attempting to determine effects of hemoglobin and antibodies against it, on hematopoesis, each was injected into the blastocoel of R. pipiens embryos, Shumway stages 8-9; morphological and histological observations were made on their subsequent development. The following results were noted; 1) Specific antibodies against adult R.

pipiens hemoglobin were obtained; 2) Synthesis of heme and globin do not necessarily take place simultaneously, since globin was present in all stages of development, including body cavity eggs, whereas heme could not be detected by the methods used until later stages (Shumway stage 21); 3) there was no consistent specific effect, either of an inhibitory or enhancing nature, of hemoglobin or of antibodies against it, on the differentiation of erythrocytes and of hemoglobin.

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INTRODUCTION

Of all the problems facing biologists today that of cellular differentiation or specialization is, in all probability, the central and most elusive one. Since all stages of development of an organism seem to be under the influence of genes, an understanding of developmental mechanisms at a molecular level will undoubtedly elucidate, to a degree, the problem of gene action.

The designation of a group of cells as differentiated reflects the morphological and functional properties of these cells, and also their pattern of combination in specific tissues. These properties in turn are dependent on the structural and enzymatic proteins present in the cells and on their surfaces.

In an attempt to elucidate to some extent the mechanisms responsible for differentiation and growth, there has been a great deal of research and discussion over the past fifteen or more years concerning the effect of substances from differentiated cells on the growth and differentiation of homologous embryonic cell types. Some investigators claim to have demonstrated that substances from differentiated cells exert a specific inhibitory effect on the differentiation and growth of the homologous embryonic cell types, while others claim the opposite

effect for these substances, i.e. an enhancement of differentiation and growth. On the other hand, a number of researchers have reported specific inhibition of growth and differentiation by treating embryos with antibodies produced against substances from differentiated cells. Interpreting differentiation on the basis of current theoretical concepts, one would expect antigens and antibodies to have opposite effects, assuming them to be counterparts to one another. In light of these divergent results one may wonder whether these substances do indeed exert a controlling influence on the mechanisms of differentiation or are simply the end products of the process.

A partial explanation for these conflicting results may lie in the fact that widely differing experimental systems and experimental procedures have been used. Also, the interpretations of these results are limited by the fact that most of this work has been done using tissue homogenates, which represent diverse populations of unknown proteins against which a spectrum of antibodies may be produced.

The present investigation was based on the assumption that, if a differentiating system was exposed at a particular time to a tissue specific protein, or to antibodies against this protein, results might be obtained which would confirm the alleged effects of these substances, and perhaps contribute to an understanding of differentiation at the molecular level.

Hemoglobin was chosen as the tissue specific protein for this study for the following reasons: 1) It can be obtained easily in relatively pure form; 2) A great deal of biochemical and genetical information for this protein is available; and 3) Rose (1954) claims to have obtained bloodless larvae by culturing early embryos of R. pipiens in adult whole blood.

Because hemoglobin is the tissue specific protein chosen for this investigation; it seems useful to briefly review some of the information concerning the time and place of appearance of erythrocytes and hemoglobin in the amphibian embryo.

The term "blood islands" was applied by Pander in 1817 to the golden red masses in the area vasculosa of the 24-hour chick embryo. Since that time, the term has been very loosely applied to the blood forming regions of practically all embryos regardless of the form these regions take. There has been considerable controversy (see Stockard, 1915, and Reagan, 1917) as to the source of the "blood island" cells and their competency to contribute more than one kind of cell to the vascular system. Both Stockard and Reagan succeeded in producing embryos without circulation, and then studied the development of blood. Stockard came to the conclusion that blood development took place in a definite localized area which he called the"blood island", and that this region was the primordial locale for all blood cells. Reagan, on the other

hand, claimed that blood cells could develop anywhere from endothelium.

Federici (1926) removed the "blood island" from embryos of R. fusca in the tail bud stage and in some instances obtained larvae completely devoid of erythrocytes. In other instances, there were a few blood cells present, which he attributed to incomplete removal of the "blood island". These results were confirmed by Goss (1928) working with Ambystoma punctatum. Goss was able to maintain these bloodless embryos for 32 days.

Slonimski (1931) working with Amblystoma mexicanum, claimed to have outlined the exact boundaries of the"blood island" region, at Harrison stage 31, by using the benzidene-peroxidase test for the presence of hemoglobin. In addition, he states the critical moment for the appearance of hemoglobin is almost coeval with the separation of the cells which form the "blood island". This separation takes place at Harrison stage 30. In all the embryos tested the benzidene test was positive at this time. Slonimski emphasized that at the moment of appearance of hemoglobin in the primitive red cells, the vascular endothelium of the "blood island" was not yet formed. He further showed that primitive blood cells can self-differentiate following explantation of the presumptive "blood island" area from the neurula stage and that by removing this area larvae are obtained devoid of blood cells.

Cameron (1940) presented evidence for a distinct physico-chemical difference between cells which develop into blood cells and those which develop into endothelial cells in Amblystoma jeffersoianum by showing that these cells exhibit a differential susceptibility to x rays. In a further study, Cameron (1941) showed that the "blood island" is formed from primitive mesoblasts, consisting of large cells about 40 μ in diameter and crowded with characteristic ovid yolk platelets. These cells were commonly observed to be in mitosis, which distributed the yolk platelets equally to the two ends of the dividing cell. Cameron studied the changes in the number, size, shape, and staining reaction of yolk platelets during development. He observed eight generations of primitive erythrocytes, in the course of which the number of yolk platelets was reduced from 128 to one in each cell. All the blood cells of the prehatching embryo consisted of the primitive cells forming the "blood island" and their descendants. After hatching, which took place on about the fifteenth day, the primitive cells disappeared and other hematopoetic regions took over the production of the oval-shaped yolk-free erythrocytes of the adult.

The best description of the formation of the "blood island" in amphibians was given by Brachet (1921) working with Rana fusca. His observations extended by Fernald (1947) to Hyla regilla. According to these workers, the primary mesoderm of the later embryo is derived

from the marginal zone opposite the blastopore. The "blood island" is derived from cells in the inner portion of this zone facing the segmentation cavity. In gastrulation, the marginal and vegetal zones are carried inside the embryo and covered by the animal zone. When the blastopore is slit shaped, the mesoderm of the notochord, somites, and lateral plates form a continuous layer between the ectoderm and endoderm in the posterior region. The cells which form the "blood island" form a part of this mesodermal layer and are scattered along the midventral line from the ventral lip of the blastopore anteriorly to the free edges of the lateral plates in the region of the liver diverticulum. At the stage of the closing of the medullary folds the ventral mesoderm becomes closely applied to the endoderm so that it is impossible to separate them, but as the embryo approaches the tail bud state they separate again.

At the time the embryo first responds to stimuli (about 5 mm. length) cellular "differentiation" takes place. In some amphibians (eq. Rana fusca) there is a separation of a thick plate of cells from the ventral mesoderm, but in others (eq. Hyla regilla) cells are proliferated from the side of a thin layer of mesoderm toward the endoderm and form a loose mass. These hematopoetic cells extend along the midventral line from the anus to the liver, bifurcate and extend along both sides

of that organ. Twenty-four hours later, when the heart begins to pulsate the blood cells are put into circulation. According to Fernald (1947) the cells of the "blood island" in the five mm. stage of Hyla regilla are in grooves in the surface of the yolk mass and are separated by the developing epithelium. As the endothelium develops, a central vessel, later the subintestinal vein, is established with many lateral branches. This whole complex becomes the vitelline system. In the five-day-old embryo the cells are released into circulation. At this time they contain yolk platelets and the blood is colorless or milky. In the eight-day-old embryo (operculum complete) the cells still have yolk platelets but the blood becomes pink. During the feeding stage the blood turns red and yolk platelets disappear. During the first fifteen days of development division of the original cells is the only supply of erythrocytes, but on the sixteenth day other hemopoetic regions take over.

In addition to these observations, Fernald made seventy-one explants of the presumptive "blood island" cells from gastrulae. He maintained fifty-eight of these for eight days, and observed that they self-differentiated into primitive blood cells. He also extirpated presumptive and definitive "blood island" regions from 840 embryos; 240 from slit-shaped blastopore stage; 90 from neurulae; and 510 from tail bud and young larval stages. He obtained a very marked reduction in

blood cells in these embryos, but none were completely devoid of blood cells. The embryos which survived the operation and had not lost so much yolk as to prevent the establishment of a complete digestive tract were maintained as long as desired. The heart and blood vessels developed in normal fashion in spite of almost complete absence of blood cells. The blood cells were able to regenerate, however, and by the end of the third week turned red. He attributed the development of some blood cells to the possibility that in healing some of the presumptive nephric mesoderm grew down and away from the influence of the organizer, thereby differentiating into primitive blood cells. These in turn by mitotic divisions could increase in number.

Fernald further made cephalic and caudal fragments by severing the embryo just anterior to the presumptive "blood island" region and obtained good heart and blood vessel differentiation with complete absence of blood cells in the cephalic pieces while the caudal ones showed very good differentiation of blood cells.

Thus it appears that the primitive erythrocytes have their origin solely from the "blood island" in amphibians, and that these cells appear at about the time of tail bud and muscular response stages. According to Slonimiski (1931), hemoglobin appears in these cells just as they are produced.

The idea that substances from differentiated cells exert a specific inhibitory effect on the differentiation of undetermined cells with the same prospective fate was explicitly proposed by Rose (1952), although the idea had been hinted at much earlier. Rose visualized differentiation as resulting from the activity of a hierarchical series of gene initiated reaction systems, which are self-limiting. One reaction would have the initial advantage in all cells, but since certain cells begin the process of differentiation before others, the metabolites of the dominant reaction would diffuse into adjacent regions, inhibiting this reaction. In the latter localities, the second reaction in the hierarchical series takes place, producing metabolites which inhibit this second reaction in sub-adjacent regions, and so on, until all the reactions leading to tissue differentiation in the morphogenetic gradient have taken place.

Experimental evidence claiming to support this theory comes from work in two areas, regeneration and embryonic development. For example, evidence from work with the regenerating system of the coelenterate, Tubularia, has been adduced in favor of Rose's concept. In Tubularia, hydranths can be regenerated from any isolated part of the organism proximal to the hydranth. Rose and Rose (1941) observed that when isolates were grown in sea water, in which adult hydranths had been kept for 12 to 24 hours, prior to introduction of the isolates,

regeneration of the isolates was inhibited. These results were interpreted as indicating the existence of a diffusible substance in intact hydranths which diffused in a disto-proximal direction, and inhibited the formation of distal structures in more proximal regions. This work was repeated by Steinberg (1954), but considerable doubt was cast on it by Fulton (1959), who claimed that the inhibition observed by the previous workers was due entirely to bacterial growth in the cultures.

Rose (1955, 1957) has shown, by using grafting techniques, that if older distal structures of Tubularia are grafted onto distal regions which would normally regenerate distal structures, the latter will now form more proximal structures. Here again the interpretation is that a specific inhibitor diffusing in a disto-proximal direction inhibits more proximal regions from forming distal structures.

Brønsted (1954, 1955) has performed experiments with species of Planaria which he interprets as exemplifying specific inhibition in a hierarchical system. According to Brønsted, the anterior blastema of a regenerating planarian is pluripotent, and all parts, both lateral and medial, can form brain and sensory structures. However, after a few days the ability to form brain is localized in the mid-region. Brønsted interprets this as being due to an inhibition of lateral regions by the mid-region because when lateral regions are isolated from the medial region they are not inhibited and can continue all the way to brain production.

Lender (1955, 1956a) has demonstrated that when the central part of a planarian blastema on its way to forming brain is removed, the lateral eyes will not form. Lender (1956b) further found that extracts of heads, but not of tails, caused lateral eyes to form in regenerates, presumably by inhibiting brain development. Thus, the interpretation of these results as indicating a system of specific inhibitions hierarchically arranged, would have the same brain products which inhibited brain development in the young regenerates favoring the development of eyes, a lower member of the series.

Tucker (1959), working with the nemertean worm, Lineus vegetus, has made similar observations and interpretations. Under normal conditions in this organism sections removed from anterior, posterior, or mid-body regions will regenerate both head and tail. Tucker observed the effects of homogenates of heads, of sections of mid-body, and of tails on the regeneration of isolates from these same regions. She found, if the homogenate used was at the same level or anterior to the region being tested, head regeneration was inhibited in that region; conversely, if the homogenate was at the same level or posterior to the region being tested, tail regeneration was inhibited. Tucker interpreted these results as showing a sort of double gradient, a hierarchy of reactions extending from head to tail, controlling head regeneration, and a hierarchy of reactions extending from tail to head, controlling tail regeneration.

The above mentioned references serve to illustrate the claims that substances from differentiated cells or regions of cells exert an inhibitory effect on the regeneration of cells or tissues of the same type.

Evidence which has been interpreted in accord with Rose's hypothesis, from studies of differentiating systems in embryos in less abundant and much less convincing than that from studies on regenerating systems. Weiss (1952) cultured embryonic chick organs in tissue culture media containing extracts of whole embryos and embryos from which the homologous organ had been removed. When heart fragments were used he observed that 2 out of 333 pulsated after four days in full extract, whereas 129 out of 349 pulsated after four days in extracts of embryos from which heart had been removed. Essentially the same results were obtained with kidney, the differentiation of new tubules occurring much more numerously in extracts lacking kidney. Thus, differentiation of embryonic tissues was reduced markedly in the presence of substances from the homologous tissue.

Rose (1955) allowed fertilized eggs of Rana pipiens to develop through cleavage and blastula stages in media containing bits of adult heart, brain, or blood. Although, in many experiments the solutions were so toxic that development was arrested before any specific effects could be observed, in a small number of experiments he was able to show some specific effects. Culturing with brain caused a delay in

neurulation and sometimes neural defects, culturing with heart completely or almost completely stopped heart formation, and culturing with blood inhibited blood cell formation. Unfortunately, only four experiments of a total of 26 produced these effects. Rose felt that the inconsistency in these results was probably due to uncontrollable factors such as seasonal variation and general condition of the eggs employed.

Shaver (1954) injected embryos of R. pipiens with fractions of adult R. pipiens brain. He obtained essentially the same results as Rose, so far as effects on the morphogenesis of the nervous system were concerned, with both cytoplasmic granules and supernatant fluid from centrifugation of homogenates. The cytoplasmic granules were more effective than whole brain tissue in causing a retardation during neurulation. Owens (1960) did a thorough histological analysis of this material. He observed that in three of four experiments in which cytoplasmic granules of adult brain were injected into embryos, from 28 to 37 per cent of the embryos had solid nervous systems. Culturing of embryos in fractions of brain homogenates showed no consistent results. Owens performed additional experiments which showed that the specificity of the inhibition of differentiation of embryonic nervous tissue by adult brain is more difficult to demonstrate than the earlier experiments indicated, inasmuch as heart granules also produced an inhibition of nervous tissue in some experiments.

Clarke and McCallion (1959a) cultured embryos of R. pipiens in cell-free homogenates of adult cerebrum and heart of this species, and observed some inhibition of development of these tissues. When they cultured embryos from fertilization to yolk-plug stages, they observed only general abnormalities, which were just as prevalent in the controls as in the experimentals. However, when they cultured from yolk plug through neural tube formation, 25 of the 165 surviving embryos showed specific neural defects. These results could be duplicated by culturing embryos in homogenates of chick cerebral hemispheres. Thus, the effect was not class specific. When embryos were cultured in cell-free homogenates of chick heart, two of 124 showed specific inhibition of that organ.

Clarke and McCallion (1959b) repeated this work in the chick and observed that 42 of 115 embryos injected with cell-free extracts of adult chick cerebrum showed specific inhibition of brain. The effect here seemed to be species specific. They interpreted their results as supporting Rose's theory.

Lenicque (1959) concluded from a very extensive study on chick embryos that differentiation of embryonic tissues was inhibited by treatment with substances from the homologous adult tissue. This investigator injected extracts of blood, brain, eye, and heart from adult chickens into the germinal cavity against the inferior surface of the

blastoderm of embryos from 18 to 20 hours of age, and observed morphological, histological, and, in some cases, chemical effects on the fourth day after injection. He found it necessary in the case of blood to fractionate it and inject the various fractions; namely, sterile defibrinated whole blood, red corpuscles, cock serum, hemolyzed red corpuscles, and fractions of blood not precipitable by ammonium sulfate. Of particular pertinence to the present investigation is the observation that cock hemoglobin was found to have no specific effect on the development of blood, exhibiting instead great general toxicity. Cock serum was completely harmless and the embryos developed normally. However, blood extracts from which hemoglobin had been removed inhibited the formation of blood in a significant number of cases. The brain extracts had no inhibitory influence on development of the blood.

In the 357 eggs injected with cerebral tissues of varying origin, the heads developed abnormally in 40 per cent of them, and there was no heart or blood inhibition observed in these embryos. The results with eye extracts were not quite as clear cut even though 50 per cent of the embryos injected showed eye abnormalities, because many of the abnormalities were associated with head abnormalities in general. The cases of heart inhibition were even less clear cut than those of eye, as they were associated with a number of general abnormalities.

Lenicque also made an attempt to determine the chemical nature

of the specific inhibitors. He found that they were non-dialyzable, thermolabile, and probably not nucleoprotein.

Recently, Braverman (1961) reported on quite extensive work with the chick which seems to provide very strong evidence for that aspect of Rose's hypothesis which emphasizes a gradient of self-limiting reactions. He injected extracts of various parts of adult or embryonic organs beneath the blastoderm of one-day-old embryos. As in the case of Rose's work (1955), Braverman obtained inhibition of nervous system by injecting brain and inhibition of mesodermal structures by injecting mesodermal derivatives. But the most striking results concern the regional inhibition of nervous system which was obtained after injecting extracts from various levels along the antero-posterior axis of the nervous system. Extracts of dorsal cerebrum affected the shape of neither spinal cord nor post-telencephalic brain inhibiting only telencephalon. Embryos injected with extracts of thalamus, midbrain, or cerebellum had defects of the whole brain. Embryos treated with spinal cord extracts had defects of the spinal cord and whole brain. Thus, only extracts of spinal cord could affect the spinal cord, extracts of the dorsal cerebrum affected only the telencephalon, and extracts of the rest of the brain affected the whole brain structure. These examples of cumulative inhibition certainly seem to suggest that a hierarchy of alternatives exists. Braverman proposes a model for differentiating

systems based on the evidence regarding suppression of enzyme forming systems, evolving mainly from the field of microbiology.

As is evident from the preceding information there is considerable, if debatable, evidence in support of the idea that substances from differentiated cells exert a specific inhibitory effect on the differentiation of homologous embryonic cell types. Rose (1957) has reviewed several types of experiments which can be interpreted in keeping with this idea.

The hypothesis that substances from differentiated cells exert an enhancing effect on the growth processes in differentiating systems comes, to a great extent, from work with the chick in which adult tissues are grafted onto the chorioallantoic membrane of embryos. Murphy (1916) observed that when grafts of adult spleen, liver, and bone marrow of chicken were made on the chorioallantoic membrane, enlargement of the homologous embryonic organ of the host occurred, especially in the case of the spleen grafts. He attributed the spleen hypertrophy to the infiltration of small lymphocytes. Danchakoff (1916) also observed hypertrophy of the spleen after making chorioallantoic grafts of adult spleen. She attributed the hypertrophy to an increased proliferation of lymphoid hemocytoblasts however. The results of Sandstrom (1932) also demonstrated host spleen hypertrophy.

Minowa (1921) observed that he could specifically suppress or

enhance the differentiation and development of sex in host embryos by grafting testicular or ovarian tissue onto the chorioallantoic membrane. Grafts of ovarian tissue stimulated the development and differentiation of the female, while inhibiting in the male. The converse was true with grafts of testicular tissue.

The most extensive work on the effects of chorioallantoic grafts of adult chicken tissues on the homologous embryonic tissue is probably that of Ebert (1951, 1954, 1955). Ebert (1951) noted a striking hypertrophy of the spleen in host chicks following chorioallantoic grafts of adult spleen. This effect was class-specific, but organ-specific only in a quantitative way. In later experiments, using chorioallantoic grafts labeled with radioactive methionine, he (Ebert, 1954) noted that there was an increase in the nitrogen content of the enlarged spleen which was not correlated with an increase in DNA content. The host spleen also exhibited a higher specific radioactivity than kidney or liver. On the basis of these observations he suggested that enlargement of the spleen was due to increased protein content, and that transfer from the graft to the host was tissue-specific, involving the selective incorporation of tissue-specific protein from grafts to homologous tissues rather than the transfer of whole cells.

The work of Van Alten and Fennell (1959) indicates that the stimulatory effect of chorioallantoic grafts is not as tissue-specific as has

been suggested by Ebert. They observed that following chorioallantoic membrane grafts of adult chicken duodenum there was a marked decrease in the absolute weight of the host, a marked increase in the relative weight of the spleen, liver, and heart, and a relative weight increase in the duodenum. Further, following grafts of adult skin and brain, the spleen and liver were significantly heavier. Following liver grafts, the liver and heart showed a significant increase in weight, and in addition, spleen grafts caused a marked increase in the weight of spleen and heart. However, they found that grafting of adult duodenum caused acceleration of tissue differentiation of the host duodenum. The polysaccharides in the connective tissue, and goblet cells of the duodenum differentiated at least 24 hours earlier than in the controls.

A number of workers have used immunological techniques in the study of development. Although most of these investigators have been concerned with the appearance of new molecules during embryonic development, some have studied the effects of antisera on development. Some of these will now be considered.

Weiss (1947) immunized guinea pigs against adult chicken liver, kidney, and muscle. When these antibodies were introduced into the circulation of the developing chick, Weiss made the following observations: the weight of all experimental embryos was, in general, less than that of the controls; there were smaller livers in the embryos

injected with kidney and muscle antisera; there were reduced kidneys in the embryos injected with liver and muscle antisera; and there was positive growth stimulus for the homologous organ. On the basis of these results Weiss suggested that antibodies act as catalysts for growth, possibly by acting as molds for templates which would in turn produce tissue specific substances.

Burke, et al. (1954) studied the effects of antibodies produced against chick lens upon the differentiation of the lens and other eye structures of the chick. They were able to produce defective lenses in embryos when they injected the antiserum at from 146 to 192 hours of development. In some of these cases the lens epithelium and retina were also abnormal.

Lippman, et al. (1950) using tissue culture explants in studying the effects of antibodies against rat kidney observed that the antibodies were toxic to explants of rat kidney, heart, and brain.

One of the most extensive studies in this area is that of Ebert (1950). Ebert explanted 468 chick blastoderms in the definitive primitive streak or early somite stages to media containing normal (control) or anti-organ sera. He used a wide range of serum dilutions. He observed the following: definitive primitive streak and head process stage blastoderms explanted on media containing either anti-heart or anti-spleen serum in final concentrations under 1:80, or anti-brain

serum in concentrations under 1:30 rapidly underwent disorganization; at dilutions between 1:80 and 1:200, in the case of anti-heart or anti-spleen sera, and 1:30 to 1:75 with anti-brain sera, normal morphogenesis occurred in the almost complete absence of growth as determined by a comparison of camera-lucida drawings; at certain critical concentrations, which for anti-heart and anti-spleen sera were approximately 1:80, and for anti-brain sera 1:30, relatively specific effects of anti-organ sera were obtained; the anti-spleen and anti-heart sera affected mesodermal structures specifically, while the anti-brain serum affected nervous tissue specifically. There was a distinction between the effects of anti-spleen and anti-heart sera in that anti-heart serum caused the absence or retardation of heart whereas anti-spleen did not. Explantation to media containing control sera did not produce any of the effects just cited.

Johnson and Leone (1955) obtained a general inhibition of development in the chick embryo with antiserum prepared against actomyosin from adult chicken hearts. Due to this general inhibition of development, it was not clear as to whether there was any specific inhibition of heart, even though heart was inhibited in a number of cases.

Owens (1960) obtained some relatively convincing evidence of specific inhibition of heart development in R. pipiens by injecting antiserum produced against adult R. pipiens heart into the blastocoel of the

developing embryo. The developing hearts in 30% of all embryos which had been injected with heart antisera were inhibited as compared to 8.6% of embryos in which inhibition of hearts followed injection with control serum. Kidney antisera had a slight inhibitory effect (12.5%) on the development of heart, while brain antisera seemed to have no inhibitory effect on heart at all.

More extensive reviews of the work on the effects of antisera on embryonic development can be found in Nace (1955) and Tyler (1957).

From the previous discussion it is evident that substances from differentiated cells and antibodies against these substances have an effect on differentiation. As for the exact nature of this effect and the specificity thereof, considerable controversy and confusion exist.

The objectives of this investigation were (1) to produce antibodies against hemoglobin of R. pipiens, and to demonstrate their immunological specificity, (2) to determine where and when blood cells and hemoglobin appear in the development of R. pipiens and (3) to determine the effects of adult R. pipiens hemoglobin and antibodies directed against it on the differentiation and growth of blood cells and hemoglobin.

MATERIALS AND METHODS

A. Isolation and Characterization of Hemoglobin Solution

Adults of R. pipiens were obtained from commercial dealers in Wisconsin and Vermont. These frogs were bled by cutting the tip off the ventricle of the heart and allowing the blood to drip into a centrifuge tube coated with and containing about 1 cc. of a 1:1, 000 heparin solution. The red cells were spun down at about 2, 000 RPM and the plasma siphoned off. The cells were washed and packed three times in 0. 7% saline solution, and then mixed with a volume equal to that of the packed cells of distilled water, and 0. 4 volume of toluene. After a short period of very vigorous shaking, the solution was stored at 4° C. for 24 hours. At the end of this period, it was centrifuged again at about 2, 000 RPM and the clear red hemoglobin solution siphoned off. This solution was then spun at 4° C. in a refrigerated centrifuge at about 40, 000 x g for 20 minutes and the supernatant fluid poured off. This supernatant will be referred to as the hemoglobin solution throughout the remainder of this report. The hemoglobin solution was treated in various ways throughout this investigation depending on its use.

Two methods were used in characterizing the hemoglobin solution: namely, spectrophotometric and electrophoretic analysis. In the spectrophotometric analysis, dilutions of 1:10, 1:20, 1:50, 1:100, 1:200

1:500, and 1:1, 000 were made with distilled water, and the maximum absorption peaks at various wave lengths were noted using a Bausch and Lomb Spectronic 20. A water blank was used in these determinations.

Two types of electrophoretic analysis were performed: namely, starch-gel and microphore. The method of Smithies (1955) was employed in the starch gel electrophoresis. A barbital buffer solution at pH 8. 8, ionic strength . 06 was used. The runs were made for 21 hours with a current of 125 volts across the strips. The only modification of the method as outlined by Smithies was that the strips were stained for total protein using Buffalo Black instead of Amido Black (10B). The method of clearing and staining was that of Vesselimouth (1958).

The electrophoresis on microphore strips (obtained from the Gelman Instrument Company) was carried out in Aronsson buffer. This buffer was made by dissolving 80. 66 grams of hydroxymethyl amino-methane, 10. 46 grams of disodium ethylenediamine-tetra-acetate, and 6. 3 grams of boric acid in 8 liters of distilled water. This gave a solution buffered at pH 8. 9. Procedures for using the microphore electrophoretic supports can be obtained from the Gelman Instrument Company (Technical Information, Manual 17). The strips were stained in Buffalo Black.

B. Preparation and Characterization of Antisera

All the antisera used in this investigation were prepared by injecting antigens in Freund adjuvant mixtures by subscapular route into adult rabbits weighing from 5 to 10 pounds. The adjuvant mixtures, available from Difco Laboratories, Detroit, Michigan, are of two types, complete and incomplete. The former is composed of mannide monooleate, 1.5 ml.; paraffin oil, 8.5 ml.; and 5 mg. of killed and dried Mycobacterium butyricum while the latter lacks the Mycobacterium butyricum. For each injection 1 ml. of antigen was mixed with 1 ml. of the adjuvant mixture in a syringe until a viscous emulsion was obtained. Three-quarters of a ml. of emulsion was injected under each scapula of the rabbit. The first injection was made using the complete adjuvant and one week later another injection using the incomplete adjuvant. From three to four weeks later, the rabbit was bled from the ear and the antiserum analyzed using the Ouchterlony technique as modified by Fox (1959). If the analysis revealed the presence of antibodies no further injections were made. The rabbit was bled every two weeks for a period of eight weeks and the titer of antibodies checked at each bleeding. At the last bleeding the animal was exsanguinated by cardiac puncture. After bleeding the blood was allowed to clot and the serum poured off and centrifuged at 1,500 RPM for 10 minutes to rid it of cells. After this the serum was dialysed against 0.65% saline and frozen until

used. Rabbits were always bled prior to injection for control serum. This control serum was analyzed by the aforementioned method to make sure there were no naturally-occurring antibodies to the various antigens tested. Antibodies against the hemoglobin solution from R. pipiens were obtained from six different rabbits. Bovine and human hemoglobin (obtained from Nutritional Biochemical Company), were also used to obtain antibodies as were various organ extracts of R. pipiens. The preparation of these organ extracts, which was the same as that for preparation of antigens used in the analysis, was as follows: The organs were removed, weighed, and homogenized in a glass homogenizer in 0.85% NaCl buffered at pH 7.4 with 0.005 M KH_2PO_4 - Na_2HPO_4 buffer solution in a ratio of one gram of tissue to 5 ml. of buffered solution. The homogenate was centrifuged at 1,500 x g and the supernatant fluid used in the various analyses.

The Ouchterlony plates were made as described by Shaver (1961). In the various plates run, the number of wells varied from 4 to 7 depending on the number of antisera being analyzed and the number of antigens used in the analysis. The geometry of the plates and the various analyses will be evident from the photographs and explanations in the section on results. Each of the wells held approximately 0.15 ml. of solution and in the process of their development received about 10 doses. An attempt was made to always refill the wells before they completely dried out.

In many of the analyses it was necessary to adsorb the antisera with various antigens before placing them in the wells for analysis. This was accomplished by homogenizing the appropriate tissues in the antisera or by mixing the already prepared antigen solution in various proportions with the antiserum and allowing the mixture to stand at 4^{o} C. for 24 hours. Subsequent to the absorption the plates were run as usual.

C. Determination of the Time and Place of Appearance of Erythrocytes and Hemoglobin

Two methods were used in attempting to determine the time and place of appearance of hemoglobin: namely, the Ouchterlony technique described above and various forms of the benzidene-peroxidase test for the catalysis by the heme group of the oxidation of benzidene by H_2O_2.

Representative developmental stages, beginning with body cavity eggs through the stage when the operculum is complete (Shumway stage 25), were tested for the presence of hemoglobin by the Ouchterlony method. Specifically the stages tested were the following: body cavity eggs, dejellied uterine eggs (Shumway stage 1), dejellied mid-blastulae (Shumway stage 8), dejellied mid-gastrulae (Shumway stage 11), dejellied neurulae (Shumway stage 16), tail bud (Shumway stage 17), muscular response (Shumway stage 18), gill circulation (Shumway stage 20), and operculum complete (Shumway stage 25). Antigens were prepared by homogenizing 200 embryos in 5 cc. of 0.85% NaCl buffered with 0.005

M phosphate buffer at pH 7. 4. The homogenate was placed in the cold (4^{o} C.) for a period of 24 hours and then spun in a refrigerated centrifuge at 4^{o} C. at 5, 000 x g for 20 minutes. The clear supernatant fluid was poured off and an estimation of its total protein was made using a modification (Lowry, et al., 1951) of the Folin-Ciocaltau (1927) method. Volumes of fluid representing equal amounts of total protein of the various stages were placed in separate wells of the Ouchterlony plates surrounding a central well containing an antiserum against hemoglobin. Also adsorptions of anti-hemoglobin sera with the various developmental stages were performed, and the adsorbed antisera analyzed on agar-diffusion plates.

A number of variations of the benzidene-peroxidase test were employed. These tests were performed on the above mentioned supernatant fluids of homogenized embryos, on whole intact embryos, and on histological sections. The method used by Lenicque (1959) in which 4 ml. of homogenate were mixed with 1 ml. of saturated benzidene and 1 ml. of 3% H_2O_2, and various modifications thereof were employed. The modifications consisted of varying the number of ml. of homogenate or of benzidene and H_2O_2 used, and also by varying the percentage of H_2O_2 (30%, 1%, 0. 6%) and the brand of benzidene used (Merk, Lamotte, NBC^{o} benzidene dihydrochloride). Bing's (1931) modification of Wu's method, Slominski's (1931) method, and O'Brien's (1961) method for

benzidene-peroxidase were also employed.

The most successful and by far the most sensitive of these methods was that of O'Brien. This method employs O'-Dionisidine (3, 3'-dimethoxybenzidene) instead of the benzidene employed in the other methods. Also the appearance of a yellow to orange color is indicative of a positive test with this method; whereas, the appearance of a brown, green, or blue color is indicative of a positive test in the other methods. In employing this method on histological sections, it was necessary to counter-stain with hematoxalin after fixing and staining in the O'-Dionisidine solution. Since it was not possible to use this method on embryos fixed and embedded in paraffin, they had to be quick-frozen with acetone and dry ice and sectioned on a cryostat (Harris-International, model CT). The embryos were embedded in a puree made of chick brain tissue and frozen to a cutting block. The amount of blood in the brain tissue was not excessive and rather than invalidating the technique served as a good control.

D. The Effects of Adult Hemoglobin and Anti-Hemoglobin Serum on the Differentiation of Erythrocytes and Hemoglobin

The eggs used in these experiments were obtained by the method of Rugh (1934). Insemination and subsequent preparation of the eggs were performed according to methods described in Hamburger (1960). After

the embryos developed to mid-blastula stage (Shumway stage 8), treatment was begun.

In the nine experiments carried out in the winter and spring of 1961, the eggs were injected with and cultured in the various solutions, however, in the twelve experiments carried out in 1962 the culturing method was dropped. The solutions routinely used in the treatments were the following: Niu-Twitty (1953) solution, normal rabbit serum, hemoglobin antisera, and hemoglobin solution. Some additional antisera and antigens were used in the 1962 experiments as will be seen in the section on results. The Niu-Twitty solution and the normal rabbit sera were employed as controls, because all dilutions of the antisera and antigens were made in Niu-Twitty solution. In addition to these two types of controls, a slide of fertilized eggs untreated in any way was also used in each experiment as a check on the viability of the eggs. No batches of eggs were used in these experiments in which there was not at least 90% cleavage. In the nine experiments carried out in 1961, dilutions ranging from 1:6 to 1:15 were employed for the control sera and antisera while dilutions ranging from 1:10 to 1:100 were employed for the hemoglobin solution. In the twelve experiments carried out in 1962 all antisera and control sera were diluted 1:15, while the hemoglobin solution was diluted 1:100. For culturing the Niu-Twitty solution was diluted 1:10.

The embryos which were cultured in the various solutions were mechanically dejellied, placed in a finger bowl (about 50 in each bowl) and covered with the appropriate solutions. They were kept at 15° C. and fresh solution added daily. After gastrulation they are placed in 10% of full strength Niu-Twitty solution and kept at 18° C.

Embryos were injected with micropipettes containing the various solutions at the mid-blastula stage (Shumway stage 8), utilizing a simple micro-injection apparatus. It should be mentioned here that the micropipettes used in the twelve experiments of 1962 were pulled on a Livingston Micropipette-puller instead of by hand and the tip diameter was smaller than the ones pulled by hand. In all cases the solutions were injected into the developing blastocoel through the animal hemisphere. An attempt was made to inject approximately the same amount of material into each egg. However, as there was no way to prevent some of the material from coming back out as the micropipette was removed, it was impossible to quantitate this method exactly. After injection the eggs were placed in finger bowls filled with aerated tap water and allowed to continue development at 18° C.

The embryos were observed periodically and careful records kept of the numbers dying and of those retarded at various stages and of the abnormalities appearing. At the time of the development of gill, and of subsequent circulation (Shumway stage 20), the gill size and

circulation were observed and comparisons made in four experiments. In five experiments embryos were fixed in Smith's modification of Bouin's fluid when normally-developing embryos were in the swimming stage (Shumway stage 21). At this time a number of the embryos in all the groups had not reached this stage due to retardation and obvious abnormalities. These embryos were also fixed at this time. Photographs were taken of representative normal and abnormal embryos. Embryos from five experiments were embedded in paraffin, serially sectioned at 15 microns, mounted, stained in hematoxylin and eosin, and examined for abnormalities. The embryos, which survived until the operculum was complete (Shumway stage 25), from two experiments, were homogenized, and subjected to the benzidene-peroxidase test as employed by Lenicque (1959). Comparisons were made between the various treatments.

RESULTS

A. Isolation and Characterization of the Hemoglobin Solution

A clear red hemoglobin solution was obtained as a result of the isolation procedure employed. Owing to the fact that the yield is very low, thus requiring large volumes of frog blood, it was not deemed practical to work with crystalline hemoglobin.

The spectrophotometric analysis showed three peaks of maximum absorption. One broad peak appeared at 400-425 mμ and two lesser peaks at 540-545 mμ and 575-580 mμ respectively, which are characteristic for oxyhemoglobin. These observations indicated that the preparation of hemoglobin employed met the conventional standards of purification.

The electrophoretic analysis on starch gel strips showed one large heavily staining band at about 2 cm. in the anodal direction and a questionable band continuous with this band (Plate 1, Figures 1 and 2). It could not be determined with certainty whether this second band was a separate band or a continuation of the first band. When the hemoglobin solution was used full strength there was considerable trailing but with a dilution of 1:10 this problem was alleviated (Plate 1, Figure 1). The frog serum which was migrated simultaneously with the hemoglobin solution showed 7 or possibly 8 bands and served as a control for the technique (Plate 1, Figure 2).

PLATE 1

Electrophoretic Analysis of the Hemoglobin Solution

Figure 1 Anodal end of starch gel strips on which hemoglobin solution was migrated, stained for total protein. Sample origin at extreme left.
Top strip -- full strength hemoglobin solution.
Bottom two strips -- 1:10 dilution of hemoglobin solution, two different runs. Note one, possibly two bands.

Figure 2 Anodal end of starch gel strips on which hemoglobin solution and frog serum were migrated, stained for total protein. Sample origin at extreme left.
Top strip -- 1:10 hemoglobin solution.
Bottom strip -- 1:10 frog serum. Note seven or eight bands.

Figure 3 Microphore strips on which hemoglobin solution was migrated, stained for total protein, origin, dark staining region on left; anodal end on right.
Top strip -- Sample size 5 lambda, migrated for 45 minutes at 5 milliamperes and 145 volts. Note only one band indicated.
Bottom strip -- Sample size 15 lambda, migrated for 3 hours at 3 milliamperes and 80 volts. Note possible second band separating from first.


1
2
3

The electrophoretic analysis on microphore strips gave similar results. When the solution was allowed to migrate for three hours at a current of 3 milliamperes and 80 volts across the strips one large darkly staining band appeared at about 2. 5 centimeters in the anodal direction (Plate 1, Figure 3, bottom). Continuous with this band in the anodal direction a lighter staining area appeared. When a smaller sample size was used and allowed to migrate for 45 minutes at a current of 5 milliamperes and 145 volts across the strips only one band was observed (Plate 1, Figure 3, top). Thus, the electrophoretic analysis showed the presence of two bands when the strips were stained for total protein.

B. Production and Characterization of the Antisera

All the rabbits injected with hemoglobin produced antibodies demonstrable by the formation of precipitation lines on the Ouchterlony plates at the first bleeding, and the antibody titers remained sufficiently high throughout all of the bleedings. When frog and bovine hemoglobin solutions were reacted with normal rabbit serum no reactions were observed (Plate 2, Figures 4 and 9). The number of lines formed by reacting hemoglobin solutions with the various antisera obtained against them, varied from one to three (cf. Plate 2, Figures 4 and 8). This variation was observed not only between the antisera obtained from different rabbits, but in the same antiserum when used in different

plates. However, when two and three lines were present they were extremely close together, sometimes being almost inseparable and very weak in most instances. When run simultaneously on the same plate the anti-hemoglobin sera from different rabbits showed precipitation lines which formed lines of identity with one another (Plate 2, Figure 5). Various dilutions of a hemoglobin solution were run against one hemoglobin antiserum: namely, full strength, 1:10, 1:50, 1:100, 1:1, 000, and 1:10, 000. When the solution was used full strength, the resulting plate was so dark that the lines were obscured, and dilutions of 1:1, 000 and above showed no visible lines. Plate 2, Figure 6, shows dilution of 1:10 (Well B), 1:50 (Well C), and 1:100 (Well D). It was observed that a 1:10 dilution of hemoglobin produced precipitation zones of optimal visibility. Thus, in all subsequent plates in this analysis a dilution of 1:10 was used. When hemoglobin antiserum was adsorbed with the hemoglobin solution by allowing a mixture of equal parts to stand for 24 hours at 4° C., no precipitation lines were formed between this adsorbed antiserum and hemoglobin (Plate 2, Figure 7).

It was shown that antisera produced against R. pipiens hemoglobin did not cross-react with mammalian hemoglobin. Two such heterologous hemoglobin solutions were used: namely, bovine and human. No precipitation lines were formed between the antiserum against R. pipiens hemoglobin and the heterologous hemoglobins (Plate 2, Figure 8).

Conversely, no lines were formed between an antiserum against bovine hemoglobin and the pipiens hemoglobin solution (Plate 2, Figure 9).

The problem of showing organ or tissue specificity was somewhat more difficult as some sources of antigens used (serum, heart, kidney, muscle and brain of R. pipiens) had a considerable amount of blood in them. Owing to the fact that it was impossible to eliminate all of the hemoglobin from these antigen mixtures, a number of plates had to be run utilizing adsorption techniques to demonstrate the specificity of the hemoglobin antisera.

Brain and muscle had less hemoglobin content than the other organs tested as evidenced by the fact that only faint precipitation lines appeared when the hemoglobin antiserum was reacted against them (Plate 3, Figure 10, Plate 4, Figure 16). Conversely, only faint precipitation lines appeared when antisera against brain and muscle were reacted against the hemoglobin solution (Plate 3, Figure 11). When the hemoglobin antiserum was adsorbed with brain by allowing a mixture of equal parts of antigen and antiserum to stand for 24 hours at 4° C., the precipitation lines between the hemoglobin antiserum and the homologous antigen were not taken out (Plate 3, Figure 12), again indicating that brain does not contain sufficient blood to adsorb the antibodies against hemoglobin.

Analyses made using frog serum as antigen against antihemoglobin serum, and antiserum against frog serum vs. hemoglobin, showed that

there was no contamination of the hemoglobin solution by the serum proteins. In only one case where there was obviously a considerable amount of hemolysis of erythrocytes in separating the serum from the cells, was there any indication of precipitation lines formed between the hemoglobin antiserum and frog serum (Plate 2, Figure 4, Plate 4, Figure 17). Conversely, no precipitation lines were formed between serum antiserum and the hemoglobin solution (Plate 3, Figure 14).

Reactions of the hemoglobin antiserum with heart, kidney, and the hemoglobin solution showed lines of identity between all three (Plate 3, Figure 15). The lines formed between heart and the hemoglobin antiserum were extremely weak, while the lines between kidney and the hemoglobin antiserum were strong, in fact just as strong as the lines between the hemoglobin antiserum and hemoglobin solution. This is what oue would expect if, as was observed, much more of the blood could be washed out of the hearts than out of the kidneys. Plate 4, Figure 16, shows that no precipitation lines appeared between the hemoglobin antiserum and heart. In this case special care was taken to get all the blood possible out of the hearts before homogenizing them. On the other hand Plate 4, Figure 17, shows a very strong line between heart and the hemoglobin antiserum. In this case a considerable amount of blood was purposely left in the hearts. When the hemoglobin solution was reacted against heart antiserum in one plate (Plate 3, Figure 11) precipitation

lines were formed and in another plate (Plate 4, Figure 18) they were not. Again this indicates a variability in the amount of blood present in the heart homogenate. When hemoglobin antiserum was adsorbed with heart, the precipitation lines between the hemoglobin antiserum and the hemoglobin solution were not taken out (Plate 4, Figure 19), indicating an insufficient amount of blood in these heart homogenates to adsorb all the antibodies against hemoglobin.

In all cases where kidney was reacted against the hemoglobin antiserum, strong precipitation lines appeared (Plate 3, Figure 15, Plate 4, Figure 19), which formed lines of identity with the hemoglobin lines where the wells were appropriately arranged (Plate 3, Figure 15). Kidney antiserum, when reacted with the hemoglobin solution on the same plate with hemoglobin antisera, showed lines of identity with the hemoglobin antisera (Plate 2, Figure 5). Plate 4, Figure 20 shows that when the hemoglobin antiserum is adsorbed with kidney all precipitation lines are taken out. And finally, when kidney antiserum and kidney antiserum adsorbed with hemoglobin solution were reacted against the hemoglobin solution and kidney on the same plate, the hemoglobin line was taken out, leaving a specific kidney line (Plate 4, Figure 21). Thus, it is obvious that adsorbing the kidney antiserum with hemoglobin removes the hemoglobin line.

PLATE 2

Analysis of Antisera Prepared Against Hemoglobin Solution, I

Figure 4 Ouchterlony Plate Number VI
Well A - R. pipiens hemoglobin solution
Well B - R. pipiens hemoglobin antiserum #82
Well C - R. pipiens serum
Well D - Control serum #82

Figure 5 Ouchterlony Plate Number 61
Well A - R. pipiens hemoglobin solution
Well B - R. pipiens hemoglobin antiserum #82
Well C - R. pipiens hemoglobin antiserum #93
Well D - R. pipiens hemoglobin antiserum #94
Well E - R. pipiens hemoglobin antiserum #117
Well F - R. pipiens hemoglobin antiserum #146
Well G - R. pipiens kidney antiserum #137

Figure 6 Ouchterlony Plate Number III
Well A - R. pipiens hemoglobin antiserum #82
Well B - R. pipiens hemoglobin solution 1:10
Well C - R. pipiens hemoglobin solution 1:50
Well D - R. pipiens hemoglobin solution 1:100

Figure 7 Ouchterlony Plate Number 10
Well A - R. pipiens hemoglobin antiserum #82
adsorbed with R. pipiens hemoglobin solution
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens serum
Well D - R. pipiens kidney homogenate

Figure 8 Ouchterlony Plate Number 21
Well A - R. pipiens hemoglobin antiserum #82
Well B - R. pipiens hemoglobin solution
Well C - bovine hemoglobin solution
Well D - human hemoglobin solution

Figure 9 Ouchterlony Plate Number 30
Well A - bovine hemoglobin solution
Well B - bovine hemoglobin antiserum #98
Well C - bovine hemoglobin antiserum #102
Well D - pipiens hemoglobin solution
Well E - control serum #98

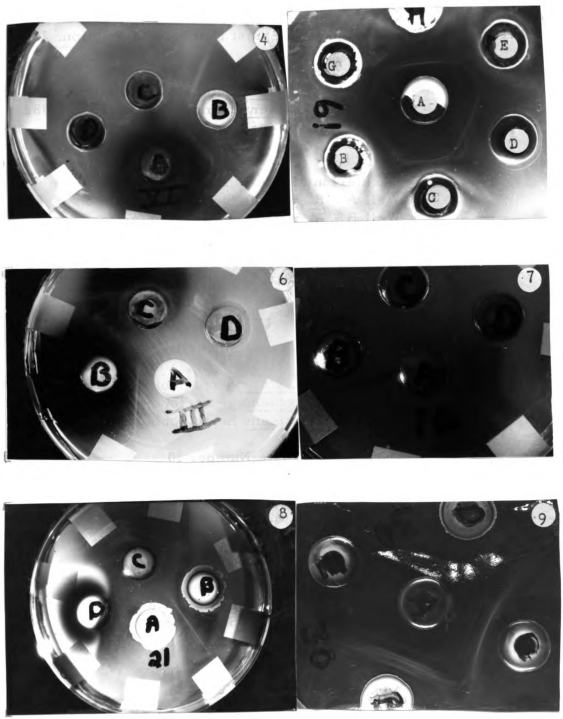

4
C
B
B
6
E
D
B
A
III
7
8
C
B
D
A
21
9

PLATE 3

Analysis of Antisera Prepared Against Hemoglobin Solution, II

Figure 10 Ouchterlony Plate Number 14
Well A - R. pipiens hemoglobin antiserum #82
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens brain homogenate
Well D - R. pipiens muscle homogenate

Figure 11 Ouchterlony Plate Number 22
Well A - R. pipiens hemoglobin solution
Well B - R. pipiens hemoglobin antiserum #82
Well C - R. pipiens heart antiserum
Well D - R. pipiens serum antiserum
Well E - R. pipiens muscle antiserum
Well F - R. pipiens brain antiserum
Well G - R. pipiens kidney antiserum

Figure 12 Ouchterlony Plate Number 41
Well A - R. pipiens hemoglobin antiserum #82 adsorbed with R. pipiens brain homogenate
Well B - R. pipiens serum
Well C - R. pipiens hemoglobin solution
Well D - R. pipiens muscle homogenate
Well E - R. pipiens brain homogenate

Figure 14 Ouchterlony Plate Number 13
Well A - R. pipiens serum antiserum #33
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens serum
Well D - R. pipiens heart homogenate

Figure 15 Ouchterlony Plate Number VII
Well A - R. pipiens hemoglobin antiserum #82
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens heart homogenate
Well D - R. pipiens kidney homogenate


10
D
A


11
F
G
A
C


12
B
C
D


14
C
D
B

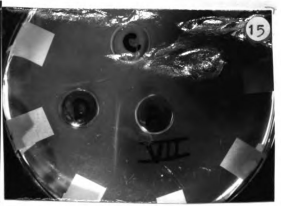

15
C
D
VIII

PLATE 4

Analysis of Antisera Prepared Against Rana pipiens Hemoglobin III

Figure 16 Ouchterlony Plate Number 11
Well A - R. pipiens hemoglobin antiserum #82
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens heart homogenate
Well D - R. pipiens brain homogenate

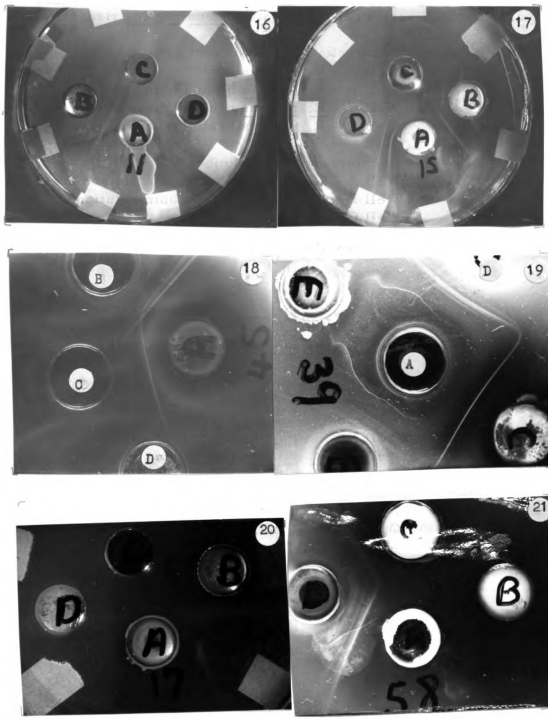
Figure 17 Ouchterlony Plate Number 12
Well A - R. pipiens hemoglobin antiserum #82
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens serum
Well D - R. pipiens heart homogenate

Figure 18 Ouchterlony Plate Number 45
Well A - R. pipiens hemoglobin solution
Well B - R. pipiens hemoglobin antiserum #117
Well C - R. pipiens kidney antiserum
Well D - R. pipiens heart antiserum

Figure 19 Ouchterlony Plate Number 39
Well A - R. pipiens hemoglobin antiserum #94
adsorbed with pipiens heart
Well B - R. pipiens heart homogenate
Well C - R. pipiens hemoglobin solution
Well D - R. pipiens kidney homogenate
Well E - R. pipiens brain homogenate

Figure 20 Ouchterlony Plate Number 17
Well A - R. pipiens hemoglobin antiserum #82
adsorbed with R. pipiens kidney
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens kidney homogenate
Well D - R. pipiens muscle homogenate

Figure 21 Ouchterlony Plate Number 58
Well A - R. pipiens kidney antiserum
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens kidney antiserum adsorbed with
R. pipiens hemoglobin solution
Well D - R. pipiens kidney homogenate


16
C
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18
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19
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20
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D
A
21
C
B

Thus, these results strongly indicate that the antibodies formed against the R. pipiens hemoglobin solution are complementary only to it, as the cross reactions with heterologous tissues can be shown to be proportional to the hemoglobin content of those tissues.

C. Determination of the Time and Place of Appearance of Erythrocytes and Hemoglobin

Using the Ouchterlony technique to determine the time of appearance of the hemoglobin molecule during development, it was observed that precipitation lines formed when body cavity eggs and all subsequent stages tested were reacted against the hemoglobin antiserum. These components in each stage reacting with hemoglobin antiserum formed lines of identity with each other and also with hemoglobin (Plate 5, Figures 22 and 23). During the development of these plates, volumes of homogenates representing equivalent amounts of total protein from the various stages were placed in the respective wells (7.5 mgs.). The intensity of the lines indicates that the reacting material was approximately the same concentration in all the stages tested. As can be seen from Plate 5, Figure 25, an antiserum to a homogenate of body cavity eggs reacted very weakly with the hemoglobin solution and the lines completely disappeared when the antiserum was adsorbed with the hemoglobin solution. This adsorption was performed by mixing equal parts of antigen and antiserum and allowing the mixture to sit for 24 hours at 4° C.

A number of plates (Plate 5, Figures 24, 26, 27, Plate 6, Figures 28, 29, 30 and 31), on which hemoglobin antiserum and hemoglobin antiserum adsorbed with various stages were reacted with the hemoglobin solution and the same stages as those used for adsorption, were run. The adsorptions were carried out by homogenizing the stages in the antisera in a ratio of 1 gram of the embryos to 5 c. c. of the antiserum and allowing the mixture to stand at 4^{o}C. for 24 hours before centrifuging. It can be seen from observing these figures that the precipitation lines are present in essentially the same patterns on all the plates and that the adsorption of the hemoglobin antisera using the preparations of adsorbing antigens cited above did not remove enough of the antibodies complimentary to the hemoglobin to inhibit the reactions. However, when the hemoglobin antiserum was adsorbed with larger quantities of antigens from developmental stages, the complimentary components can be removed. For example, Plate 6, Figure 32, illustrates the results of adsorbing hemoglobin antiserum with a large excess of antigen prepared from body-cavity eggs. This is evidenced by the lack of precipitation lines between the well containing the adsorbed antiserum (Well C) and those containing the hemoglobin solution and the body-cavity eggs (Wells B and D respectively). The excess adsorbing antigen reacted with the unadsorbed hemoglobin antiserum as is seen by the presence of lines between these two wells.

When the usual variations of the benzidene-peroxidase test (cf. page 28) were applied to homogenates, whole embryos, and sections of the same stages which were used in the agar-diffusion plates, just cited, no positive test was observed until the stage when the operculum is complete (Shumway stage 25). However, when O'Brien's (1961) technique, using 3, 3' dimethoxybenzidene instead of benzidene, was employed, positive tests for hemoglobin were obtained at the swimming stage (Shumway stage 21-22) on homogenates, on whole embryos and on histological sections. Plates 7 and 8 show sections through the heart-forming and "blood island" region of embryos in the tail bud and muscular response stages (Shumway stages 17-18). There is no yellow or orange color indicating hemoglobin in these sections. As can be seen (Plate 8, Figure 46) the technique was valid as the hemoglobin in the surrounding brain tissue, in which the embryos were embedded for sectioning, took the stain very readily. Plate 8, Figures 47 and 48 show a definite positive test in the heart region of embryos in the swimming stage (Shumway stages 21-22). Thus, it is apparent that no positive test for the presence of the heme group of the hemoglobin molecule could be obtained until the primitive blood cells were put into circulation.

At the time the blood cells appeared in circulation their structure was very dissimilar to that of adult blood cells. They appeared as large

spherical cells containing a nucleus and numerous yolk platelets (Plate 9, Figure 51). By the time the organism reached the larval stage (Taylor and Kollros stage 1) there were no blood cells with yolk platelets, the circulating cells being typical of the adult type, i. e. : being much smaller, nucleated, and elliptical in shape (Plate 9, Figure 50). When O'Brien's technique was employed on sections through the liver of these larvae, a positive test for hemoglobin was observed in several cells (Plate 9, Figure 49). The size and shape of these cells indicates that the liver at this time is hematopoetic. Thus, the indications are that the primitive cells containing yolk-platelets of the earlier embryo disappear as new hematopoetic regions take over producing cells structurally similar to adult erythrocytes.

PLATE 5

Determination of the Time of Appearance of the Hemoglobin Molecule. Analysis of Ouchterlony Technique, I

Figure 22 Ouchterlony Plate Number 68
Well A - R. pipiens hemoglobin antiserum #146
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens uterine egg homogenate
Well D - R. pipiens blastula homogenate
Well E - R. pipiens gastrula homogenate
Well F - R. pipiens neurula homogenate
Well G - R. pipiens tail bud homogenate

Figure 23 Ouchterlony Plate Number 80
Well A - R. pipiens hemoglobin antiserum #129
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens body cavity egg homogenate
Well D - R. pipiens blastula homogenate
Well E - R. pipiens gastrula homogenate
Well F - R. pipiens neurula homogenate
Well G - R. pipiens tail bud homogenate

Figure 24 Ouchterlony Plate Number 74
Well A - R. pipiens hemoglobin antiserum #129
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens hemoglobin antiserum #129 adsorbed with R. pipiens blastula homogenate
Well D - R. pipiens blastula homogenate

Figure 25 Ouchterlony Plate Number 71
Well A - R. pipiens body cavity egg antiserum
Well B - R. pipiens body cavity egg homogenate
Well C - R. pipiens body cavity egg antiserum absorbed with R. pipiens hemoglobin solution
Well D - R. pipiens hemoglobin solution

Figure 26 Ouchterlony Plate Number 76
Well A - R. pipiens hemoglobin antiserum #129
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens hemoglobin antiserum adsorbed with R. pipiens neurula homogenate
Well D - R. pipiens neurula homogenate

Figure 27 Ouchterlony Plate Number 75
Well A - R. pipiens hemoglobin antiserum #129
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens hemoglobin antiserum adsorbed with gastrula homogenate
Well D - R. pipiens gastrula homogenate

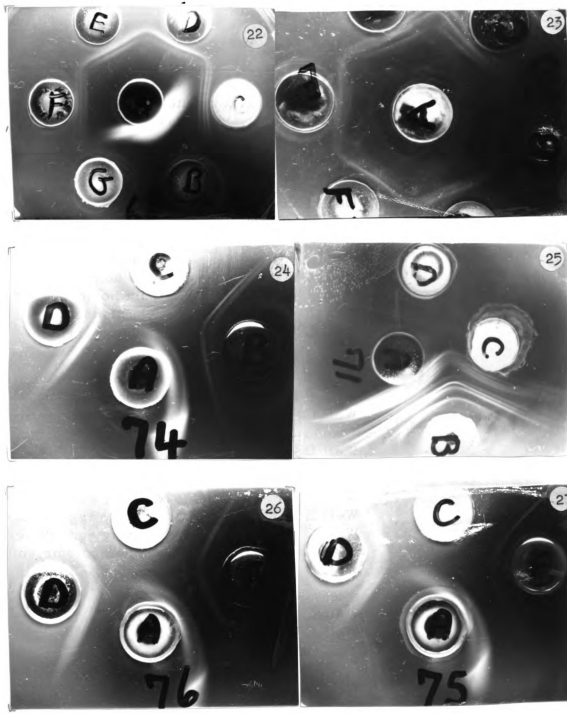
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PLATE 6

Determination of the Time of Appearance of the Hemoglobin Molecule. Analysis by Ouchterlony Technique, II.

Figure 28 Ouchterlony Plate Number 77
Well A - R. pipiens hemoglobin antiserum #129
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens hemoglobin antiserum #146 adsorbed with R. pipiens tail bud homogenate
Well D - R. pipiens tail bud homogenate

Figure 29 Ouchterlony Plate Number 78
Well A - R. pipiens hemoglobin antiserum #146
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens hemoglobin antiserum adsorbed with R. pipiens muscular response homogenate
Well D - R. pipiens muscular response homogenate

Figure 30 Ouchterlony Plate Number 79
Well A - R. pipiens hemoglobin antiserum #129
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens hemoglobin antiserum adsorbed with Shumway stage 18-19 homogenate
Well D - R. pipiens Shumway stage 18-19 homogenate

Figure 31 Ouchterlony Plate Number 86
Well A - R. pipiens hemoglobin antiserum #129
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens hemoglobin antiserum adsorbed with Shumway stage 25 homogenate
Well D - R. pipiens Shumway stage 25 homogenate

Figure 32 Ouchterlony Plate Number 88
Well A - R. pipiens hemoglobin antiserum #129
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens hemoglobin antiserum adsorbed with an excess of R. pipiens body cavity egg homogenate
Well D - R. pipiens body cavity egg homogenate

Figure 33 Ouchterlony Plate Number 94
Well A - R. pipiens hemoglobin antiserum #129
Well B - R. pipiens hemoglobin solution
Well C - R. pipiens globin solution
Well D - R. pipiens kidney homogenate

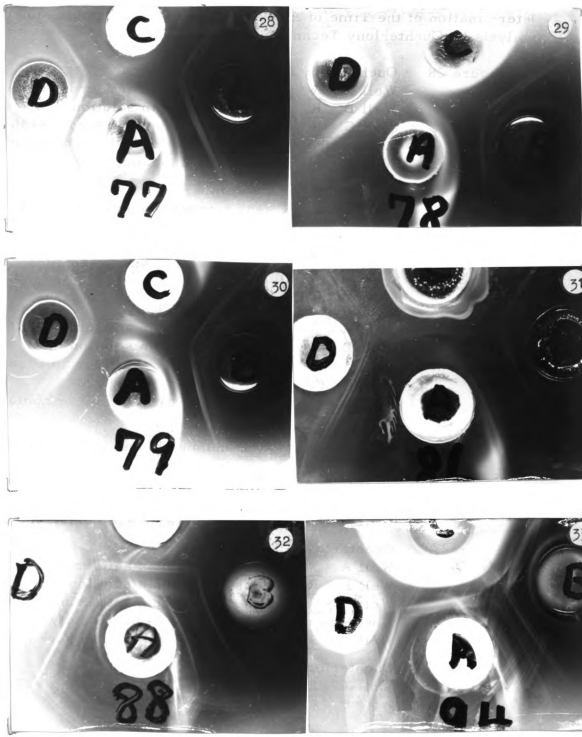
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PLATE 7

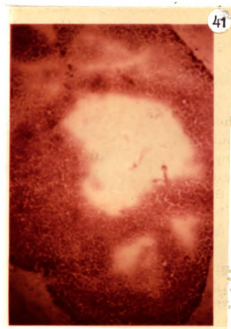
Determination of the Time and Place of Appearance of Hemoglobin by Applying the Benzidene-Peroxidase Test (O'Brien's, 1961) to Histological Sections, I

Figure 41 x. s. through the heart-forming region of a R. pipiens embryo in a tail bud stage (stage 12). Note the absence of a yellow or orange stain in this section. Heart forming region is at lower right neural tube at upper left, gut in the center.

Figure 42 x. s. through the "blood island" forming region of the same embryo as above figure. Note area of aggregation in ventral mesoderm (bottom) and lack of yellow or orange stain.

Figure 43 x. s. through the heart forming region of a R. pipiens embryo in the muscular response stage (stage 18). Heart forming region (bottom); gut, center. Note absence of positive test for hemoglobin.

Figure 44 x. s. through "blood island" region of the same embryo as in Figure 43. Note lacunae forming in ventral and lateral mesoderm. Note presumptive blood cells peripheral to lacuna on left. No indication of positive test.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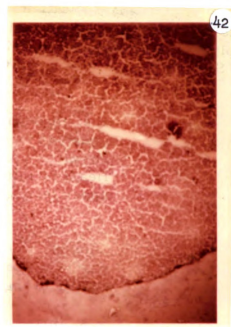
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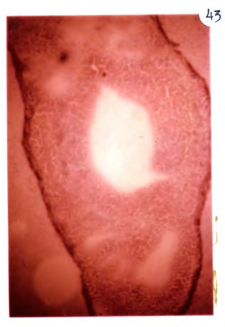
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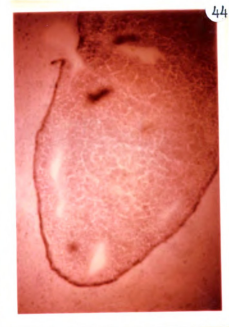
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PLATE 8

Determination of the Time and Place of Appearance of Hemoglobin by Applying the Benzidene-Peroxidase Test (O'Brien, 1961) to Histological Sections, II

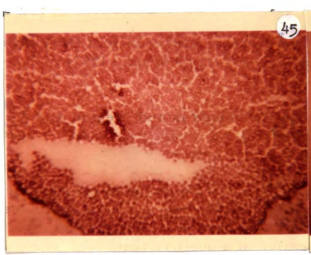
Figure 45 x. s. through the "blood island" region of a R. pipiens embryo in the muscular response stage (stage 18). Note lack of yellow or orange stain.

Figure 46 Blood vessel in the brain tissue in which embryos were embedded for sectioning. This blood vessel was found on the same section as Figure 45. Note orange stain indicative of a positive test.

Figure 47 x. s. through the heart region of a R. pipiens embryo in Shumway stage 21-22. Note orange stain in heart region. Section through posterior portion of ventricle.

Figure 48 Same embryo as Figure 47, x. s. through heart region just as the bulbus is coming in. Note orange stain in the heart region.

PLATE 8



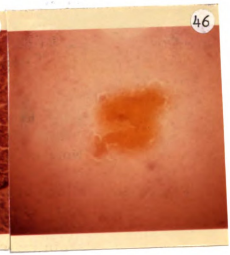


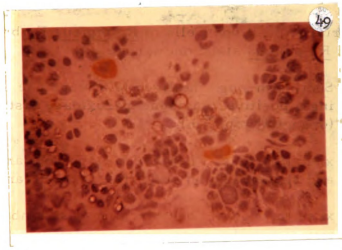
PLATE 9

Morphological Variation in Blood Cells of R. pipiens Embryos (Shumway stage 21) and R. pipiens stage one larvae.

Figure 49 Section through the liver of a stage one larva showing a positive benzidene-peroxidase test in some cells (orange cytoplasm).

Figure 50 x. s. through the heart of a stage one larva, note size and shape of blood cells (Hematoxylin and Eosin).

Figure 51 x. s. through the heart region of an embryo of Shumway stage 21. Note large, spherical blood cells laden with yolk-platelets (Hematoxylin and Eosin).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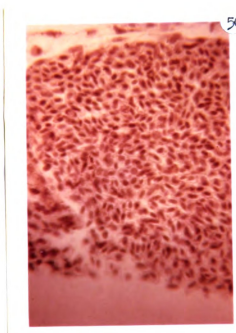
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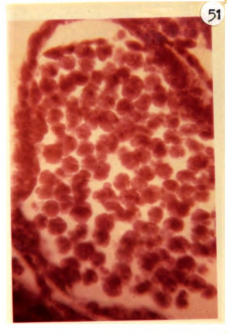
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D. The Effects of Adult Hemoglobin and Antibodies Produced Against It on the Differentiation of Erythrocytes and Hemoglobin

The results of the nine experiments carried out in the winter and spring of 1961 were pooled and the data are given in table form (Table 1, page 64). In Table 1, the numbers and percentages of embryos dying at certain developmental stages and those still living at Shumway stages 22-25 are presented. Embryos which became retarded at a certain stage and never attained the morphological characteristics of the next stage were classified as dying in the stage in which they became retarded, even though they may have been fixed and sectioned before they actually died. As is evidenced by the high percentage of the fertilized controls which were living at Shumway stage 22-25, untreated eggs were evidently viable.

All treatments employed produced significant decreases in the viability of the embryos. For instance, by culturing in Niu-Twitty solution the viability was reduced to 76.50% as compared to 92.38% in the fertilized controls. Using the chi-square test for heterogeneity, this difference proved to be highly significant. Since the percentage living to stages 22-25 after culturing in Niu-Twitty solution is considerably larger than after the other treatments, it is apparent that all the latter treatments were even more deleterious. It is equally obvious that treatment

by culturing embryos in the various solutions was much less detrimental than injecting them. For instance, when the number living in the group cultured in the hemoglobin solution is compared to the number living in the group injected with the hemoglobin solution, a chi-square of 47.75 was obtained, indicating a difference significant beyond the .01 level. Since this was the least significant difference obtained in comparing the culturing technique with the injection technique, it is evident that embryos are more susceptible to treatment by the injection technique. The fact that appreciable numbers of embryos died soon after injection (blastula stage) indicates that considerable mechanical damage may have taken place. Further evidence of this will be seen later.

The number of embryos living in each experimental group was significantly different from their control groups. For example, chi-square values greater than 100, (indicating highly significant differences), were obtained in each case when the group injected with control serum was compared with the group injected with the hemoglobin antiserum, and also, when the group injected with hemoglobin solution was compared to the group injected with Niu-Twitty solution. However, the differences between experimental groups were not significant. For example, when the group injected with a hemoglobin solution was compared with the group injected with the hemoglobin antiserum, a chi-

square of .9513 was obtained which would be expected between 30-50% of the time by chance alone. However, the greatest lethality in the group injected with the hemoglobin solution was in the gastrula stage, whereas in the group injected with the hemoglobin antiserum it was in the tail bud and muscular response stages.

One would expect substances which are generally toxic to produce their effects relatively early in development, while more specific effects would be produced later. For example, the fact that more embryos cultured in hemoglobin antisera died at the gastrula stage than at tail bud and muscular response stages, would indicate an interference with some basic metabolic pathway operating at the former time. Embryos injected with hemoglobin antisera, however, had a level of mortality at the tail bud and muscular response stages, to be expected if some specific effect peculiar to that stage was involved. Since the "blood islands" appear at around the tail bud and muscular response stages, one might conclude that substances specifically affecting them were contained in the hemoglobin antisera. When the number of embryos dying at the tail bud and muscular response stages in the group injected with hemoglobin antisera was compared with that in the group injected with control serum, a chi-square of 80.21 was obtained, indicating a significant difference beyond the .01 level. Thus, the indications from these prelim-

Table 1. --Inhibition of the Development of R. pipiens Embryos by Treatment with Adult R. pipiens Hemoglobin and Antisera against It. Pooled Data from Nine Experiments (1960-1961)

Treatment	Number Dying						No. living Sh. 22-25	Percentages Dying					% living Sh. 22-25
	T	B	G	N	TB & MR	Sh 20-21		B	G	N	TB & MR	Sh. 20-21	
F.C.	1,103	18	31	21	14	0	1,019	.63	2.81	1.90	1.30	0	92.38
NTSI	1,109	196	125	86	53	0	649	17.67	11.27	7.75	4.78	0	58.52
NTSC	349	0	45	22	15	0	267	0	12.90	6.30	4.30	0	76.50
CSI	1,187	148	316	191	193	5	334	12.47	26.6	16.10	16.26	.42	28.14
CSC	324	0	136	25	19	2	142	0	41.98	7.72	5.86	.62	43.83
HAI	1,405	203	384	197	443	10	168	14.45	27.33	14.02	31.53	.71	11.96
HAC	333	0	103	41	15	0	174	0	30.93	12.31	4.50	0	52.25
HSI	1,190	186	511	185	98	14	196	15.63	42.94	15.55	8.24	1.18	16.47
HSC	324	0	160	26	24	5	109	0	49.38	8.02	7.41	1.77	33.64

T = Total number of embryos
B = Blastula
G = Gastrula
N = Neurula
TB & MR = Tail Bud and Muscular Response Stages
SH = Shumway Stages

FC = Fertilized Control
NTSI = Niu-Twitty Solution Injected
NTSC = Niu-Twitty Solution Cultured
CSI = Control Serum Injected
CSC = Control Serum Cultured
HAI = Hemoglobin Antiserum Injected
HAC = Hemoglobin Antiserum Cultured
HSI = Hemoglobin Solution Injected
HSC = Hemoglobin Solution Cultured

inary experiments were that the injection technique was superior to the culturing technique for producing what could possibly be interpreted as due to specific effects.

Twelve experiments were carried out in 1962 in which the culturing technique was dropped, and in addition to the treatments employed in the 1961 experiments, some heterologous antisera were also employed. The results of these experiments were pooled and the data are given in Table 2, (Page 67).

The results from these twelve experiments were not consistent with the results from the first nine experiments, as can readily be seen by comparing Tables 1 and 2. None of the treated embryos in the 1962 experiments died in the blastula stage, and the percentages dying as gastrulae were consistently smaller than in 1961. This may be accounted for by the fact that the micropipettes used in the 1962 experiments were machine-pulled rendering the tip diameter considerably smaller, so that less mechanical damage was done to the embryo. Even though there were significant differences at the .01 level between the treated embryos and the fertilized controls in the 1962 experiments, the chi-square values were not nearly as large as those in the 1961 experiments. For instance, when the group injected with Niu-Twitty solution was compared to the fertilized control group in the 1962 experiments a chi-

square of 18.7 was obtained, whereas the same comparison in the 1961 experiments gave a chi-square of 340.1. When the group injected with control serum was compared with the group injected with Niu-Twitty solution in the 1961 experiments, a chi-square of 45.74 was obtained which indicates a significant difference beyond the .01 level. However, as compared to a chi-square of 214.93 obtained from the same comparison in the 1961 experiments, this chi-square is relatively small. Contrary to what was expected on the basis of the 1961 experiments, the group injected with the hemoglobin antisera exhibited even better development than the group injected with control serum. In addition, there was no significant difference in the number of embryos dying at the tail bud and muscular response stages between the groups injected with antiserum and control serum as a chi-square of .23146 was obtained. A chi-square this large would be expected 50-70% of the time by chance alone. Thus, the apparent specific effect of the hemoglobin antiserum obtained in the 1961 experiments, was not corroborated in the 1962 experiments.

Of the 21 experiments carried out in both years, the embryos which survived in the first four experiments were observed until they reached stage 1 larvae. Comparisons between gill development, circulation through the gills, and the time at which the blood turned red in the

Table 2. --Inhibition of the Development of R. pipiens Embryos by Treatment with Adult R. pipiens Hemoglobin and Antisera Against It. Pooled Data of Twelve Experiments (1961-1962)

Treatment	Number Dying					No. living Sh. 22-24	Percentage Dying				Percentage Living Sh. 22-25
	T	B	G	N	TB & MR		B	G	N	TB & MR	
FC	709	16	15	24	25	629	2.26	2.12	3.39	3.53	88.72
NT	548	0	47	36	30	435	0	8.58	6.57	5.47	79.38
CS	710	0	135	88	48	439	0	19.01	12.39	6.76	61.83
APH	810	0	114	121	69	506	0	14.07	14.94	8.52	62.47
PH	629	0	143	164	61	261	0	22.73	26.07	9.70	41.49
ABovH	205	0	35	12	8	150	0	17.07	5.85	3.90	73.17
AHumH	121	0	16	0	9	96	0	13.22	0	7.44	79.34
ABR	135	0	8	7	5	115	0	5.93	5.19	3.70	85.19

T = Total number of embryos
B = Blastula
G = Gastrula
N = Neurula
TB & MR = Tail Bud and Muscular Response Stages
Sh = Shumway Stages

FC = Fertilized Control
NT = Niu-Twitty Solution
CS = Control Serum
APH = pipiens hemoglobin antiserum
PH = pipiens hemoglobin solution
ABovH = Bovine hemoglobin antiserum
AHumH = Human hemoglobin antiserum
ABR = pipiens brain antiserum

embryos after the various treatments were made. There were no noticeable differences between them. In all the embryos observed, the gills developed normally, circulation of a colorless fluid containing cells appeared around Shumway stages 20-21, and the circulating fluid turned pink about Shumway stage 23 and red in stage 1 larvae.

The histological examination of the embryos which were living when normal embryos were in Shumway stage 21, from five experiments, revealed only general defects. Plates 10 and 11 show the morphological appearance of the normal embryo of this stage and the types of abnormalities which appeared in the various groups. Most of the embryos which were abnormal were edematous to some degree and exhibited very poor gill formation or were completely lacking gills. Many of them had warty growths on the ventral surface. The histological examination of these embryos revealed that the heart was completely absent or greatly retarded. Plates 12, 13 and 14 show the types of abnormalities observed. The most common defect was of heart formation, which was usually combined with a general retardation of growth. Many of these embryos also showed open or solid neurocoels, and solid digestive tracts. The results of these five experiments were pooled and are given in Table 3.

PLATE 10

Morphological Abnormalities Resulting from Injection Treatments, I (These are typical abnormalities which appeared in all treatments employed.)

Figure 34 Normal swimming larva (Shumway stage 21) representing stage of embryos developing normally at the time of analysis, dorsal view.

Figure 35 Same embryos as above, side view.

Figure 36 Embryo injected with hemoglobin solution.

Figure 37 Embryo injected with Niu-Twitty solution.


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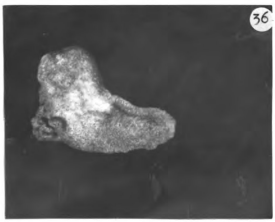
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PLATE 11

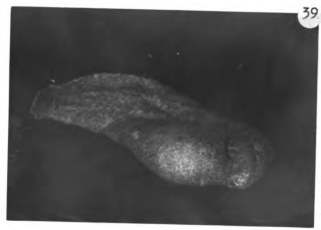
Morphological Abnormalities Resulting from Injection Treatments, II (These are typical abnormalities which appeared in all treatments employed.)

Figure 38 Embryo injected with hemoglobin antiserum.

Figure 39 Embryo injected with control serum.

Figure 40 Embryo injected with control serum. This abnormality was found in only one experiment in which every embryo injected with control serum showed it.

PLATE 11



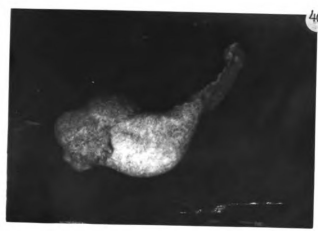


Table 3. --Pooled Results of Histological Analysis of Embryos of Five Experiments

Treatment	Total Number of Embryos	Number Showing General Abnormalities	Percentage Showing General Abnormalities
Fertilized Control	492	13	2.64
Niu-Twitty Injected	245	10	4.08
Control Serum	355	34	9.58
Hemoglobin Antisera Injected	305	33	10.82
Hemoglobin Solution Injected	146	10	6.85

Not only were no specific effects on the development of blood cells by the hemoglobin antisera and hemoglobin solution observed, but also there were no significant differences between them and their respective controls in regard to the general abnormalities produced. Plate 12, Figures 53 and 54 and Plate 13, Figures 55 and 56 show that embryos lacking hearts exhibit very good blood cell formation in the hematopoetic regions. When the number of embryos exhibiting general abnormalities in the group injected with hemoglobin antiserum was compared with the group injected with control serum by the chi-square test for heterogeneity, a chi-square of .15805 was obtained. A chi-square this large would be expected about 70% of the time by chance alone.

PLATE 12

Types of Histological Abnormalities Exhibited by Treated Embryos, I

Figure 52 x. s. through the heart region of a normal embryo (Shumway stage 21).

Figure 53 x. s. through the heart region of an embryo injected with hemoglobin antiserum. Note thickened neural tube, almost solid gut, and greatly reduced and abnormal heart.

Figure 54. x. s. through hematopoetic region of the same embryo as Figure 53. Note well developed blood cells typical of primitive type.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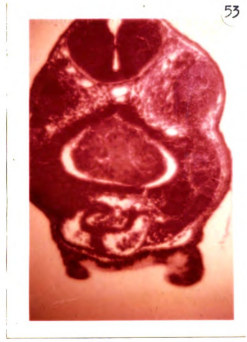
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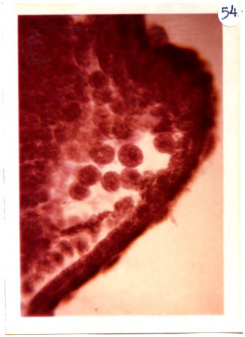
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PLATE 13

Types of Histological Abnormalities Exhibited by Treated Embryos, II

Figure 55 x. s. through heart region of an embryo injected with R. pipiens hemoglobin solution. Note that heart is practically absent, gut practically solid, very little mesenchyme in head region.

Figure 56 x. s. through hematopoetic region of same embryo as Figure 55. Note large number of blood cells at right of liver.

Figure 57 x. s. through heart region of an embryo injected with Niu-Twitty solution. Note heart (lower left) practically absent, very poor differentiation in general.


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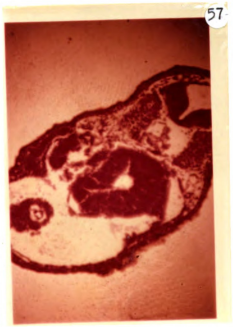
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PLATE 14

Types of Histological Abnormalities Exhibited by Treated Embryos, III

Figure 58 x. s. through heart region of an embryo injected with control serum. Note complete absence of heart.

Figure 59 x. s. through forebrain of same embryo as Figure 58. Note open neurocoel.

Figure 60 x. s. through heart region of an embryo untreated (fertilized control). Note heart absent, neurocoel solid.


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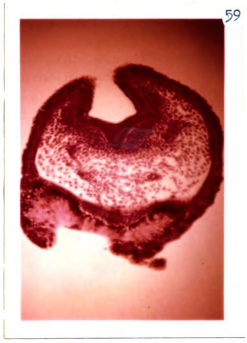
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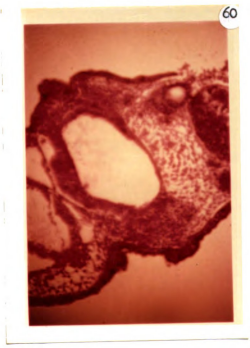
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A comparison between the group injected with the hemoglobin solution and the group injected with Niu-Twitty solution revealed a chi-square of .92874 which would be expected 30-50% of the time by chance alone.

The results of applying the benzidene-peroxidase test to the embryos which survived until Shumway stage 25 from two experiments are given in Table 4 below.

Table 4. --Comparison of Hemoglobin Content of Embryos in Shumway Stage 25 as Reflected by Optical Density After Applying the Benzidene-Peroxidase Test.

Treatment	Experiment Number	
	V Optical Density	VI Optical Density
Fertilized Controls	.215	.192
Niu-Twitty Solution	.210	.215
Control Serum	.205	.227
Hemoglobin Antiserum	.206	.225
Hemoglobin Solution	.211	.245

In experiment V, twelve embryos from each group were used whereas in experiment VI, Fifteen from each group were used. As in obvious from Table 4, the optical densities of the treatments in each experiment are probably as close as experimental error would allow. There is certainly no indication of inhibition of hemoglobin formation by the antigen or antiserum in either experiment.

DISCUSSION

A. Isolation and Characterization of the Hemoglobin Solution

As has been pointed out in the section on results, it was not practical in this investigation to crystallize the hemoglobin, as the number of frogs which would have had to be sacrificed for such an undertaking would have been enormous. However, the indications from the spectrophotometric and electrophoretic analysis were that the hemoglobin solution was relatively pure. The peaks of maximum absorption which were observed on spectrophotometric analysis (400-425 mμ, 540-545 mμ, 575-580 mμ) correspond almost exactly with those listed by Fruton and Simmonds (1959) for oxyhemoglobin. They state that all porphyrins exhibit a broad peak or band at around 400 mμ termed the Soret band, and in addition, oxyhemoglobin shows two peaks of maximum absorption at 543 mμ and 578 mμ respectively.

The appearance of not more than two and possibly only one band in the electrophoretic analysis, when the strips were stained for total protein, is also indicative of a relatively pure solution. It is known that there are small amounts of enzymes in hemolysates of red cells of other species which on incubation with the proper substrates show up as bands on electrophoretic strips. If these were present in the frog hemoglobin solution, they were not visualized in the staining for total protein. The

appearance of 7 or 8 bands when the frog serum was migrated indicated that the technique employed was adequate for protein separation. As for the number of bands which appeared in the electrophoresis of the frog hemoglobin, there are a number of reports on electrophoretic analysis of normal adult human hemoglobin showing one, two and three components (eq. Kunkel, 1958, Derrien, 1958). These workers report that purified normal adult human hemoglobin (hemoglobin A) has one major component, which forms a large band, and two minor components, one faster moving and one slower moving, which form somewhat vague minor bands. Thus, if an analogous situation exists in frog hemoglobin, the appearance of more than one band would not necessarily preclude the possibility that the solution was relatively pure. In addition, the fact that antibodies produced against the frog hemoglobin solution seemed to exhibit specific complementarity for the antigen indicates a relatively pure solution.

B. Production and Characterization of Antisera

As mentioned in the results, the production of antibodies against frog hemoglobin by the Freund adjuvant technique was very successful, as all the rabbits injected produced strong titers by the time of the first bleeding. Even though the titers seemed to drop somewhat in the later bleedings they were still sufficient to form visible precipitation lines

when reacted with the hemoglobin solution.

The analysis of the antisera produced against the adult R. pipiens hemoglobin solution very strongly suggests that the antibodies were specific for R. pipiens hemoglobin. For example, the observed amounts of blood present in the various tissues employed in the analyses allowed fairly accurate predictions of whether precipitation lines would form against hemoglobin antiserum on the agar-diffusion plates. As an illustration, one would expect extremely weak lines to form between brain or muscle homogenates and the hemoglobin antisera, or between brain and muscle antisera and the hemoglobin solution, owing to the small amount of hemoglobin present in washed minces of these tissues. Observations of agar-diffusion plates utilizing these reagents supported these expectations. Absorption of the hemoglobin solution by brain and muscle homogenates would not be expected to affect the formation of lines between hemoglobin antiserum and hemoglobin solution, and this is precisely what the results of this test show.

Two possibilities could account for the reactions on the plates between the hemoglobin antisera and frog serum. 1) If there was contamination of the hemoglobin solution by the serum proteins, or 2) if a considerable amount of hemolysis of the red cells had taken place in separating the serum from the cells. The results indicate that the former

was not the case since antiserum against frog serum showed no precipitation line formation when reacted with hemoglobin solution. As for the second possibility, only one case in which there was obvious hemolysis of red cells in separating them from the serum, showed any reaction on plates between frog serum and hemoglobin antiserum.

One would expect extremely weak lines, if any, to appear when heart homogenates were reacted with the hemoglobin antisera, if precautions had been taken to remove all the blood possible from the hearts before homogenizing them. If these precautions were not taken, one would expect rather strong lines to appear. By the same token, one might expect variable results when heart antisera were reacted with the hemoglobin solution, and also variable results when the hemoglobin antisera were adsorbed with heart. Again, the results bear out these predictions, as was pointed out in describing the analyses where hearts were deliberately either exsanguinated as much as possible, or not exsanguinated at all.

In the case of kidney which has an extremely high content of hemoglobin, and from which it is extremely difficult to wash out of the blood would expect precipitation lines to appear when the kidney brei was reacted with hemoglobin antisera, which would be identical with the lines between the hemoglobin solution and its antiserum. For the same

reasons, one would expect that an antiserum against kidney would produce precipitation lines when reacted with the hemoglobin solution, and that these lines would form identities with those between hemoglobin and hemoglobin antiserum. If the precipitation lines formed between hemoglobin antiserum and kidney antigen were indeed identical with those between the hemoglobin solution and hemoglobin antiserum, then one would expect that adsorbing the hemoglobin antiserum with kidney antigen would remove the lines formed between the hemoglobin antisera and the hemoglobin solution. Conversely, one would also expect the adsorption of kidney antiserum with hemoglobin solution to remove the line identical with that between hemoglobin solution and hemoglobin antiserum and leave a specific kidney line. Again the results of the plate made to test these expectations fully confirmed the predictions just described.

The fact that the number of precipitation lines which appeared between hemoglobin and hemoglobin antisera varied from 1 to 3, parallels a similar observation made in the immunological analysis of normal adult human hemoglobin (Hemoglobin A) by Chernoff (1959). He attributed these results to the fact that normal, slow, and fast components of hemoglobin A, sometimes separated and sometimes did not. In addition to this explanation of the multiple components of the frog hemoglobin, there is also the possibility that the frog hemoglobin molecule breaks down into smaller units with differential rates of diffusion, thus forming separate lines

very close to one another. This seems even more likely when it is recalled that the frog hemoglobin solution was prepared in distilled water rather than in a buffer solution and that it was frozen and thawed during the period when these analyses were being made. The fact that six of the hemoglobin antisera showed lines of identity with one another when reacted with the hemoglobin solution on the same plate indicated a remarkable consistency of these preparations, as it is not at all uncommon for different rabbits to exhibit differential reactions to the same antigen in respect to antibody formation. On the basis of the predictions made and the results obtained from these analyses, it would appear that antibodies with a high degree of specific complementarity to adult R. pipiens hemoglobin were obtained.

C. Determination of the Time and Place of Appearance of Erythrocytes and Hemoglobin

The fact that the homogenates of body cavity eggs and all subsequent stages tested showed precipitation lines identical with one another and with those formed between the adult hemoglobin solution, and the hemoglobin antiserum at first would seem to indicate the presence of a tissue-specific protein before the differentiation of the tissue for which it is characteristic. Previous investigators have demonstrated the presence in eggs and early embryos of antigens identical with protein in adult tissues. For example, Cooper (1948) obtained antisera against

the washed yolk-platelet fraction of frog eggs and showed that it reacted with adult frog serum. Flickinger and Nace (1952) showed that the oocytes of Rana temporaria contain antigens like those in the serum of the adult. Considering the various extracts and antisera used by Cooper and by Flickinger and Nace, there seems to be little doubt that antigens very much like the adult serum proteins are present throughout the life history of the amphibian. Nace (1953) has duplicated these findings in the chick and makes the suggestion:

> ". . . the proteins of the yolk form a source of serum protein available to the embryo until its own synthetic mechanism is established, and secondly, that such proteins, originally derived intact from the maternal organism may provide the models (templates) for serum protein synthesis by the embryo."

The whole problem of heterosynthesis versus autosynthesis is thoroughly reviewed by Schechtman (1955), and considerable evidence from a number of areas is cited in favor of the idea of the heterosynthesis of macromolecules by the maternal organism which are put into the embryo. Flickinger and Rounds (1956) have shown quite clearly using radioactive phosphorus, that the site of synthesis of the yolk proteins of the egg is the liver in the hen.

However, it would be impossible to say definitely on the basis of the technique used in the present analysis that the antigens of the various stages tested which formed precipitation lines with the hemoglobin antiserum had a molecular structure identical to that of the adult hemoglobin

molecule. The fact that adsorption of adult hemoglobin antiserum with large quantities of homogenates of body cavity eggs removed the precipitation lines between the adult hemoglobin solution and the adult hemoglobin antiserum certainly indicates that there must be a number of active sites common to both molecules, but this result cannot be interpreted to prove that they are identical. In fact, the results of the benzidene-peroxidase tests show that they cannot be identical, since no positive test for the heme group of the hemoglobin molecule could be obtained until Shumway stages 21-22. O'Brien's technique employing 3, 3' dimethoxybenzidene instead of benzidene is an extremely sensitive test, in fact, much more sensitive than the agar-diffusion technique. Thus, it appears that the synthesis of the heme portion of the hemoglobin molecule in R. pipiens does not take place until relatively late, while the globin portion of the molecule or a molecule with a very similar configuration is present even before fertilization. The question of whether this globin molecule is put into the egg by the maternal organism or is synthesized in the egg cannot be answered at this time, but it is evident that the synthesis of heme and globin is not necessarily coeval in the embryo. This same conclusion was reached by Wilt (personal communication, 1962) working with the chick. Wilt observed that the globin was present in the chick egg from 0 hours of development on, but heme synthesis did not begin until about 24-30

hours of development when the blood turned red.

Thus, it appears that the heme group is not necessary in the antigen-antibody complexing of the hemoglobin molecule and antibodies against it. When the adult R. pipiens hemoglobin was separated into its two components, heme and globin, by acetone-HCl treatment (10 vol. 1% HCl in acetone added to the hemoglobin solution) and the globin filtered out, washed, redissolved, and reacted on an agar-diffusion plate against a hemoglobin antisera, precipitation lines were formed. Plate 6, Figure 33 shows that definite precipitation lines were formed between the globin solution and the hemoglobin antiserum, even though there was a non-specific precipitation in the agar which obscured them to some extent. This was probably due to the fact that once the globin was separated from the heme and washed several times in the acetone-HCl solution, it was dissolved in a medium of greater alkalinity than that of the agar. Wilt (personal communication) also observed that the heme group of the hemoglobin molecule in the chick was unnecessary for the antigen-antibody complexing of the two molecules.

The observations made in this study on the development of erythrocytes were to a great extent similar to those made by Fernald (1947) working with Hyla regilla which are discussed in the introduction. In R. pipiens the primitive blood cells begin to appear in the "blood island" region at the tail bud and muscular response stages and are

put into circulation after the heart begins to beat (Shumway stage 21). These cells are laden with yolk platelets and are colorless. At Shumway stage 25, the blood appears pink in color turning red in the feeding stage (Taylor and Kollros stage 1 larvae). The erythrocytes at this time are devoid of yolk platelets and morphologically similar to adult red blood cells. Sections through the liver of these larvae indicated that this organ is hematopoetic at that time. The present observations were also in accord with those of Cameron (1949) working with Amblystoma jeffersonianum. Cameron described eight generations of primitive blood cells containing yolk platelets which disappear about 24 hours after hatching and are replaced by yolk-free cells coming from other hematopoetic regions.

Frieden (1961) presented very good evidence for an alteration in the properties and biosynthesis of hemoglobin at metamorphosis in the anuran, Rana grylia. He has shown by paper electrophoresis that the one hemoglobin band present in tadpole red cell hemolysates begins to fade during metamorphosis and is replaced as the major band by a second band which begins to appear at that time. In addition he identified a non-heme-containing precursor in tadpole red cell hemolysates which was converted into a new hemoglobin during metamorphosis. The appearance of this new hemoglobin was accompanied by demonstrable Fe^{59} uptake.

Considering the results of the present study, in addition to the other works mentioned above, the indications are that there are at least two and possibly three types of hemoglobin present during the life cycle of amphibians. The fact that an antiserum against adult R. pipiens hemoglobin reacted with body cavity eggs and all subsequent stages up to Shumway stage 25, indicates the presence of a globin, structurally similar to or possibly identical with that in adult hemoglobin, in eggs and earlier stages which is localized in the primitive erythrocytes when they appear. The fact that these primitive cells disappear and are replaced by cells from other hematopoetic regions might suggest the synthesis of a new hemoglobin (fetal hemoglobin). However, a shift in the site of synthesis would not necessarily imply a change in the substance synthesized. As no immunological analysis was made of the hemoglobin during the larval stages, nothing definite can be stated on this point. The work of Freiden on amphibians and of numerous workers on other organisms, clearly indicates a change in the structure of the hemoglobin molecule at metamorphosis. Thus there is evidence that in anurans there exist a primitive hemoglobin, a fetal hemoglobin, and an adult hemoglobin. However, the primitive hemoglobin might be the same as fetal hemoglobin or the same as adult hemoglobin. As it is virtually impossible to bleed amphibian embryos at the time these primitive cells are present, unfortunately

no electrophoretic analysis can be made of the hemoglobin present at that time. However, in this study, since the globin present in the egg and all subsequent stages up to Shumway stage 25 exhibited lines of identity on agar-diffusion plates with the adult hemoglobin solution when reacted against an antiserum against adult hemoglobin solution, the indications are that this "primitive" hemoglobin might be identical to the globin of the adult hemoglobin molecule.

The idea of three types of normal hemoglobin has also been postulated by workers on organisms other than amphibians. Halbrecht and Klibonski (1956) postulated a primitive embryonic hemoglobin in the human. They recovered a ten week old embryo from an intact extra-uterine pregnancy and compared the hemoglobin from it to that of cordal hemoglobin (fetal hemoglobin) and adult hemoglobin, by determining the amount of alkaline-resistant hemoglobin present and by paper electrophoresis. They found the hemoglobin from this ten-week old embryo to differ from that of cordal blood and adult hemoglobin. Also Allison (1955) has obtained evidence in the mouse for a primitive hemoglobin different from fetal hemoglobin and adult hemoglobin. He asserts that the processes leading to the formation of the three normal hemoglobin types (P, F, and A) seem to be under independent genetic control. Gene f in the mouse, which when present in double dose, severely retarded the production of fetal hemoglobin but

had no influence on the synthesis of the primitive and adult hemoglobin types. This gene in double dose produced a severe hypochronic anemia in late fetal life but a complete recovery occurred after birth.

In accord with Nace's (1953) suggestion concerning the serum proteins mentioned previously in this section, it seems to be possible, if not probable, that the globin, which results from a breakdown of hemoglobin, possibly in the liver of the maternal organism, is put into the yolk platelets of the developing oocyte. This globin could well be identical to the globin in the adult hemoglobin molecule as the immunological analysis seems to indicate. At the time when heme synthesis takes place (Shumway stage 21-22) this globin may be made available by yolk solubilization and may complex with the heme being synthesized to form the hemoglobin of the primitive erythrocytes. This hemoglobin would disappear with the primitive cells containing it, as other hematopoetic regions take over, and fetal hemoglobin would be synthesized in these new sites. Then at metamorphosis, the fetal hemoglobin would be replaced as the major hemoglobin by adult hemoglobin.

D. The Effects of Adult Hemoglobin and Antibodies Produced Against it on the Differentiation of Erythrocytes and Hemoglobin

As was pointed out in the introductory section, one group of investigators believes that substances from differentiated cells exert a

specific inhibitory effect on the growth and differentiation of the homologous embryonic cell types, whereas others feel that such substances specifically stimulate growth and differentiation. The most convincing evidence for the former point of view comes mainly from the work on regenerating systems. Whether a regenerating system can validly be assumed to be the same in regard to mechanisms of differentiation as an embryonic system remains to be seen. The evidence for specific inhibitory effects of adult tissue substances on differentiating systems in embryos is much less convincing due to the great possibility of non-specificity in these experiments.

The bulk of the evidence which has been used to support the idea, that substances from differentiated cells specifically stimulate the processes of differentiation and growth in homologous embryonic cell types, comes mainly from experiments in which chicken spleen was grafted onto the chorioallantoic membrane of the chick embryo, the spleen of which showed subsequent hypertrophy. In light of the mixed content of cells in the spleen, it is thought by many workers that it is not a typical tissue. Also, the work of Van Alten and Fennell (1959) strongly indicates that numerous tissues when placed on the chorioallantoic membrane will bring about spleen hypertrophy in the host chick embryo. Thus, in this case also there seems to be a lack of demonstration of specific reactions.

A number of workers employing antisera against substances from differentiated cells claim to have obtained specific inhibition of the differentiation and growth of the homologous embryonic cell types. If an antigen-antibody type of mechanism is involved in the processes of differentiation and growth, then these results would seem to be contradictory to the idea that substances from differentiated cells exert a specific inhibitory effect, as one would expect antigen and antibody to have opposite effects. Some of the experimental results in favor of this idea are quite convincing (Ebert, 1950, Owens, 1961). However, the bulk of the evidence again suffers from an insufficiently rigorous demonstration of specificity. One of the major shortcomings of this work is that most of it has been done with highly heterogeneous antigen-mixtures which are not easily characterized.

It was hoped in the present study that by using a tissue-specific protein which could be prepared in relatively pure state and also antibodies against it, a more precise test of the contrasting concepts of differentiation mentioned above, could be obtained. Even though the results of the present study did not fulfill this expectation, they do emphasize the fact that the number of uncontrollable variables in this and similar studies make a valid interpretation of the results of such experiments extremely difficult. The most obvious of these variables was that between different frogs used in regard to the susceptibility of

their eggs to the treatments. A comparison of Tables 1 and 2 very clearly shows a variability between eggs used in two different years. In addition to this annual variability there was very obviously a variability in the eggs of different frogs during the same year.

Table 5 below shows the variability in susceptibility of eggs from different frogs to treatment with the same control serum.

Table 5. --An Example of the Variability Among the Eggs from Five Different Female Frogs in Susceptibility to Treatment with Control Serum from Rabbit No. 102

	Frog Number				
	16	17	19	20	21
Percentage Living at Shumway Stages 22-25	91	81.5	85.5	69	0

On the other hand there was very little variability when the eggs from the same frog were treated with different control sera, as is shown in Table 6 below.

Table 6. --An example of the Uniformity of Susceptibility of the Eggs from the Same Frog to Treatments with Four Different Control Sera

	Control Serum Number			
	117	157	146	129
Percentage Living at Shumway Stages 22-25	80.4	83.6	79.4	75

The chi-square test for heterogeneity showed that there was no significant difference between the numbers living in the various groups of Table 6.

Whether this variability between the eggs is the result of genetic factors or environmental factors or a combination of both cannot be determined from this study. In any event, the variability of eggs from different females was an uncontrollable factor in this study and lacking genetically pure lines of frogs, an unavoidable one.

Another uncontrollable variable in this study was the amount of material injected into the blastocoel of each embryo. Although the amount originally injected was approximately the same, there was no way of controlling the amount which came back out when the micropipette was removed.

The above mentioned variables made it virtually impossible to determine if there was an effect in the nine experiments carried out in 1961 dependent upon the dilutions (1:6, 1:8, 1:10, 1:15) which were employed for the control sera and antisera. Table 7 will point this out.

By comparing the results of experiments in which the same antiserum was used, it is obvious that other variables are operating here which make it virtually impossible to assess the effects of the dilution. For instance, by comparing experiments 1 and 2 it seems as though the greater dilution results in the poorest development. By comparing experiments 6, 7, and 8 in which the same antiserum was employed and the same dilution in experiments 6 and 7, it appears that there might

have been a slight dilution effect when the percentages of embryos living are compared, but when the percentages of embryos dying at tail bud and muscular response stages are compared, it is obvious that some other variable was operating, as the percentages of embryos dying after treatment with the same dilutions varied from 28-50%.

Table 7. --Effects of Injecting Antisera Against Hemoglobin on the Development of R. pipiens Embryos in the Nine Experiments of 1961. Dilution Range 1:6 - 1:15

Experiment No.	Antiserum No.	Dilution	Percentage Dying at Tail Bud and Muscular Response	Percentage Living Shumway Stages 22-25
1	82	1:6	34	28.9
2	82	1:8	38	16.7
3	93	1:8	19.21	3.5
4	93	1:10	30	13.4
5	94	1:8	4.4	3.4
6	117	1:10	28	16
7	117	1:10	50	15.9
8	117	1:15	53	24.5
9	94	1:15	35	1.03

The hemoglobin solution exhibited a high general toxicity for the embryos as is evidenced (Tables 1 and 2) by the number blocked at gastrula stage. In the nine experiments of 1961, dilutions of 1:10, 1:20, 1:50, 1:100, were employed in an effort to determine at which concentration this general toxic effect was eliminated. Table 8

below shows the difficulty involved in assessing the effect of dilution of the hemoglobin.

Table 8. --Effect of Injecting Hemoglobin Solution on the Development of R. pipiens Embryos in the Nine Experiments of 1961. Dilution Range 1:10 - 1:100

	Experiment Number								
	1	2	3	4	5	6	7	8	9
Dilution	1:10	1:20	1:50	1:50	1:50	1:50	1:100	1:100	1:100
Percentage Living at Shumway Stages 22-25	19.2	10.8	10.9	5.8	10.9	36.5	21.8	26	15.5

Again the variability between the eggs from different frogs is obvious, making it virtually impossible to determine if there was a dilution effect.

The only indication of a specific effect in this study was the high percentage of embryos dying in the tail bud and muscular response stages, after injection with antisera against hemoglobin in the nine experiments of 1961. However, when a number of these embryos were fixed and sectioned before they died, there were large numbers of primitive blood cells present in the hematopoetic regions (Plate 12, Figure 54, Plate 13, Figure 56). Most of them, as was suggested from their morphological appearance, showed abnormalities of the heart and nervous system. Neither the observations on the development of gills and on the circulation through them, nor the observations on the time

at which the blood turned red, gave any indication of a specific effect on erythrocytes of hemoglobin or of antibodies against it. Furthermore, the comparisons of the amount of hemoglobin as determined by means of the benzidene-peroxidase test (Lenicque, 1959) in treated embryos reaching Shumway stage 25, gave no indication of a specific effect by either the hemoglobin solution or antibodies against it.

Thus, the results of this study, at first sight, would seem to indicate that the appearance of hemoglobin in R. pipiens is the result of differentiation or specialization via other pathways than these envisaged by concepts which invoke the participation of a tissue-specific protein on its complementary molecule in the mechanism leading to its differentiation. However, a number of objections could be raised to this interpretation by those adhering to one or the other points of view previously mentioned, concerning the validity of this study in deciding the issue. Considerable doubt could be raised as to whether blood is a typical differentiating tissue, especially when the appearance of hemoglobin is used as a criterion for differentiation or specialization, since the site of its synthesis and the kind of hemoglobin synthesized seem to vary in the ontogenetic cycle. It may be, as Lenicque (1959) appears to have demonstrated, that there is some other tissue-specific protein in red blood cells than hemoglobin which exerts a controlling influence on their differentiation. This tissue specific protein might be present in only very small

concentrations, too small to be picked up on the electrophoretic analysis used in this study, and too small to elicit antibody production, or it might produce non-precipitating antibodies which would not have been apparent in this study. The present results, however, certainly bear out Lenicque's observations on the toxicity of hemoglobin or its breakdown products for developing embryos. Thus, Lenicque might say that this general toxic effect of hemoglobin masked the specific effect of the substance present in much smaller concentration.

The same objection could be raised in regard to the antisera used since normal control sera appeared to be quite toxic generally to the developing embryos. But the fact remains that a high percentage of the embryos in all the treatments in the 1962 experiments developed normally. One would think that if a specific effect was being exerted that it would show up in the absence of a general toxic effect. The uncontrollable variables mentioned previously, namely, variability in the eggs from different females, and variable amounts of material injected, could also be sources of criticism of this study. In addition, there may be some question as to whether the injected material was able to get to the site where it could produce a specific effect and if it remained active long enough to produce this effect. Since the position of the presumptive "blood island" cells has been shown to be in the marginal zone opposite the blastopore, immediately next to the segmentation

cavity would easily have access to them. Since the globin or some relatively large globin precursor was shown to be present in body cavity eggs and all subsequent stages, it seems likely that antibodies injected into the blastocoel would react with them. If this globin precursor intervened in more developmental processes than the synthesis of hemoglobin, then this might account for the more generalized effects observed.

Although the results of this study cannot be used in support of one or another of the proposed mechanisms responsible for differentiation, it is believed that the approach used is sound and that by employing an experimental system more suited to this type of study much light can be shed on the mechanisms of differentiation at the molecular level.

SUMMARY

1. In spite of the fact that a number of studies have been carried out concerning the effects of substances from differentiated cells, and of antisera against these substances, on the differentiation and growth of homologous embryonic cell types, there remains considerable confusion concerning the results. This confusion is due in part, at least, to the fact that widely different experimental systems and procedures have been used in these investigations. Also, meaningful interpretations have been hindered by the fact that highly heterogenous antigen and antibody mixtures have been employed in these studies. The purposes of the present study were to attempt a clarification of this problem by treating the same differentiating system with a relatively pure tissue specific protein and antibodies against it, and also to make observations on the time and place of appearance of the tissue-specific protein (hemoglobin) employed.

2. A hemoglobin solution was isolated from adult R. pipiens blood and analysed by spectrophotometric and electrophoretic analysis. That the solution was relatively pure was indicated by the spectrophotometric analysis which revealed maximum absorption peaks which coincided with those known for oxyhemoglobin, and the electrophoretic analysis revealed the presence of not more than two and possibly only one

band, when the strips were stained for total protein. The results of the electrophoretic analysis are discussed in light of similar results on hemoglobins from other species.

3. Antibodies were produced in the rabbit by employing Freund's adjuvant technique and analysed by the agar-diffusion technique of Ouchterlony. The analyses of the antisera revealed them to be specific for R. pipiens hemoglobin, in regard to the heterologous hemoglobins and tissue antigens used in the analysis. Where there were cross reactions with heterologous tissue antigens, they were shown to be due to the blood in those tissues, as the hemoglobin content was proportional to the strength of reaction obtained.

4. The agar-diffusion technique of Ouchterlony and the benzidene-peroxidase test were employed to determine the time and place of the appearance of hemoglobin in development. The agar-diffusion technique revealed cross reactions between the hemoglobin antisera and antigens from representative stages of development beginning with body cavity eggs through Shumway stage 25. However, a positive benzidene-peroxidase test for hemoglobin could not be obtained until Shumway stages 21-22, even though the primitive erythrocytes appeared much earlier (Shumway stages 17 and 18). It is concluded from these observations that the synthesis of heme and globin is not necessarily coeval in the

developing embryo. The possibility of the heterosynthesis of globin by the maternal organism and its subsequent passage into the developing egg is discussed in light of the previous work done in this area. Also the possibility of the presence of two or three different kinds of hemoglobin during the ontogeny of R. pipiens is discussed.

5. Embryos of R. pipiens in the mid-blastula stage were treated with the hemoglobin solution and with antisera prepared against it. Two methods of treatment were employed: injection of the embryos with hemoglobin solution and with antisera prepared against it, and culturing the embryos in the presence of these substances. No specific effects of either the hemoglobin solution or the antisera prepared against it on the development of the primitive erythrocytes or hemoglobin could be shown. There were a number of general abnormalities produced but there was no significant difference between the number of these in the experimental groups and in the control groups. The difficulty of drawing definite conclusions from this study is discussed in light of the uncontrollable variables in the experiments. Obvious points of objection to the study are mentioned and discussed. If any generalization could be made, it is that substances from differentiated cells or their complementary molecules are simply the end products of differentiation and play no intrinsic role in the mechanisms leading to their differentiation.

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