THE EFFECTS OF ADULT TISSUE HOMOGENATES, AND OF ANTISERA AGAINST ADULT TISSUES, ON EMBRYONIC DEVELOPMENT IN THE FROG

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THE EFFECTS OF ADULT TISSUE HOMOGENATES, AND OF ANTISERA AGAINST ADULT TISSUES, ON EMBRYONIC DEVELOPMENT IN THE FROG

by

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ABSTRACT

A study was made of the relative effects of tissue fractions of adult organs of Rana pipiens and of antisera prepared against homogenates of the same organs on the differentiation of homologous organs in embryos of this species. Experimental treatment was performed in 2 ways: culturing embryos in the presence of the tissue fractions or antisera, and injecting embryos with these same substances.

Most of the work with tissue fractions was done with the nervous system. The results of these studies suggest that: (1) the nervous system is rather poor for such studies since it is easily affected by a variety of treatments and the types of effects produced on this system are not easily interpreted, (2) the injection technique is superior to the culturing method for producing specific effects on the developing nervous system, and (3) the fraction of cytoplasmic granules produces more consistent effects on the development of the nervous system than other cytoplasmic fractions.

The most consistent effect on embryonic development of antisera prepared against adult organs was a marked inhibition of heart development by anti-heart serum. Thirty per cent of all embryos which were injected with anti-heart serum showed this inhibitory effect. Anti-kidney serum appeared to have some inhibitory effect on the embryonic heart while anti-brain serum had no inhibitory effect on the developing heart at all.

Analysis of antisera prepared against adult brain, heart, and kidney of frog showed that these organs have several common components. The antigenic properties of kidney and heart are quite similar and these

differ considerably from the antigenic properties of brain. Using adsorption techniques it was possible to demonstrate specific components in most of the antisera.

Preliminary studies in which embryos were injected with antisera prepared against protein extracts of heart and skeletal muscle were somewhat inconclusive although some heart inhibition was noted.

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INTRODUCTION

In recent years there has been considerable discussion concerning the effects of substances from differentiated cells on the differentiation of homologous embryonic cell types. Some investigators claim to have demonstrated that these substances have an inhibitory effect on the differentiation of embryonic cells, whereas others believe they have shown that growth processes are stimulated in the affected cells. Reports that antisera produced against various types of differentiated cells have an inhibitory effect upon the differentiation of homologous embryonic cell-types, would lead to the conclusion that the complementary substances in adult tissues could not possibly have an inhibitory effect, since antigens and antisera should have opposite effects (see Weiss 1947, Ebert 1955).. Perhaps one of the major reasons that this controversy has not been settled is that investigators holding opposing views have worked with different experimental systems and have used widely different experimental procedures. The present investigation was begun with the idea that a meaningful test of these opposing hypotheses might be made by subjecting the same differentiation system to substances from fully differentiated tissue, and to antisera prepared against these tissues.

A short discussion of the development of these different points of view is in order here. The hypothesis that substances from differentiated cells can inhibit the differentiation of undetermined cells with the same prospective fate, is based on a proposal by Rose (1952) that differentiation is caused by a hierarchy of self-limiting reaction systems. He suggests that differentiation depends upon a series of gene-initiated reaction systems which are successively inhibited by the collection of

specific inhibitors. According to this theory, one differentiating reaction system, assumed to have an initial advantage, produces metabolites, the concentrations of which are self-limiting. When the concentrations of these substances reach a critical level, differentiation in this system ceases, and the next differentiating system in the hierarchal series has the advantage and the differentiation of another tissue is thereby initiated.

The evidence adduced for the theory mentioned above has been derived from experiments in which tissues are allowed to differentiate in the presence of adult or fully developed tissues. Experimental tests have been performed in two general areas: namely, regeneration and embryonic development. Extensive work has been done with the regenerating system of the coelenterate, Tubularia. In Tubularia, any portion of the organism proximal to the hydranth can, if isolated, reorganize and regenerate hydranths. Rose and Rose (1941) found that when isolates were grown in sea water which had previously been subjected to adult hydranths for 12-24 hours, regeneration of the isolates was inhibited. This inhibition was attributed to the action of a diffusible substance produced in the hydranths which in the normal situation diffuses down the stem and prevents more proximal parts from forming distal portions. Thus, the presence of this substance in the 'inhibitory water' produced the same results as if an intact hydranth had been on the isolated portions. Consequently, no regeneration occurred. Steinberg (1954) studied this phenomenon in great detail and substantiated the findings mentioned above. However, Fulton (1959) has re-examined the whole problem and has shown rather conclusively that the inhibition is due entirely to bacterial growth in the cultures.

Using grafting techniques, Rose (1955, 1957) has shown that regeneration of distal structures in an isolate can be inhibited in the presence of older distal structures. He proposes that differentiation in Tubularia depends upon a specific inhibitor which acts in a distoproximal direction. Thus, more proximal regions never form distal structures as long as normal distal structures are present.

Lender (1956) has demonstrated a similar situation in the regeneration of the flatworms, Polycelis nigra (Ehrb.) and Dugesia lugubris (O. Schm.). He cut the heads from these organisms and allowed them to regenerate in the presence of homogenates of other individuals of the same species. Under the influence of raw homogenates of heads, the brain fails to regenerate to the normal extent, although the eyes and other tissues regenerate normally. Similar homogenates of tails, on the other hand, had little or no inhibitory effect. Centrifugation of the homogenates showed the inhibitory effect to be in the supernatant fluid causing Lender to propose that the inhibitor is a diffusible chemical substance, derived from the brain of planarians.

Recently, Tucker (1959) has done a similar and very extensive study with the nemertean worm, Lineus vegetus. Under normal conditions, sections removed from anterior, posterior, or mid-body regions of the worm will regenerate both head and tail. Tucker studied the effects of homogenates of heads, mid-body, and tails on the regeneration of pieces isolated from these same regions. She found that head homogenates inhibit head regeneration in isolates from all regions; mid-body homogenates inhibited head regeneration in mid-body and posterior but not in anterior isolates; and tail homogenates inhibited head regeneration only in isolates from the posterior region. Conversely, tail homogenates inhibited tail regeneration in all isolates; mid-body

homogenates inhibited tail regeneration in mid-body and posterior isolates; and head homogenates inhibited tail regeneration only in anterior isolates. Similar homogenates of Cerebratulus californiensis, another nemertean worm of the same family, produced about the same effect on regeneration of Lineus vegetus as did the homogenates of the latter species. However, homogenates of Amphiporus formidibalis, a worm belonging to a different order, had no effect upon regeneration in L. vegetus. Tucker proposes that anterior regeneration in L. vegetus is governed by a hierarchy of regions extending from the head to the tail. Posterior regeneration, on the other hand, is governed by a similar hierarchy extending from tail to head.

Saetren (1956) has studied the effects of homologous organs in the regeneration of liver and kidney in the rat. Following removal of portions of the liver and kidney of the same animal, the abdominal cavity was injected with macerates of either kidney or liver. The mitotic count in the homologous organ was greatly decreased whereas the heterologous organ was not affected. This worked equally well with both kidney and liver.

Using micro-dissection and grafting techniques, Tartar (1958) has shown that oral structures will inhibit the regeneration of new oral structures in Stentor (Ciliata, Protozoa).

Thus, it appears that substances from differentiated cells or regions of cells have an inhibitory effect on homologous differentiating systems in regenerating cells or tissues.

The results of experiments on differentiating systems in embryos have not proven as satisfactory as those on regenerating organisms. Weiss (1952) cultured embryonic chick organs in tissue media prepared with extracts of whole embryos or of embryos from which the homologous organ had

been removed. Using heart fragments, it was observed that 2 out of 333 pulsated after 4 days in full extract, whereas 129 out of 349 pulsated after 4 days in extract of embryos lacking hearts. With kidney fragments (metanephros), 74 out of 1007 differentiated new tubules in full extract whereas 176 out of 1006 did so in medium lacking kidney. Weiss proposed that these results indicated that differentiation is reduced markedly in the presence of a homologous organ.

Rose (1955) reported on experiments in which he cultured embryos of Rana pipiens in the presence of adult tissues. Dejellied, fertilized eggs were placed in culture dishes containing bits of adult tissue and allowed to develop. In a small number of experiments Rose was able to demonstrate some specific effects. The effects were two-fold; retardation of development at a specific stage and specific malformations of homologous embryonic structures. Brain-treated embryos were delayed at the neural plate stage, heart-treated embryos at the neural tube and early tailbud stages, and blood-treated embryos were delayed following the tailbud stage. Specific effects on the nervous system in embryos treated with adult brain were: poor morphology of the central nervous system, neural canal incomplete or missing, and a random arrangement of cells in the neural tube. Heart treatment resulted in a reduction in size, in varying degrees, of the heart. This was accompanied by a failure of outgrowth of the external gills and the tail. The most striking effect in the blood-treated embryos was the absence of blood cells, even though a good functional heart was present. These results seem quite convincing except for the fact that only 4 of a total of 26 experiments produced such results. Rose feels that the lack of consistent results

from one experiment to another was probably due to a number of uncontrolled factors such as seasonal variation and general condition of the eggs employed in the experiments.

Recently, Clarke and McCallion (1959a) repeated Rose's experiments on embryos of Rana pipiens using homogenates of brain and heart of this species. They claim to have obtained specific effects on the nervous system with brain homogenates. Heart-treated embryos did not show any neural defects but 2 out of 124 embryos did show hearts which were reduced in size. They interpret their results to be in keeping with Rose's theory.

It might be noted here that Spiegel (personal communication) has perfprmed experiments of the same nature as those of Clarke and McCallion. When frog embryos were cultured with breis and extracts of brain, spinal cord, heart, liver, lung, blood, skeletal muscle, kidney, and ovary, no cases of specific inhibition of the homologous tissues were noted. He did obtain some evidence of retardation of development, as did Rose, but found that he could duplicate these results with rabbit serum albumin. Thus he believes the retardation is not a specific effect but is probably due to differences in concentration of protein in the tissue fractions employed as "inhibitors."

Shaver (1954) injected embryos of Rana pipiens with fractions of adult R. pipiens brain. He was able to show that Rose's results with whole brain tissue could be duplicated with both cytoplasmic granules and supernatant fluid of brain, insofar as external morphogenesis of the nervous system was concerned. Cytoplasmic granules appeared to be more effective than whole brain tissue in producing retardation during

neuralation. The present investigation was begun with a thorough histological analysis of this material, the results of which will be presented later.

Recently, Braverman (1958 a and b) reported briefly on work with the chick in which extracts of adult organs were injected beneath the blastoderm of 1-day-old embryos. These preliminary reports state that brain affected the developing nervous system and mesodermal derivatives affected mesodermal structures, such as somites. Further statements concerning this work must await a complete and detailed report. Clarke and McCallion (1959b) have also reported briefly on experiments in which homogenates of adult tissues of the chick were injected into the yolk of early embryos. They also report specific effects on the nervous system with homogenates of adult brain.

Evidence for the alternative hypothesis, that substances from fully differentiated cells stimulate growth processes in differentiating systems, comes mostly from work with grafts of adult tissues onto the chorioallantoic membrane of the chick. Murphy (1916) observed that chorioallantoic grafts of adult chicken spleen, liver and bone marrow induced enlargement of homologous organs of host embryos. This was especially striking in the case of spleen grafts. This spleen hypertrophy was attributed to an increased infiltration of small lymphocytes. These observations were confirmed by Danchakoff (1916), although she attributed the hypertrophy to increased proliferation of lymphoid hemocytoblasts. The results of Sandstrom (1932) also demonstrated host spleen hypertrophy. Minoura (1921) transplanted ovarian and testicular tissue of the chick to the chorioallantoic membrane and observed that development and differen-

tiation of one sex was stimulated by grafts of the gonads of the same sex and inhibited by grafts of the gonads of the opposite sex.

An extensive investigation of the problem of the effects of chorioallantoic grafts of adult chicken tissue on homologous tissue of the embryo was carried out by Ebert (1951, 1954, 1955). He observed a very marked spleen hypertrophy in host chicks following grafts of adult chick spleen (Ebert, 1951). In other experiments, Ebert (1954) noted that the nitrogen content of enlarged spleen was increased and he concluded that spleen enlargement was directly related to increased protein content but that increase in DNA content was not correlated with the increase in protein. Because of these findings, Ebert believes that hypertrophy of the host spleen represents a true increase of spleen tissue and not merely an increase of certain lymphocytes as was suggested by some earlier investigators. As a result of using grafts containing S^{35}-labelled methionine, Ebert further concluded that the hypertrophy was due in large part to a "selective incorporation into the proteins of the host tissue of tissue-specific components from the transplant."

The results of the chorioallantoic graft experiments support in general the idea that substances from differentiated cells have a stimulatory effect on homologous differentiating systems. Unfortunately, most of this work has been done with the spleen which is not believed to be a typical organ by many workers. For example, Van Alten and Fennell (1959) made the following observations from their work with chorioallantoic grafts: (1) the host spleen was enlarged following grafts of adult spleen, duodenum, brain, and skin, and (2) the host liver and heart were enlarged following spleen, liver and duodenal grafts. It appears from this study

that the stimulatory effect of chorioallantoic grafts is not tissue-specific as had been suggested by Ebert.

A slightly different approach to this problem was taken by Andres (1955) who injected suspensions of chick mesonephros and liver into 6-day chick embryos, intravenously. With mesonephros, host mesonephros exhibited increases in mitotic indices. Liver showed some increase but not as great as that of mesonephros. When liver was injected, the host liver had a slight increase in mitotic index but mesonephros had a slight decrease. Andres proposes that adult tissues have a stimulatory effect on homologous embryonic tissues in the chick.

In the last few years, an increasing number of investigators have used various immunological techniques in studying embryonic development. Most of these studies have been concerned with the embryonic appearance of molecules similar to those present in the adult. Nace (1955) and Tyler (1955, 1957) give excellent reviews of the work in this area. A relatively small number of investigators have studied the effects of antisera on the development of the embryo.

Burke, et al. (1944) injected chick lens antiserum into chick embryos and studied its effects upon differentiation of the lens and other eye structures. The age of the embryos injected ranged from 146 hours to 337 hours. Using adult lens antisera, it was noted that the lenses were affected in embryos ranging in age from 146 to 192 hours at the time of injection. The authors attributed failure to affect lens beyond this age as due to the hardness already attained by the lens. In addition to the effect on the lens, it was observed that some of the neighboring tissues, such as the lens epithelium and the retina, were not

completely normal. Using antiserum against 160-hour lens, the authors could not detect any morphological defects in the lens or other eye structures.

Parkes (1946) studied the effects of amphibian larval antiserum on the normal amphibian larva. Antisera were prepared against 2 species of frogs, Xenopus laevis and Rana temporaria, and 1 species of toad, Discoglossus pictus. It was found that with concentrations of the Xenopus antiserum greater than 1:50 all larvae of this species died within a short while. The same larvae lived well in normal rabbit serum of concentrations up to 1:25. Decreasing the concentration increased the time the larvae would live in the antiserum. If the antiserum was absorbed with tadpole extract, no adverse effect was noticed. Parkes was also able to produce some lethal effects on the Xenopus larvae with heterologous antisera, but of somewhat lesser intensity than was noted with the homologous antiserum. Flickinger and Nace (1952) found that an antiserum prepared against the supernatant fluid resulting from centrifugation at 18,000 x g of homogenates of hatched Rana fusca larvae (Shumway stage 20) caused a cytolysis of the lateral epidermis of the same stage when demembranated embryos were grown in the antiserum. Grundwaldt (1949) studied the effects of antisera against 3 separate fractions of newly hatched chicken brain on growth in culture of nerve cord from 9-day and 13-day-old chick embryos. The 3 fractions consisted of a saline-soluble fraction, an alcohol-soluble fraction, and an alcohol-insoluble fraction. Antisera prepared against the saline-soluble and the alcohol-insoluble fractions inhibited outgrowth in cultures of both 9-day and 13-day-old cord. On the other hand, the alcohol-soluble fraction affected only 13-day-old cord.

Grundwaldt concluded that 2 different antigens were produced during development of the chick brain; one, an alcohol-insoluble antigen appears as early as the ninth day; the second, which is alcohol-soluble, develops between the ninth and thirteenth day of incubation.

Lippman, et al. (1950), studied the effects of rat anti-kidney serum on tissue culture explants. A large number of embryonic tissues from the rat and a few from the chick were used to test the antiserum. The antiserum was toxic to explants of rat kidney, heart, and brain. It was also toxic to some of the chick explants which were tested.

Ebert (1950) has done extensive work in connection with the effects of antisera on development. He used antisera prepared against heart, spleen, and brain of the chick. Embryos to be tested with the antiserum were explanted according to the method of Spratt (1947). Normal development could be attained by growing embryos on culture media containing adult blood serum of the chick. These explanted embryos served as controls. The antisera had very pronounced effects on the embryos. At high concentrations, the antisera were all lethal or produced extremely abnormal effects on the embryos. At lower concentrations, Ebert noticed that differentiation proceeded in the complete absence of growth. This was a non-specific reaction since it was produced by certain concentrations of all 3 antisera.

With concentrations of the antisera lower than those producing the non-specific reactions, growth continued but specific defects were noted. Anti-spleen and anti-heart sera affected mesodermal structures, whereas anti-brain serum affected nervous tissue. The anti-heart and anti-spleen sera caused a blockage in somite and lateral plate mesoderm differentiation. The morphogenesis of the nervous system in these antisera was basically

complete, but the central nervous system was somewhat abnormal. Embryos cultivated in anti-heart serum never developed pulsating hearts, while those in anti-spleen serum did. Embryos treated with anti-brain serum had specific defects in the forebrain region. Ebert thinks this may reflect a general effect on nervous tissue, but is expressed only in the anterior end of the embryo due to some type of antero-posterior gradient.

Johnson and Leone (1955) report that antiserum prepared against actomyosin from adult chick hearts inhibits morphogenesis of the heart in chick embryos, but the degree of specificity is not clear since there is also general inhibition of development. Another example of what appears to be a general lethal effect of antisera on developing chick embryos, is the work of Nettership (1953).

Gluecksohn-Waelsch (1957) studied the effects of antisera on development by immunizing virgin female mice and subsequently mating them. The embryos from mice so treated were studied to determine any effects which may have been produced. Two inbred strains of mice were used for experimental animals whereas mice from a strain which was not inbred were used for tissue donors. Virgin female mice were given doses of mouse brain and heart antigens. Subsequently, samples of blood were taken to determine whether an antiserum had been produced by the injected mice. Then the mice were bred, and the embryos were allowed to develop for 10 days. The mothers were then sacrificed and the embryos were studied for specific abnormalities. The embryos from mice injected with brain emulsion were observed to have abnormal nervous systems in some cases. These abnormalities consisted of suppression of differentiation

of nervous tissue, microcephaly, and abnormalities of closure of neural folds. Embryos from mice injected with heart emulsions showed no effects of the treatment. These results are suggestive of a transmission of molecules from the mother to the embryo which produce specific morphogenetic effects in the case of the nervous system, but it was not possible to demonstrate that the antibodies present in the mother were able to enter the embryo.

Thorough reviews of the work on the effects of antisera on embryonic development can be found in Nace (1955) and Tyler (1957). It is apparent that evidence exists that substances from adult tissues and antisera against these substances have an effect on homologous differentiating systems. However, there is considerable confusion as to exactly what these effects are.

The objectives of the present investigation were: (1) a detailed analysis of some previous experiments (Shaver 1954) on the differentiation of the nervous system of embryos of Rana pipiens which had been cultured in or injected with fractions of adult brain of R. pipiens, (2) additional experiments in which early embryos of R. pipiens were treated with adult tissue fractions, employing additional tissue types, in an effort to secure more comparable data, (3) a study of the effects on the differentiation of embryonic tissue of antisera prepared against homologous adult tissue, and (4) an investigation of the effects of antisera prepared against specific proteins from the adult frog heart on the development of the embryonic heart.

MATERIALS AND METHODS

The leopard frog, Rana pipiens, served as the experimental animal for all experiments. These frogs were obtained from commercial dealers in Wisconsin and Vermont.

The adult tissues with which embryos were cultured or injected were prepared in the following manner. Hearts and brains were removed from adult frogs, weighed, and suspended in a 0.005 M KH_2PO_4-Na_2HPO_4 buffer solution at pH 7.4 in the ratio of 1 gram of tissue to 10 ml. of buffered solution. The tissues were then homogenized at 4°C, either in a glass homogenizer or a Virtis homogenizer, model 23. The homogenates were centrifuged at 2,500 x g at 4°C to remove connective tissue, unbroken cells, and other debris. The resultant supernatant fluid was centrifuged at 18,000 x g. The particles thrown down at this force are referred to in the experiments as cytoplasmic granules. These were washed by centrifugation and resuspended in a volume of phosphate buffer equal to the volume of the supernatant fluid. The supernatant fluid from the centrifugation at 18,000 x g was used without further dilution. In the experiments reported by Shaver (1954), the histological analyses of which are reported below, the procedure for obtaining fractions of tissue homogenates was identical to that described above except that Holtfreter's solution was employed as the homogenization medium. Holtfreter's solution is a dilute Ringer's solution, buffered with $NaHCO_3$ at pH 7.2.

The eggs employed were obtained after artificially induced ovulation resulting from injection of pituitary glands of frogs, (Rugh 1934). Sperm suspensions for inseminating the eggs were prepared in the following way. Testes were removed from pithed male frogs and crushed in 10 per cent

full strength Holtfreter's solution, 5-7 ml. per testis. The suspension was then filtered through glass wool to remove excess testicular tissue. Eggs were placed on 1 x 3 inch glass slides and flooded with sperm suspension. After 10 minutes the excess sperm suspension was poured off, the eggs placed in a finger bowl filled with aerated tap water, and allowed to develop at room temperature to the 32-128 cell stage (Shumway stages 7-8) at which time treatment was begun. This stage was found to be most suitable for 2 reasons: (1) injection of any material usually produces a large percentage of gross abnormalities in earlier stages, and (2) treatment at later stages does not appear to affect early morphogenesis significantly.

Embryos were treated with tissue fractions or antisera in 2 ways. In some experiments, embryos were dejellied and cultured in the presence of the tissue fractions or antisera. In these experiments the developing embryos were kept at about 12°C and fresh tissue fractions or antisera were added daily. Following gastrulation, the developing embryos were placed in 10 per cent full strength Holtfreter's solution and kept at 20°C. In experiments of Shaver (1954 and personal communication), it had been noted that there were no apparent differences in bacterial growth, under these conditions, and when an antibiotic (chloromycetin) was used. Due to this fact, and because antibiotics are generally quite toxic to embryos, they were not employed in these experiments.

In other experiments the tissue fractions or antisera were injected into the embryo at Shumway stage 7 or 8. Injection of tissue fractions or antisera into embryos was performed with a micro-injection apparatus, consisting of a 10 cc. syringe to which polyethylene tubing of small dia-

meter was attached by a special adapter. A micropipette (tip diameter approximately 0.02 to 0.05 mm.) made by drawing out a capillary tube of 1 mm. outside diameter over a small flame, is inserted in the free end of the tube. The syringe was filled with water prior to the insertion of the pipette, an air space being left to keep the material being injected from coming in contact with the water. An attempt was made to inject the material into the small blastocoele which was beginning to form when the embryos were injected. In all cases injections were made in the animal hemisphere. The embryos injected with tissue fractions were kept at 12°C for 2 days and then allowed to develop at 20°C. The 2-day cold period was eliminated in the experiments in which antisera were injected into embryos.

The embryos were observed periodically and all changes and abnormalities were recorded. Embryos subjected to fractions of brain were fixed at the early tailbud stage (Shumway stages 17-19), while embryos which had developed in the presence of fractions of heart were allowed to develop into swimming larvae (Shumway stages 21-24) before being fixed. Embryos in early stages (Shumway stages 17-19) were fixed in Smith's modification of Tellyesnicky's fluid, while those in later stages were fixed in Smith's modification of Bouin's fluid. The embryos were embedded, serially sectioned at 13 microns, mounted, stained in hematoxylin and eosin, and examined for abnormalities. In the experiments where the embryos appeared to have enlarged notochords, the diameter of the notochord of each embryo was measured with an ocular micrometer.

Organs which were to be used as antigens were removed, weighed, and homogenized in 0.85% NaCl buffered at pH 7.4 with 0.005 M phosphate

buffer. The homogenate was centrifuged at 1200 x g and the supernatant fluid was used for injection into rabbits.

The first antisera prepared against these antigens were produced by giving a male rabbit 5 intravenous injections during a 10-day period, 1 every other day. The first 2 injections were 0.5 ml. of antigen each; the third 1 ml.; the fourth 1.5 ml.; and the fifth was 2 ml. . Two weeks following these injections each animal was given an anamnestic injection of 2 ml. of antigen intravenously. Seven days later the rabbit was bled from the ear and a precipitin titer was run. It the titer was at least 1:64 the rabbit was bled as soon as possible by heart puncture. If the concentration of antibodies was not high enough, the rabbit was given another 2 ml. injection of antigen and a titer was run again in 1 week. This procedure was carried out until the desired concentration of antibodies was obtained.

Most of the antisera used were prepared by using the Freund adjuvant technique. The adjuvant mixtures, available from Difco Laboratories, Detroit, Michigan, are of 2 types, complete and incomplete. The complete adjuvant mixture is composed of mannide monooleate, 1.5 ml.; paraffin oil, 8.5 ml.; and 5 mg. of killed and dried Mycobacterium butyricum. The incomplete adjuvant mixture lacks the Mycobacterium butyricum. Each rabbit received 3/4 ml. under each scapula of an emulsion consisting of equal parts of antigen and complete adjuvant mixture. A week later an injection using incomplete instead of complete adjuvant mixture was similarly made. From 3 to 4 weeks later, the rabbit was bled from the ear and the antiserum was analyzed using the Ouchterlony technique as modified by Fox (1959). If the analysis indicated the presence of antibodies, the rabbit was exsan-

guinated by cardiac puncture as soon as possible. Otherwise, the animal was given a second injection with the incomplete adjuvant-antigen mixture.

The Ouchterlony technique was performed in the following way. A 2% agar solution in 0.85% NaCl buffered with 0.005 M phosphate buffer at pH 7.4 was prepared, filtered, and measured out in 30 ml. quantities. Eight 1 5/8 x 1/2 inch strips of filter paper (Whatman #1) were arranged equidistantly around the lip of the male half of a Petri dish with a small portion lying on the bottom of the dish. The dish was then sterilized by dry heat at 120°C for 12 hours. Following sterilization, 30 ml. of sterile agar were poured into the dish and the agar was allowed to harden. After hardening, a template was placed over the agar, wells were cut and the pieces of agar left in the wells were removed. A drop of agar was then placed in each well to seal the bottom. Each of the wells held 0.15 ml. of solution. In most cases each plate was given a total of 10 doses of 0.15 ml. in each well. For most of the anaylses performed, the antiserum being analyzed was placed in the center well (labelled An in Plate 4, figure 12), the homologous antigen was placed in well 2, and other antigens being used for comparative purposes were placed in wells 1 and 3 (see Plate 4, figure 12). In some plates a comparison of 3 antisera was accomplished by placing the antisera in wells 1, 2, and 3, while the antigen was placed in the center well. For the analysis of complex systems involving numerous antigenic components, the Bjorklund (1952) specific inhibition test was employed. In this procedure, the antigen which was used for inhibition was placed in the antiserum well in doses of 0.15 ml. until the desired number of inhibiting doses were absorbed. Subsequent to the last dose of inhibiting antigen, the plates were run as usual.

Protein extractions from muscular tissues were carried out with a modification of Mommaert's (1958) procedure for the preparation of myosin. Extractions were made of heart and skeletal muscle from both adult chicken and frog. Skeletal muscle and heart ventricles were removed from freshly killed animals and placed immediately into ice water. After thorough cooling, the muscle was weighed, mixed with 3 ml. of Guba-Straub solution (0.3 M KCl, 0.10 M KH_2PO_4, and 0.05 M K_2HPO_4) per gram of tissue, and minced with the Virtis homogenizer. After extracting for 15 minutes with constant stirring the material was centrifuged to remove the muscle residue. The solution was diluted with 10 volumes of cold, deionized water (added slowly with constant stirring) and left in the cold until the protein settled to the bottom, usually about 3 hours. The supernatant fluid was siphoned off as far as was practicable and the protein collected by centrifugation. The protein was then dissolved in as small amount of 0.6 M KCl as possible. This solution was diluted with 10 volumes of cold, deionized water in the same manner as before. The protein was allowed to settle in the cold and was collected by centrifugation after siphoning off the supernatant fluid. The protein was then dissolved in a KCl solution, buffered at pH 6.5-6.8, the concentration of which was 0.4 M with respect to KCl and contained KH_2PO_4 and K_2HPO_4 in equimolar proportions, to bring the ionic strength up to 0.5.

The procedure for preparation of antisera against the protein extracts of muscular tissue was identical with that already described for the antisera against homogenates of adult frog heart and brain. With the chicken material, a concentration of 1 volume of packed protein to 5 volumes buffered KCl was sufficient to elicit an immune response in the rabbit. How-

ever, a concentration of 1 volume of the protein from frog muscle to 2 volumes of buffered KCl was necessary to produce the desired immune reaction which was analyzed with the Ouchterlony technique. The treatment of embryos with the antisera against muscle protein was essentially the same as that described for the antisera against adult frog heart and brain except that no culturing experiments were performed. Fixation, histological preparation, and microscopic examination of the embryos were the same as that for the organ antisera.

In cataloging the results, all abnormalities were noted but special attention was placed on the heart. Each embryo was scored as either having a normal-sized heart or as possessing an inhibited or reduced heart. At first, an attempt was made to catalog the hearts as normal, slightly inhibited, and greatly inhibited. The "slightly inhibited" category was later dropped after it was decided that this fine distinction could not be made with any degree of accuracy.

RESULTS

A. Effects of Adult Tissue Fractions on Embryonic Development.

Most of the results of treating developmental stages with adult tissue fractions were obtained with frog brain. They represent in part a microscopic examination of embryos produced in experiments performed by Shaver (1954), in which embryos which were cultured in tissue fractions of adult brain showed no consistent effect on the development of the nervous system.

In experiments in which embryos were injected with fractions of adult frog brain, however, the neurocoele was very much reduced or absent in many of the treated embryos. This condition, termed "solid nervous system" (Plates 1-3, pages 29-33), extended throughout the entire length of the brain and spinal cord in some embryos, but in others was localized in a particular region of the nervous system. No correlation could be made between the location of this abnormality and the tissue fractions employed. However, the fraction of cytoplasmic granules produced the effect with greater frequency and with more consistency from one experiment to another. In 3 of 4 experiments in which cytoplasmic granules of adult brain were injected, from 28 to 37 per cent of the embryos had solid nervous systems. A fourth experiment gave completely negative results. The data from these experiments are given in Table 1 (page 22).

It can be seen from Table 1 that the development of the nervous systems of embryos treated with blood cells from adult frogs were normal. However, the number of embryos so treated was extremely small.

Table 1.--Effects of Tissue Fractions of Adult Brain on the Developing Embryonic Nervous System

Treatment	Total Number of Embryos				Number of Embryos With Solid Nervous Systems				Per Cent of Embryos With Solid Nervous Systems			
	Exp 1	Exp 2	Exp 3	Totals	Exp 1	Exp 2	Exp 3	Totals	Exp 1	Exp 2	Exp 3	Av.
Fertilized Control	9	22	1	32	0	0	0	0	0.0	0.0	0.0	0.0
Phosphate Buffer	1	13	12	26	0	1	0	1	0.0	7.7	0.0	3.8
Whole Brain	11	11	13	35	2	0	1	3	18.2	0.0	7.7	8.6
Brain Supernatant Fluid (cult.)	5	15	2	22	1	3	0	4	20.0	20.0	0.0	18.2
Brain Supernatant Fluid (inj.)	10	30	31	71	3	2	0	5	30.0	6.7	0.0	7.0
Brain Granules (cultured)	6	15	10	31	1	1	1	3	16.7	6.7	10.0	9.7
Brain Granules (injected)	16	25	7	48	6	9	2	17	37.5	36.0	28.6	35.4
Blood (cultured)	--	--	3	3	-	-	0	0	--	--	0.0	0.0
Blood (injected)	--	--	7	7	-	-	0	0	--	--	0.0	0.0

The only other effect on the embryos which was noted was an apparent increase in the size of the notochord in embryos injected with supernatant fluid of adult brain homogenates. This was very marked in Experiment 2 and was noted to a lesser extent in Experiment 3. In Experiment 2 the mean diameter of the notochord in embryos injected with supernatant fluid was 129.18 microns as compared to 97.86 microns for the controls. The range of notochord diameters was 70-180 microns for the embryos injected with supernatant fluid, whereas the range for the controls was 80-120 microns. Plate 3, figure 11 (page 33) shows an embryo with an extremely enlarged notochord. The values obtained from an analysis of individual measurements (Table 2, page 24) suggest that the notochords were either grossly affected or were not affected at all. It is possible that this size difference may represent merely a general delay in development. Most of the embryos with enlarged notochords still had unclosed neural folds, indicating that their development was retarded. As the notochord differentiates, its diameter becomes less and consequently a greater diameter of the notochord may represent no more than general delay in development.

Additional experiments were performed by the writer during the winter of 1958. The data from some of these experiments, given in Tables 3 and 4 (page 25), indicate that the specific inhibition of differentiation of embryonic nervous tissue by adult brain may be more difficult to demonstrate than the earlier experiments suggested, inasmuch as the embryonic nervous system can be shown to be affected by other agents.

In Table 3 data from experiments are presented in which a comparison was made between the effects of tissue fractions from heart and brain of adult frog on the development of the homologous embryonic tissues. In

Table 2.--Experiment 2 Notochord Size (diameter in mocrons) of Individual Embryos Treated With Adult Brain Tissue Fractions.

Fertilized Control (21 emb.)	Injected PO_4 (13 emb.)	Injected Granules (22 emb.)	Injected Supernate (22 emb.)	Cultured Granules (13 emb.)	Cultured Supernate (15 emb.)	Whole Brain (10 emb.)
100	100	120	180	150	90	90
100	80	85	100	120	130	90
110	110	100	150	120	90	90
120	110	90	100	90	110	100
100	90	130	140	85	80	80
120	80	105	120	90	135	100
120	90	90	100	80	95	150
80	110	130	100	110	110	130
120	110	140	140	100	95	120
110	100	105	140	110	100	110
95	90	125	160	120	40	
90	80	100	120	105	90	
90	160	80	90	65	130	
90		105	120		100	
90		120	110		80	
80		110	70			
80		110	170			
100		100	170			
100		100	100			
80		80	120			
80		120	180			
		110	140			
$\bar{X}$ 97.86	100.77	107.14	128.18	103.93	98.33	106.00

$P < .05$

Table 3.--Effects of Tissue Fractions of Adult Brain and Heart on the Differentiation of the Embryonic Brain and Heart

Treatment	Total Number of Embryos		Number of Embryos With Abnormal Nervous Systems		Number of Embryos With Abnormal Hearts	
	Exp 5	Exp 6	Exp 5	Exp 6	Exp 5	Exp 6
Fertilized Control	20	-	0	-	0	-
Phosphate Buffer (c)	--	6	-	0	-	N
Phosphate Buffer (i)	18	15	0	3	0	2
Brain Granules (c)	--	6	-	0	-	N
Brain Granules (i)	20	6	3	3	N	N
Brain Supernate (c)	--	6	-	1	-	N
Brain Supernate (i)	--	6	-	3	-	N
Heart Granules (i)	24	12	0	3	1	4
Heart Supernate (i)	--	7	-	3	-	1

Table 4.--Abnormalities of the Nervous System Produced by Treatment of Embryos with Antisera Prepared Against Adult Organs

Treatment	Total Number of Embryos	Number of Embryos With Abnormalities of the Nervous System
Fertilized Control	24	3
Holtfreter's Solution (i)	5	0
Normal Rabbit Serum (c)	21	5
Normal Rabbit Serum (i)	17	4
Brain Antiserum #11 (c)	10	1
Brain Antiserum #11 (i)	12	5
Heart Antiserum #2 (c)	11	4
Heart Antiserum #2 (i)	23	1

c = cultured
i = injected
N = no observation

Experiment 5 (Table 3) it appears that nervous tissue development is affected by adult brain granules, but not by heart granules. However, in Experiment 6 (Table 3) it can be seen that fractions of both heart and brain affect the developing nervous system. Due to the fact that embryos which were treated with fractions of adult brain were fixed at earlier stages than the embryos treated with adult heart fractions, it was not possible to assess the effects of adult brain on heart differentiation, since at the stage when these embryos were fixed (Shumway stages 17-19) heart development is just commencing. However, the effect of heart granules on the developing heart in Experiment 6, and to a lesser degree, in Experiment 5 was clearly inhibitory inasmuch as heart development was greatly suppressed.

Table 4 (page 25) presents results of an experiment in which embryos were treated with antisera against adult tissues. It will be noted that both normal rabbit serum and antisera prepared against adult frog brain and heart produced abnormalities of the developing nervous system. These abnormalities were of the same nature as those which were produced by the tissue fractions of adult frog brain. Results such as those listed in Table 4, showing effects on the developing nervous system, were observed periodically in the experiments with antisera prepared against various adult organs.

The results presented above, together with some suggestions from preliminary experiments on the effects of antisera prepared against adult organs, led to the re-orientation of the experimental program towards the following objectives: (1) A study of the effect of experimental treatments on the differentiation of the heart. This organ would seem to

present the possibility for a more definite assessment of the experimental treatment, inasmuch as inhibition of its development can apparently occur without concomitant effects on other organs, and (2) A study of the effects of antisera prepared against various organs of adult frog on embryonic development. Similar studies on the chick had demonstrated specific inhibitions of organ differentiation, and it seemed desirable to extend this type of approach to the frog, in view of the claims made (Rose, etc.) that presence of the adult tissue in itself could produce specific inhibition of differentiation.

PLATE 1

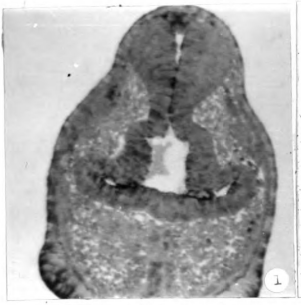
Examples of "Solid" Mid-brains as Produced by Injection of Embryos with Tissue Fractions of Adult Brain.

Figure 1 x.s. through the mid-brain of a normal embryo from the fertilized controls of Experiment 2

Figure 2 x.s. through the mid-brain of an embryo from Experiment 1 which had been injected with cytoplasmic granules of adult frog brain

Figure 3 x.s. through the mid-brain of an embryo from Experiment 3 which had been injected with cytoplasmic granules of adult frog brain

Figure 4 x.s. through the mid-brain of an embryo from Experiment 1 which had been injected with supernatant fluid from adult frog brain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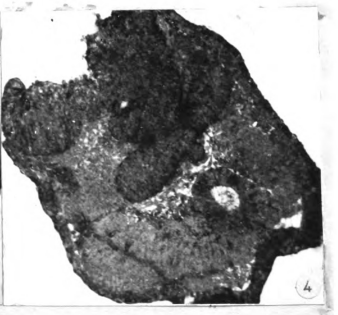
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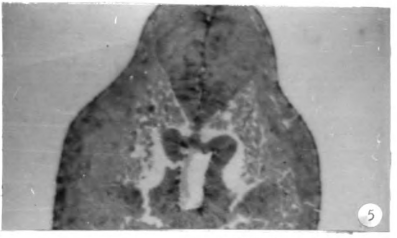
PLATE 2

Examples of "Solid" Hind-Brains as Produced by Injecting Embryos With Tissue Fractions of Adult Frog Brain

Figure 5 x.s. through the hind-brain of an embryo from the fertilized controls of Experiment 2 (Same embryo as in Figure 1)

Figure 6 x.s. through the hind-brain of an embryo from Experiment 3 which had been injected with cytoplasmic granules of adult brain

Figure 7 x.s. through the hind-brain of an embryo from Experiment 1 which had been injected with supernatant fluid of adult brain



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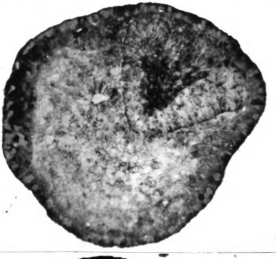

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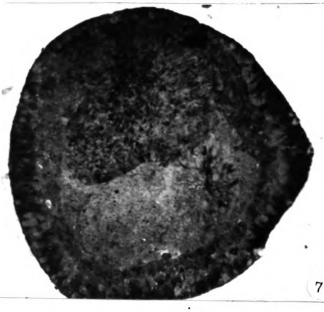

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PLATE 3

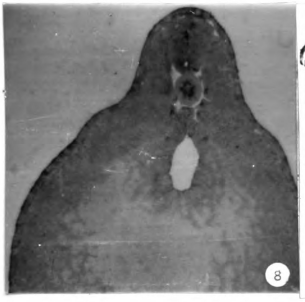
Abnormalities of the Spinal Cord and Notochord as Produced by Injecting Embryos with Tissue Fractions of Adult Frog Brain

Figure 8 x.s. through the mid-abdominal region of an embryo from the fertilized controls of Experiment 2 (same embryo as Figures 1 & 5).

Figure 9 x.s. through the mid-abdominal region of an embryo from Experiment 1 which had been injected with supernatant fluid from adult frog brains

Figure 10 x.s. through the mid-abdominal region of an embryo from Experiment 1 which had been injected with cytoplasmic granules from adult frog brains

Figure 11 x.s. through the mid-abdominal region of an embryo from Experiment 1 which had been injected with supernatant fluid from adult frog brains. Note the size of the notochord as compared to the control (Figure 8).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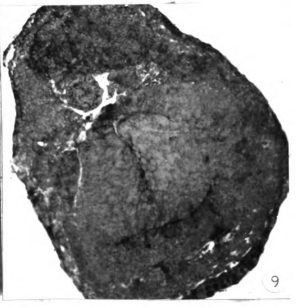
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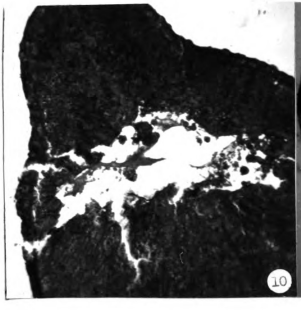
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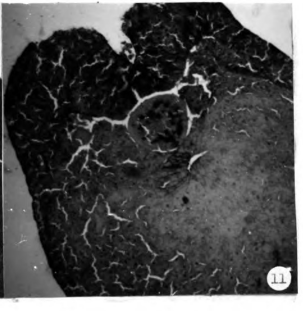
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B. Effects of Antisera Prepared Against Adult Organs on the Differentiation of Embryonic Tissue.

1. Analysis of Antisera

The analysis of the antisera prepared against adult brain, kidney, and heart showed that these organs have several complementary factors in common (Plates 7-10). In fact, it was impossible to determine with certainty that any of the antisera had specific components without the use of adsorption techniques. From analyses in which several antisera prepared against the same adult organ were compared, it was seen that these antisera contained several common components, but some antisera possessed components which were not present in other antisera prepared against the same organ (Plate 10, Figures 45 and 46). Presumably, this is due to the well-established fact that the antibody-producing mechanisms of individual rabbits react differently to the same antigen.

With adsorption techniques, it was seen that the antigenic properties of heart and kidney are similar and that these differ considerably from the antigenic properties of brain (Plates 7-10). This seems to be largely due to the presence of more factors in antisera prepared against heart and kidney than are present in anti-brain sera. For instance, when anti-kidney or anti-heart sera were adsorbed with brain antigen, these antisera still possessed several components against homogenates of heart and kidney (see Plate 8, Figures 28, 31 and 34). On the other hand, when anti-brain sera were adsorbed with heart or kidney, no more than one component could be demonstrated in these antisera. (Plate 10, Figure 43).

Of the 4 anti-heart sera tested, 2 (#10 and #52) showed single specific components when adsorbed with kidney. Anti-heart serum #53 appeared to have a specific component when adsorbed with brain but this disappeared when this serum was adsorbed with kidney antigen (see Plate 8, Figures 31 and 32). It is interesting to note that antiserum #53 produced less heart inhibition than any of the other anti-heart sera employed. However, anti-heart serum #49 produced the highest per cent of inhibition (36.8%) of any of the anti-heart sera used and it showed no specific line in the Ouchterlony plate when adsorbed with kidney.

All of the anti-kidney sera seems to have specific components when adsorbed with brain (see Plate 9, Figures 37 and 40; Plate 8, Figure 34). However, when heart was used for adsorption, only 2 of the anti-kidney sera (# 14 and #26) seemed to have a specific component. This may be due to the fact that the heart homogenate was a very poor material for adsorption tests. In nearly every test which was made the heart homogenates that were used for adsorption clogged the antiserum well to the extent that the antiserum being tested did not diffuse into the agar nearly as rapidly as the antigens. In many instances the tests were discontinued before completion because the antisera would not diffuse at all. Attempts to overcome this difficulty by centrifuging the homogenates at greater forces and using the supernatant fluid for adsorptions were not successful. For these reasons, it is felt that the negative tests involving adsorption with heart antigen may not necessarily represent the true situation.

Table 5 (Page 37) gives a complete list of all the adsorption tests performed. It can be seen that most antisera seemed to have some degree of specificity. However, most plates were given only 6 doses of inhibiting antigen, while a total of 5 doses of the same antigen was given to the plate following adsorption. Consequently, it is very likely that the specific lines which appeared in the Ouchterlony plates represent no more than quantitative differences of antigenic substances in the various organs employed in the tests.

2. Effects of Antisera on Differentiation of Embryonic Tissues

a. External Manifestations of Treatment with Antisera

Embryos treated with antisera prepared against homogenates of adult organs were observed from the time of treatment (late cleavage to early blastula, Shumway stages 7-8) until they were fixed (5-6 days following treatment). In most experiments, all the embryos, including those treated with phosphate buffer and control sera, showed some general abnormalities, but nothing specific was observed. However, there were a few cases in which certain characteristic abnormalities were noted in the embryos treated with antisera prepared against adult organs.

In Experiment 20, about 57% of embryos injected with a 1:8 concentration of kidney antiserum #14 were extremely edematous. Control serum, antiserum against adult brain, and antiserum against adult heart did not produce this effect at all. This syndrome is characterized by extreme distention of the central body wall due to the presence of accumulated fluid in the body cavity. Although antiserum #14 was used in a total of 5 experiments, Experiment 20 was the only

Table 5.--The Presence of Specific Factors in Anti-Organ Sera as Determined by the Technique of Adsorption

Antiserum	Adsorbed With			
	Heart	Kidney	Brain	Blood Serum
Heart Antiserum #2	-	0	+	0
Heart Antiserum #10	-	+	+	-
Heart Antiserum #49	0	-	0	0
Heart Antiserum #52	-	+	+	0
Heart Antiserum #53	-	-	+	0
Kidney Antiserum #14	+	-	+	-
Kidney Antiserum #26	+	-	+	0
Kidney Antiserum #47	-	-	+	0
Kidney Antiserum #57	-	-	+	0
Brain Antiserum #11	+	0	-	0
Brain Antiserum #17	+	+	-	+
Brain Antiserum #56	-	-	-	0

+ = Presence of lines in the Ouchterlony plate which appeared specific

- = Absence of specific lines in the Ouchterlony plate

0 = No test made

one in which the edematous condition was observed. Three other kidney antisera (#26, #47, and #57) were used in other experiments, and none of these produced this condition in any experiment. It is possible that the particular embryos used in Experiment 20 were extremely sensitive to antiserum prepared against adult kidney.

Histological examination of the edematous embryos of Experiment 20 showed that the pronephros was present. Unfortunately, the extreme edema had either distorted the tissues or had resulted in such poor fixation that a thorough histological examination was impossible. In some of the embryos, the cells comprising the walls of the pronephric tubules seemed loosely arranged and the lumen of the tubule appeared enlarged. Plate 12, Figure 62 shows a cross-section through the pronephros of one of the edematous embryos. In many of these embryos, the heart was very much reduced in size, as well. This appears to be a secondary effect of the edema, since the heart was not affected in any embryo of this group that was not edematous. Also the heart was normal in edematous embryos if the edema was confined to the regions of the abdomen posterior to the pericardial space. Table 6 below gives the data from Experiment 20 in regard to the edematous condition.

Table 6.--Experiment 20. Edema in Embryos Treated With Adult Organ Antisera.

Treatment	Total Number of Embryos	Number of Embryos with Edema	% of Embryos with Edema
Fertilized Controls	52	0	0.0
Phosphate Buffer	49	3	6.5
Control Serum #10	2	0	0.0
Anti-heart Serum #10	27	0	0.0
Anti-heart Serum #17	19	0	0.0
Anti-kidney Serum #14	40	23	57.5

Another type of characteristic abnormality which was externally identifiable was noted in larvae of 2 experiments (10 and 12) in which embryos were cultured in and injected with a 1:8 concentration of an antiserum prepared against adult frog heart (Anti-heart serum #2). A high percentage of embryos cultured in this antiserum showed a very marked abnormality of the spine. The curvature of the spine was so abnormal that the tip of the tail was located directly beneath the abdomen. Unfortunately, no pictures were taken of the external appearance of these embryos. Histological examination of the abnormal embryos in Experiment 10 showed that most of them had a very specific defect in the central nervous system. At the juncture of the hind-brain and neural tube, the nervous tissue was greatly enlarged and considerably disorganized. There were often 2 or more neurocoels throughout the affected region. Examples of this abnormality are shown in Plate 12, Figures 59 and 60. Embryos in Experiment 12 were not analyzed because poor fixation made sectioning impossible. The data from these 2 experiments are summarized in Table 7 below.

Table 7.--Curvature of the Spine as Caused by Culture in Heart Antiserum. (data from Experiments 10 and 12)

Treatment	Total Number of Embryos	Number of Embryos with Abnormal Spines (external appearance)
Fertilized Controls	22	0
Control Serum	34	2
Holtfreter's Solution	12	0
Heart Antiserum #2	31	13

In another experiment (11), embryos were cultured in dilutions of the same antiserum (#2) of 1:16 and 1:32. The effect noted above was

not observed in this experiment at all. Nothing can be said of the specificity of this effect since antiserum prepared against heart was the only one used in these experiments. It is entirely possible that most antisera prepared against other adult organs would affect the nervous system in this way, under similar conditions.

b. Histological Analysis of Embryos Treated with Antisera

The most consistent and significant morphogenetic effect noted in the histological analysis of larvae developed from embryos into which antisera against adult organs were injected, was the inhibition of the development of the heart by the antisera prepared against homogenates of adult frog heart. This inhibitory effect ranged from a slight decrease in size of the heart to an extreme condition in which the heart was completely absent (see Plate 11). Usually, all other internal structures, including the pericardial space, were normal. Externally, these embryos showed stunted gills, varying degrees of failure of outgrowth of the tail, and in some cases a very rough epidermis. In addition, there seemed to be a greater tendency among these embryos to be slightly edematous. However, these external abnormalities did not prove to be reliable criteria of heart abnormality, since they were noted in embryos in which the heart was essentially normal.

As was mentioned earlier, an attempt was made at first to classify the affected embryos into 2 groups, slightly or extremely inhibited. Due to the lack on an adequate criterion upon which to make this distinction, the "slightly inhibited" group was dropped and these embryos were classified as normal. Consequently, in all the results which are listed below only those embryos in which the heart was either drastically reduced in size or absent are classified as being affected.

In these experiments, dilutions of the antisera ranging from 1:4 to 1:32 were employed. From preliminary experiments it was thought that this would be sufficient to show a dilution effect. However, when the data were tabulated and analyzed with the chi-square test for heterogeneity, it was found that there were no significant differences in the percentages of inhibition produced by the dilutions of heart antisera in the range given above. Thus, in Table 8 all embryos which were treated with the same antiserum are listed together, the results of treatment with the various dilutions having been pooled. In some experiments only one concentration of the several employed was used. In Experiments 10, 13, and 14 a concentration of 1:8 was used, while in Experiments 18 and 19 a dilution of 1:6 was employed. It will be noted in Experiment 17 (Table 8) that 5 of 32 embryos treated with kidney antiserum #14 had inhibited hearts. All but one of these 5 were in the group treated with a 1:4 concentration. Weaker concentrations of this antiserum apparently were ineffective in producing heart inhibition. This was the only case noted in the experiments of a decrease in inhibitory effect with a decrease in concentration of the antiserum.

From Table 8 it will be noted that of 8 experiments, data from 4 experiments (10, 15, 18, and 19) were statistically significant beyond the 0.01 level; data from 2 experiments (16 and 17) were significant beyond the 0.05 level; and data from 2 experiments (13 and 14) were not statistically significant when the chi-square test for heterogeneity was used. It can be seen (Text figure 1, page 44) that the developing hearts in 30% of all embryos which had been injected with

heart antisera were inhibited as compared to the hearts of embryos injected with control sera (8.6% inhibition). Kidney antisera had a slight inhibitory effect (12.5%) on the development of the heart, while brain antisera appeared to have no inhibitory effect on the heart at all. Phosphate buffer, when injected alone, seemed to be inhibitory to the developing heart to a very slight extent. However, this inhibition appears to be due to a general cytotoxic effect, since many of the embryos were extremely abnormal and many died. The addition of a tissue homogenate or rabbit blood serum tended to decrease this apparent toxicity of the phosphate buffer.

Apparently, the period at which embryos were treated was very critical in the experiments involving injection of antisera into embryos. Several experiments were performed in which embryos were injected from late blastula through early tailbud stages. No inhibition of organ structure or function was noted in these experiments.

The antisera were adsorbed with homologous antigens in an attempt to remove the inhibitory effect. Unfortunately, in 2 experiments in which embryos were treated with adsorbed antisera, the unadsorbed antisera did not produce any appreciable inhibition of heart development. Consequently, it was impossible to show that the inhibitory activity could be removed by adsorption. However, in these experiments with adsorbed antisera, heart inhibition was noted in a few embryos treated with heart antisera which had been adsorbed with brain. On theoother hand, there were no cases of inhibition among the embryos treated with anti-heart serum adsorbed with heart. From these preliminary studies, it appears that adsorption of the heart antisera with heart antigen removes the inhibitory effect but adsorption with brain antigen does not.

Table 8.--Inhibition of Heart Development in Embryos Injected with Antisera Against Adult Organs

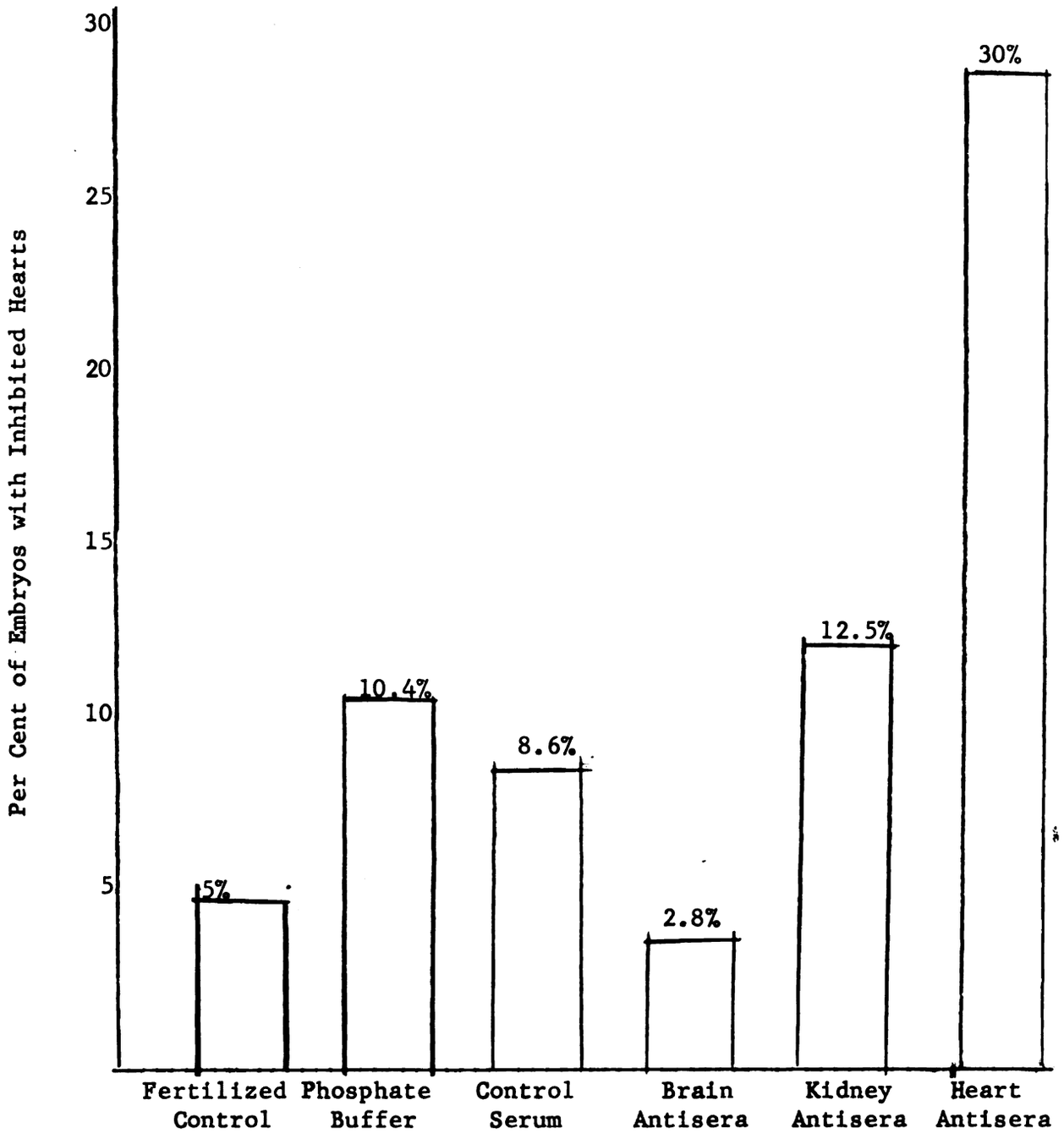
Treatment	Experiment Number 10			13			14			15			16			17			18			19			Totals		
	T	N	%	T	N	%	T	N	%	T	N	%	T	N	%	T	N	%	T	N	%	T	N	%	T	N	%
FC	14	0	0.0	12	1	8.3	12	0	0.0	12	1	8.3	12	0	0.0	12	1	8.3	40	3	7.5	25	1	4.0	139	7	5.0
PO_4	-------			11	2	18.2	12	1	8.3	11	1	9.0	11	3	27.7	12	1	8.3	7	0	0.0	13	0	0.0	77	8	10.4
CS#2	5	0	0.0	32	2	6.3	12	1	8.3	--------			--------			--------			-------			-------			49	3	6.1
CS#10	-------			-------			-------			44	2	4.5	40	5	12.5	12	2	16.7	-------			-------			96	9	9.4
CS#26	-------			-------			-------			--------			--------			--------			-------			13	2	15.4	13	2	15.4
CS#47	-------			-------			-------			--------			--------			--------			30	3	10.0	-------			30	3	10.0
CS#53	-------			-------			-------			--------			--------			--------			38	3	7.9	-------			38	3	7.9
CS#57	-------			-------			-------			--------			--------			--------			29	2	6.8	-------			29	2	6.8
BAS#11	-------			10	0	0.0	12	0	0.0	--------			--------			--------			-------			-------			22	0	0.0
BAS#17	-------			-------			-------			11	1	9.0	--------			11	0	0.0	-------			-------			22	1	4.5
BAS#56	-------			-------			-------			--------			--------			--------			34	1	2.9	-------			34	1	2.9
KAS#14	-------			-------			-------			--------			--------			32	5	15.6	-------			-------			32	5	15.6
KAS#26	-------			-------			-------			--------			--------			--------			26	2	8.3	14	1	7.1	38	3	7.9
KAS#47	-------			-------			-------			--------			--------			--------			36	4	11.1	16	3	18.8	52	7	13.5
KAS#57	-------			-------			-------			--------			--------			--------			38	5	13.2	-------			38	5	13.2
HAS#2	23	9	39.1	24	5	20.8	12	2	16.7	--------			--------			34	13	38.2	-------			-------			93	29	31.2
HAS#10	-------			-------			-------			30	10	33.3	47	13	27.7	33	12	36.4	-------			-------			110	35	31.8
HAS#49	-------			-------			-------			--------			--------			--------			21	8	38.1	17	6	35.3	38	14	36.8
HAS#52	-------			-------			-------			--------			--------			--------			36	8	22.2	-------			36	8	22.2
HAS#53	-------			-------			-------			--------			--------			--------			23	4	17.4	-------			23	4	17.4
P=	<.01			.30			.30			<.01			<.05			<.05			<.01			<.01			<.01		

FC = Fertilized Controls
PO_4 = Phosphate Buffer
CS = Control Serum
BAS = Brain Antiserum
KAS = Kidney Antiserum
HAS = Heart Antiserum

T = Total Number of Embryos
N = Number of Embryos with Inhibited Hearts
% = Per Cent of Embryos with Inhibited Hearts

TEXT FIGURE 1

Inhibitory Effect of Organ Antisera On Heart Development
Accumulation of data from all experiments and all antisera which were employed



C. Effects of Antisera Prepared Against Muscle Proteins on Embryonic Development.

It was thought at the beginning of the investigation that it would be possible to extract myosin from frog heart and skeletal muscle by the same procedure as was used for extraction of myosin from mammalian muscle. The original procedure used for protein extraction was one suggested by Dr. Karl Guthe, University of Michigan. According to Guthe, this procedure produces myosin from rabbit muscle which is 98% pure without employing any special procedures for removing contaminants such as actin and actomyosin. However, when a method for removing actomyosin was added to this procedure, practically all material resulting from the protein extraction of frog muscle was removed, suggesting that this protein was essentially actomyosin. Recent investigations (deVillafrance, 1956; Laufer, 1959) have shown that standard procedures for the isolation of myosin from mammalian muscle produce mostly actomyosin when applied to frogs and salamanders. Due to the uncertain identification of the materials extracted from the muscle tissues employed, the antigens are referred to below as "muscle protein."

Analysis of the antisera prepared against the muscle proteins showed that all of the antisera contained more than a single complementary component in common with protein extracts of muscle or with homogenates of organs. For example, antiserum #20, prepared against protein extracted from heart muscle of the frog, was seen to have only a single strong component against frog heart muscle protein and exhibited no reaction with frog skeletal muscle protein and chick heart muscle protein (Plate 5, Figure 15). However, when other tissues from the frog, such as brain and kidney, were tested against antiserum #20,

there was some reaction between the antiserum and these organs (Plate 5, Figure 16).

Antiserum #15, prepared against frog skeletal muscle protein, exhibited 2 complementary components against skeletal muscle protein of the frog (Plate 4, Figure 13) and 1 against frog kidney (Plate 4, Figure 14). The antisera prepared against proteins extracted from chick heart and skeletal muscle contained numerous complementary components to their respective antigens as well as to chick liver and skeletal muscle homogenates (Plate 5, Figure 17; Plate 6, Figures 18-20).

Although there were no cross reactions between the antigens and antisera from frog heart and skeletal muscle proteins, such cross reactions were observed between the antigens and antisera from the chick heart and skeletal muscle proteins. No cross reactions of antigens and antisera of the frog with the antigens and antisera of chick were ever observed. However, a comparison was made of extractions of proteins from skeletal muscle from 3 species of frogs, Rana pipiens, R. clamitans, and R. sylvatica, and these contained identical complementary components to antiserum #15 prepared against skeletal muscle protein of R. pipiens (no photograph was made of this observation). It would appear from this result that the proteins extracted from these species of the genus Rana are very similar in antigenic properties. However, proteins extracted from the skeletal and cardiac muscle of Rana pipiens are different in nature from chick proteins of similar origin.

Embryos treated with the antisera prepared against muscle proteins showed no detectable external abnormalities which were not observed in

the controls. Microscopic examination of the embryos treated with these antisera showed abnormalities similar to those which were observed in embryos which were treated with antisera prepared against heart. The most consistent of the abnormalities were varying degrees of inhibition of heart development. Four experiments (#21-#24) were performed in which the effects of the antisera prepared against muscle protein on the developing heart were studied. Table 9 lists the number of embryos with inhibited heart development which were observed in these experiments. It will be seen from the table that there was little consistency from one experiment to another. For instance, in Experiment 21 antiserum #20, prepared against frog heart muscle protein, produced 34.5% inhibition of the heart but in other experiments this antiserum showed no appreciable effect upon the developing heart. When the data were analyzed by the chi-square test for heterogeneity, the results were significant beyond the 5 per cent level in only one experiment (#24). Even when the data were pooled (Table 8, column marked "totals"), the results were not statistically significant.

Table 9.--Inhibition of Heart Development by Antisera Prepared Against Adult Muscle Proteins.

Treatment	Experiment Number												Totals		
	21			22			23			24					
	T	N	%	T	N	%	T	N	%	T	N	%	T	N	%
Fertilized Control	12	0	0.0	12	0	0.0	11	0	0.0	12	0	0.0	47	0	0.0
Phosphate Buffer	--	-	--	10	3	30.0	11	0	0.0	11	0	0.0	32	3	9.4
Holtfreter's Solution	8	1	12.5	--	-	--	--	-	---	--	-	--	8	1	12.5
Control Serum	--	-	--	5	0	0.0	9	2	22.2	9	0	0.0	23	2	8.5
Serum #15 (FSM)	30	5	16.7	--	-	--	--	-	--	24	5	20.8	54	10	18.5
Serum #18 (CSM)	22	5	22.7	22	4	18.2	23	3	13.0	24	2	8.3	92	14	15.2
Serum #20 (FHM)	29	10	34.5	--	-	--	24	1	4.2	24	0	0.0	67	11	16.4
Serum #23 (CHM)	27	6	22.2	23	2	8.7	24	4	16.7	24	6	25.0	98	18	18.4

T = Total number of embryos
N = Number of embryos with hearts inhibited
% = Per cent of embryos with hearts inhibited
FSM = Frog skeletal muscle protein
CSM = Chick skeletal muscle protein
FHM = Frog heart muscle protein
CHM = Chick heart muscle protein

PLATE 4

Analysis of Antisera Prepared Against Muscle Proteins I

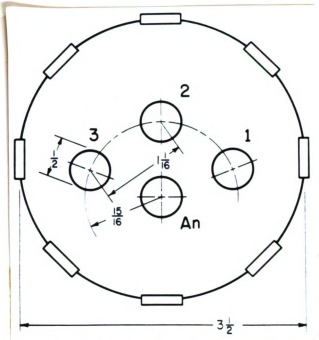
Figure 12 Geometry of the Ouchterlony Plates used in these analyses, (after Fox, 1959).

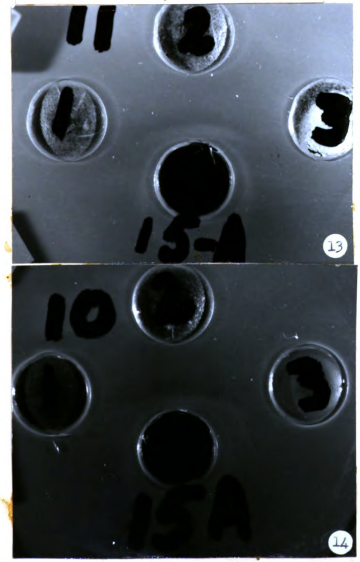
Figure 13 Ouchterlony Plate No. 11
Antiserum #15-prepared against protein extract from skeletal muscle of the frog

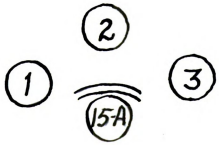
Well 1--Frog heart muscle protein
Well 2--Frog skeletal muscle protein
Well 3--Chick skeletal muscle protein

Figure 14 Ouchterlony Plate No. 10
Antiserum #15-prepared against protein extract from skeletal muscle of the frog

Well 1--Frog brain Homogenate
Well 2--Frog skeletal muscle protein
Well 3--Frog kidney homogenate


2
3
1
An
3 1/2


15-A
13
10
15A
14


2
1
3
15-A

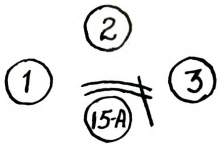

2
1
3
15-A

PLATE 5

Analysis of Antisera Prepared Against Muscle Proteins II.

Figure 15 Ouchterlony Plate No. 15
Antiserum #20-Prepared against frog heart muscle protein

Well 1--Frog skeletal muscle protein
Well 2--Frog heart muscle protein
Well 3--Chick heart muscle protein

Figure 16 Ouchterlony Plate No. 14
Antiserum #20-Prepared against frog heart muscle protein

Well 1--Frog brain homogenate
Well 2--Frog heart muscle protein
Well 3--Frog kidney homogenate

Figure 17 Ouchterlony Plate No. 13
Antiserum #18-Prepared against chick skeletal muscle protein

Well 1--Chick heart muscle protein
Well 2--Chick skeletal muscle protein
Well 3--Frog skeletal muscle protein

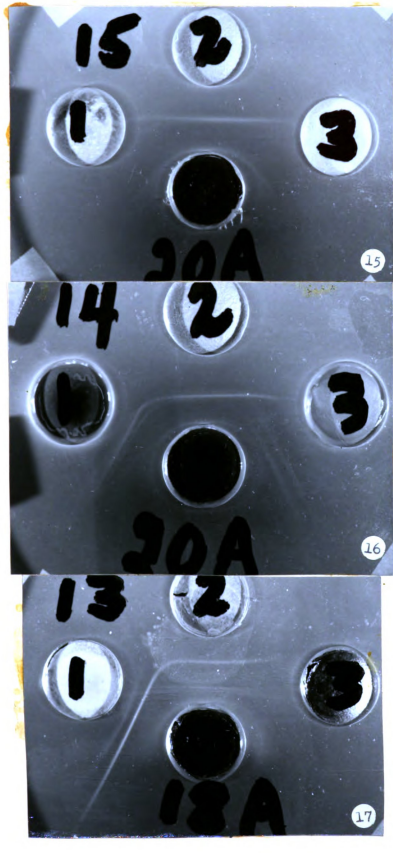

15
2
1
3
20A
15
14
2
1
3
20A
16
13
2
1
3
18A
17

PLATE 6

Analysis of Antisera Prepared Against Muscle Proteins III.

Figure 18 Ouchterlony Plate No. 12
Antiserum #18-Prepared against chick skeletal muscle protein

Well 1--Chick skeletal muscle homogenate
Well 2--Chick skeletal muscle protein
Well 3--Chick liver homogenate

Figure 19 Ouchterlony Plate No. 17
Antiserum #23-Prepared against chick heart muscle protein

Well 1--Chick skeletal muscle protein
Well 2--Chick heart muscle protein
Well 3--Frog heart muscle protein

Figure 20 Ouchterlony Plate No. 16
Antiserum #23-Prepared against chick heart muscle protein

Well 1--Chick skeletal muscle homogenate
Well 2--Chick heart muscle protein
Well 3--Chick liver homogenate


12
2
1
3
18A
18


17
2
1
3
23A
19


16
2
1
3
20

PLATE 7

Analysis of Antisera Prepared Against Homogenates of Adult Organs I

Figure 21 Ouchterlony Plate III-A
Antiserum #11-Prepared against frog brain homogenate

Well 1--Frog heart homogenate
Well 2--Frog brain homogenate
Well 3--Frog liver homogenate

Figure 22 Ouchterlony Plate I-A
Antiserum #2-Prepared against frog heart homogenate

Well 1--Frog heart homogenate
Well 2--Frog brain homogenate
Well 3--Frog liver homogenate

Figure 23 Ouchterlony Plate No. 53
Antiserum #10-Prepared against frog heart homogenate

Well 1--Frog brain homogenate
Well 2--Frog heart homogenate
Well 3--Frog kidney homogenate

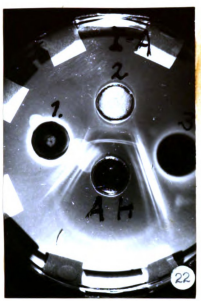
Figure 24 Ouchterlony Plate No. 38
Same as Plate No. 53 (Figure 23) except the antiserum well received inhibiting doses of brain homogenate prior to testing the antiserum.

Figure 25 Ouchterlony Plate No. 43
Same as Plate No. 53 (Figure 23) except the antiserum well received inhibiting doses of kidney homogenate prior to testing the antiserum.

Figure 26 Ouchterlony Plate No. 82
Antiserum #49-Prepared against adult frog heart

Well 1--Frog kidney homogenate
Well 2--Frog heart homogenate
Well 3--Frog skeletal muscle homogenate

21



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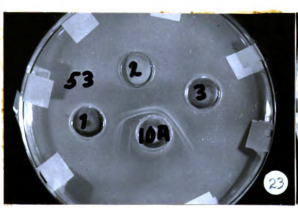


23

24

25

26

PLATE 8

Analysis of Antisera Prepared Against Homogenates of Adult Frog Organs II

Figure 27 Ouchterlony Plate No. 37
Antiserum #52-Prepared against frog heart homogenate
Well 1--Frog brain homogenate
Well 2--Frog heart homogenate
Well 3--Frog kidney homogenate

Figure 28 Ouchterlony Plate No. 40
Same as Plate No. 37 (Figure 27) except the antiserum well received inhibiting doses of brain homogenate prior to testing the antiserum

Figure 29 Ouchterlony Plate No. 46
Same as Plate No. 37 (Figure 27) except the antiserum well received inhibiting doses of kidney homogenate prior to testing the antiserum. Although difficult to see on the photograph, there is a single line between the antiserum well and well 2.

Figure 30 Ouchterlony Plate No. 30
Antiserum #53-Prepared against frog heart homogenate
Well 1--Frog brain homogenate
Well 2--Frog heart homogenate
Well 3--Frog kidney homogenate

Figure 31 Ouchterlony Plate No. 25
Same as Plate No. 30 (Figure 30) except that the antiserum well received inhibiting doses of brain homogenate prior to testing the antiserum. Note the line between the antiserum well and well 2 which appears to be specific.

Figure 32 Ouchterlony Plate No. 44
Same as Plate No. 30 (Figure 30) except the antiserum well received inhibiting doses of kidney homogenate prior to testing the antiserum. There were no lines in this plate.

Figure 33 Ouchterlony Plate No. 34
Antiserum #47-Prepared against frog kidney homogenate
Well 1--Frog brain homogenate
Well 2--Frog kidney homogenate
Well 3--Frog heart homogenate

Figure 34 Ouchterlony Plate No. 41
Same as Plate No. 34 (Figure 33) except that the antiserum well received inhibiting doses of brain homogenate prior to testing the antiserum.

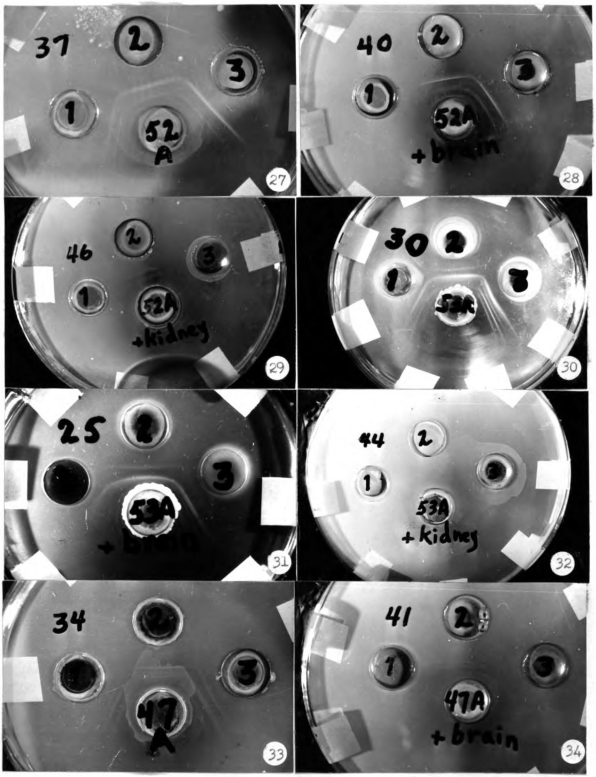

37
2
3
1
52
A
27
40
2
3
1
52A
+brain
28
46
2
3
1
52A
+kidney
29
30
2
1
3
52A
30
25
2
3
52A
31
44
2
1
3
53A
+kidney
32
34
2
3
47
A
33
41
2
1
3
47A
+brain
34

PLATE 9

Analysis of Antisera Prepared Against Homogenates of Adult Frog Organs III

Figure 35 Ouchterlony Plate No. 33
Well 1--Frog brain homogenate
Well 2--Frog kidney homogenate
Well 3--Frog heart homogenate

Figure 36 Ouchterlony Plate No. 26
Same as Plate No. 33 (Figure 35) except the antiserum well received inhibiting doses of brain homogenate prior to testing the antiserum.

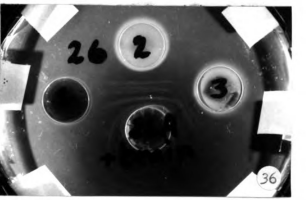
Figure 37 Ouchterlony Plate No. 28
Same as Plate No. 33 (Figure 35) except the antiserum well received inhibiting doses of heart homogenate prior to testing the antiserum. Notice the line which appears to be specific for kidney.

Figure 38 Ouchterlony Plate No. 20
Antiserum #14-Prepared against frog kidney homogenate
Well 1--Frog brain homogenate
Well 2--Frog kidney homogenate
Well 3--Frog heart homogenate

Figure 39 Ouchterlony Plate No. 35
Antiserum #57-Prepared against frog kidney homogenate
Well 1--Frog brain homogenate
Well 2--Frog kidney homogenate
Well 3--Frog heart homogenate

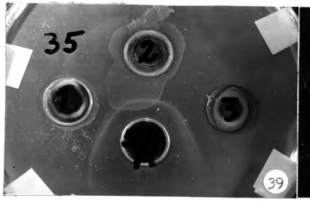
Figure 40 Ouchterlony Plate No. 42
Same of Plate No. 35 (Figure 39) except the antiserum well received inhibiting doses of brain homogenate prior to testing the antiserum.


33
2
1
3
35


26
2
3
36


2
1
3
28
26A
+ heart
37


20
2
1
38


35
2
3
39


42
2
3
1
51A
+ brain
40

PLATE 10

Analysis of Antisera Prepared Against Homogenates of Adult Frog Organs IV

Figure 41 Ouchterlony Plate No. 22
Antiserum #17-Prepared against frog brain homogenate
Well 1--Frog heart homogenate
Well 2--Frog brain homogenate
Well 3--Frog blood serum

Figure 42 Ouchterlony Plate No. 23
Same as Plate No. 22 (Figure 41) except that the antiserum well received inhibiting doses of frog blood serum prior to testing the antiserum.

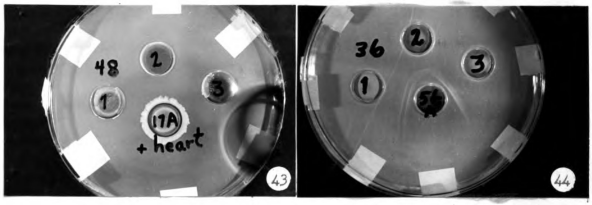
Figure 43 Ouchterlony Plate No. 48
Same as Plate No. 22 (Figure 41) except that the antiserum well received inhibiting doses of heart homogenate prior to testing the antiserum. Also well 3 contained kidney homogenate instead of blood serum. Although difficult to see there is a line between the antiserum well and well 2.

Figure 44 Ouchterlony Plate No. 36
Antiserum #56-Prepared against frog brain homogenate
Well 1--Frog kidney homogenate
Well 2--Frog brain homogenate
Well 3--Frog heart homogenate

Figure 45 Ouchterlony Plate No. 32
Center well--frog heart homogenate
Well 1--Anti-heart serum #10
Well 2--Anti-heart serum #52
Well 3--Anti-heart serum #53

Figure 46 Ouchterlony Plate No. 31
Center well--frog kidney homogenate
Well 1--Anti-kidney serum #14
Well 2--Anti-kidney serum #26
Well 3--Anti-kidney serum #47


22
2
41
13
2
+ serum
42


48
2
1
3
17A
+ heart
43
36
2
3
1
44

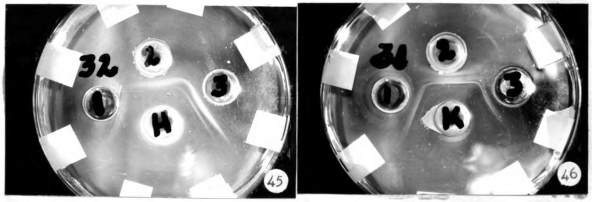

32
2
1
3
H
45
34
2
1
3
K
46

PLATE 11

Inhibition of Heart Development Resulting From Treatment of Embryos with Antisera Prepared Against Adult Organs of the Frog

Figure 47 x.s. through the heart of a control embryo from Exp. 19

Figure 48 x.s. through the heart region of an embryo from Exp. 15 which was injected with anti-heart serum #10

Figure 49 x.s. through the heart region of an embryo from Exp. 10 which was injected with anti-heart serum #2

Figure 50 x.s. through the heart region of an embryo from Exp. 19 which was injected with anti-heart serum #49

Figure 51 x.s. through the heart region of an embryo from Exp. 15 which was injected with anti-heart serum #10

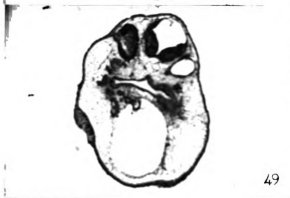
Figure 52 x.s. through the heart region of an embryo from Exp. 19 which was injected with anti-heart serum #49

Figure 53 x.s. through the heart region of an embryo from Exp. 10 which was injected with anti-heart serum #2

Figure 54 x.s. through the heart region of an embryo from Exp. 19 which was injected with anti-kidney serum #47

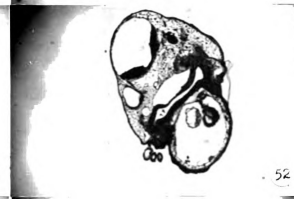

47


48


49


50


51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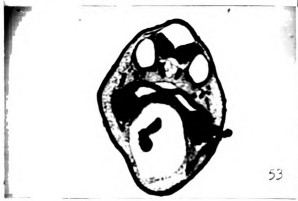
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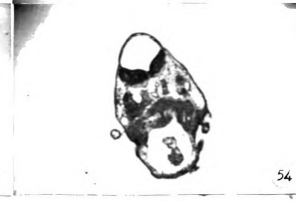

54

PLATE 12

Heart Inhibition and Other Abnormalities as Produced by Treatment of Embryos with Antisera Prepared Against Homogenates of Adult Organs and Protein Extracts of Skeletal and Heart Muscle

Figure 55 x.s. through the heart region of a normal control from Exp. 21

Figure 56 x.s. through the heart region of an embryo from Exp. 21 which was injected with antiserum #15 prepared against frog skeletal muscle protein

Figure 57 x.s. through the heart region of an embryo from Exp. 21 which was injected with antiserum #20 prepared against frog heart muscle protein

Figure 58 x.s. through the heart region of an embryo from Exp. 21 which was injected with antiserum #20 prepared against frog heart muscle protein. Note the double heart. Slightly anterior to this section the pericardial cavity was divided.

Figure 59 x.s. through the hind-brain of an embryo from Exp. 10 which had been cultured in anti-heart serum #10. Note the double neurocoele.

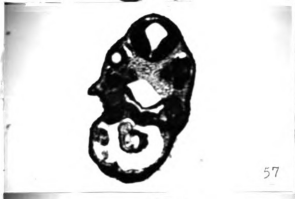
Figure 60 x.s. through another embryo which had been treated in the same manner as the embryo in Figure 59. Note once again the extra neurocoels.

Figure 61 x.s. through the pronephros of a control embryo from Exp. 20

Figure 62 x.s. through the pronephros of an edematous embryo from Exp. 20 which was treated with anti-kidney serum #14. The pronephric tubules seemed to be enlarged.


55


56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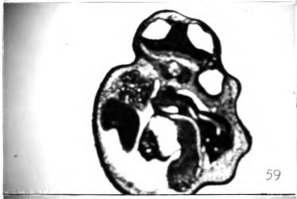
58


59


60


61


62

DISCUSSION

A. Effects of Adult Tissue Fractions on Embryonic Development

As has been pointed out in the introductory section, one group of investigators believes that substances from adult tissues stimulate the growth of homologous embryonic tissue, whereas others feel that such substances have inhibitory effects on the differentiation of the corresponding developing system. The bulk of the evidence which has been used to support the former idea has been derived from experiments in which chicken spleen was grafted onto chorioallantoic membranes of the chick embryo. There are many earlier observations which strongly suggest that spleen is not a typical differentiating tissue, due to its mixed content of cells. Furthermore, it appears that almost any tissue, when placed on the chorioallantoic membrane, can produce spleen hypertrophy in the host chick embryo (Van Alten & Fennell, 1959).

Experiments which have been devised to corroborate the alternative hypothesis, namely that substances from adult tissues may be inhibitory to homologous differentiating tissue, also appear to suffer from a lack of sufficient demonstration of specific reactions. This lack of specificity can be seen in the results of attempts to inhibit the morphogenesis of the nervous system. In the first place, the developing nervous system is affected by various types of treatments, as has been shown by experiments described in this paper, making it difficult to ascribe any specificity to effects upon the nervous system of materials from adult nervous tissue. Furthermore, the types of abnormalities described in the reports of Rose (1955), Clarke and McCallion (1959 a and b), Braverman (1959a), and the analyses described earlier in this paper are

difficult to interpret. One of the common abnormalities described in most of this work is a condition in which the neurocoele is greatly reduced or lacking. Whether this abnormality is the result of an inhibition of differentiation of nervous tissue or is actually a proliferation of additional nervous tissue is difficult to determine. Rather than being an example of specific inhibition, the increase in cells which results in "solid nervous system" might as well serve as an example of hypertrophic growth. To evaluate properly the results from experiments involving the effects of substances from adult tissue upon homologous differentiating tissues, it is necessary to be able to distinguish between the processes that initiate the differentiation of an organ and the ensuing processes which result in its growth.

For a number of reasons, therefore, the developing nervous system appears to be a very poor system to use in attempting to determine the effects of substances from adult tissues on homologous embryonic tissues. Other differentiating systems would appear to provide more definite criteria for such studies, as for example, the developing heart.

In connection with the question of substances from adult tissues affecting embryonic differentiation, some conclusions may be drawn from the experiments reported in this paper which concern both the method of treating embryos and the particular fraction of the tissue cells which may be more effective. First of all, it appears that injection of adult tissue material into developing embryos is much more effective than culturing embryos in the presence of the same substances. Although Rose (1955) and Clarke and McCallion (1959a) claim to have obtained "specific inhibition" by culturing developing frog embryos in the presence of adult

tissues of the frog, Spiegel (personal communication), Shaver (unpublished), and the writer have not been able to demonstrate any specific effect upon the developing embryos when employing culturing methods. However, the results of Shaver's (1954) experiments, the microscopic analysis of which is given in this paper, show that injection of the substances of adult tissues into developing embryos has a very pronounced effect in the case of the nervous system. Also, in the work with antisera against adult tissues, it was found that the injection procedure was superior to the culturing technique. It is possible that the substances from adult tissues may never enter the embryo when the culturing method is employed, since it is known that the surface of the developing frog embryo is highly impermeable to most substances (see Holtfreter, 1943). Recent investigations concerning treatment of frog embryos with various chemicals have also demonstrated that the injection technique is superior to the culturing procedure (Grant, 1960).

Another interesting observation from the present work with nervous tissue is that the fraction of cytoplasmic granules of adult brain was more effective in producing specific abnormalities on the differentiating nervous system than any of the other centrifugal fractions employed. Several explanations of this observation are possible. The cytoplasmic granule fraction may have a higher content of effective materials, either in their native state or through adsorption in the homogenization process. Possibly, the particulate fraction may release materials such as enzymes of the respiratory type, which could change the normal metabolic processes of the early embryo. Thus the problem of what effect substances from adult tissues have on homologous differentiating tissues in the embryo, as well as the mode of action of such substances, remains unsolved.

B. Effects of Antisera Prepared Against Adult Organs on the Differentiation of Embryonic Tissues

The analysis of the antisera prepared against adult organs of the frog suggests that there is not as much antigenic difference between organs from the same organism as would be desirable for studies concerned with the effects of organ antisera on embryonic development. As has been reported earlier in the paper, antisera prepared against adult frog brain, kidney, and heart exhibit several common components. It appears that there is little difficulty in showing differences between antigenic properties between an ectodermal derivative such as brain and a mesodermal derivative. However, considerable difficulty is encountered when one attempts to show specific differences in antigenic properties between 2 mesodermal derivatives such as heart and kidney. Such differences have been demonstrated in this study but the technique employed does not rule out the possibility that these differences could be quantitative rather than qualitative. On the other hand, the inability to demonstrate specific differences in antigenic properties does not conclusively prove that there are no differences in biochemical constitution of these organs. It is possible that the antibody-producing mechanism of the rabbit is not sensitive to the substances involved in specific chemical differences in organ composition or if specific differences do occur, the substances responsible for these differences may be present in amounts too small to elicit an immune response in the rabbit. When the frog embryo is treated with the antisera prepared against adult organs, however, it responds differently to the various antisera, indicating that there are either quantitative or qualitative differences, or both, in the antisera.

The most interesting observation made in the studies reported in this paper was that concerning the inhibition of heart development. This work bears out the earlier work of Ebert (1950) who found that antisera prepared against adult chick heart inhibited the development of the heart in explanted chick embryos. The work with the frog has an added advantage since the embryos can be grown in their normal environment.

Although the results of the experiments in which embryos were treated with antisera prepared against adult organs are highly significant statistically inasfar as inhibition of heart development was concerned, the question arises as to why the response is not the same in embryos developed from eggs of the same female or in embryos originating from eggs of different females. There are a number of possible explanations for this. First of all, the experimental procedures may account for a considerable amount of the variation. With the injection procedure employed, it was not possible to inject the same amount of material into each embryo and even if it were, there was no way to determine how much of the injected material remained in the embryo. The other possible source of individual differences in response to treatment with antisera is that the embryos may differ genetically and this heritable difference may cause a variation in the response of the embryos. Many workers have found that eggs from different females respond differently to the same experimental treatment. Whether this is due to genetic differences or to differences in uncontrolled environmental variables is not apparent.

In the experiments with organ antisera it would be desirable to know exactly what factor or factors in the antisera were producing the inhibition of heart development. Although factors specific for heart

were demonstrated to be present in some of the heart antisera, attempts to correlate heart inhibition with these factors were not successful. Furthermore, specific factors were not demonstrated in some of the heart antisera which were very effective in producing heart inhibition. There is the possibility that specific factors in some antisera may be non-precipitating and consequently would not be seen with Ouchterlony technique.

With the techniques employed in the experiments with organ antisera, it was not possible to determine what was the primary effect on the embryo which led to heart inhibition. Obviously, either processes of growth or differentiation, or both, were being affected by the treatment. If the processes of growth and differentiation were more fully understood, an evaluation of the experiments involving the effects of adult antigens or antisera on embryonic development might be more easily made.

A number of theories have been proposed within the last 20 years to account for embryonic differentiation, morphogenesis, and growth. Prominent among these proposals are the template-antitemplate theory of Weiss (1947, 1953), the auto-antibody concept of Tyler (1947, 1955), the specific inhibition theory of Rose (1952, 1957), and the "building block" hypothesis of Ebert (1954). Whether the above concepts can adequately explain differentiation and growth remains to be seen, but it seems pertinent to discuss the present work with organ antisera in connection with these ideas.

The template-antitemplate theory of Weiss states that growth and differentiation are controlled by paired substances, an intracellular

"template" for further reproduction of cells, and an accessory diffusible "anti-template" capable of inactivating the former. As the anti-templates accumulate in the surrounding medium, they reach a critical concentration and growth ceases. The evidence used in support of this theory comes mostly from studies involving regeneration of liver in adult organisms. If such a mechanism is responsible for growth of embryonic organs, it would seem most likely that an antiserum prepared against adult tissues would contain antibodies against the intracellular templates. These antibodies, when introduced into the embryo, could inactivate the templates and produce an end result comparable to the action of the normally occurring anti-templates. Consequently, cessation of growth processes would occur long before the organ involved had reached its normal size. Unfortunately, the hypothesis as presented by Weiss is based upon a set of circumstances which may have little or nothing to do with primary differentiation processes.

The auto-antibody concept of Tyler is in many respects similar to the above-mentioned theory but it goes further to specify that the paired substances function as antigen and antibody. Tyler's extensive work with interacting substances in the egg and sperm of sea urchins, as well as indications from work with cellular aggregation and venin-antivenin systems, prompted the proposal of this theory. If growth is controlled by the production of auto-antibodies, as Tyler suggests, treatment of embryos with an exogenous source of similar antibodies would consequently cause a premature cessation of growth and differentiation. This would constitute one of the simplest explanations for the mode of action of the antisera. However, as is true with all of

these theories, there is little experimental evidence to substantiate the auto-antibody concept as specifically applicable to histogenesis of tissue cells.

At first sight, the "specific inhibition" theory of Rose seems to be controverted by the results obtained by treatment of embryos with antisera prepared against substances from adult tissues. According to this theory, the presence of substances from adult tissues should inhibit the development of homologous embryonic tissue and it would logically follow that antisera prepared against these same adult substances should stimulate growth in the embryonic tissue. One would expect the antisera to contain complementary factors to the inhibitors from adult tissues, and these factors would be expected to inactivate the inhibitors in the embryo, resulting in hypertrophic growth. However, it is possible that cells in adult tissues contain substances which have antigenic sites in common with similar substances in precursor cells of the same tissue. Thus, the antiserum might inactivate these precursor cells and this tissue would fail to differentiate.

In considering the general theory of Rose in connection with experiments of the type described by him and by others (see above) in which frog embryos exposed to adult tissues are "specifically inhibited," a basic contradiction appears to exist when these results are made to appear as correlative with results of experiments where an obvious gradient system is involved. That is, the effects noted on the regeneration of "dominant" structures in the gradient system of such forms as Tubularia and Lineus, caused by the presence of additional "dominant" structure material, seems to have little connection with the type of

inhibition noted by Rose and others on the nervous or other systems in frog embryos treated with substances from homologous adult systems. For example, one would presumably predict, on the basis of Rose's general theory, that the fore-brain and other anterior neural structures of the embryo, would be selectively inhibited by adult brain. This is not the case, either in Rose's experiments or in the tests made by other workers. Thus, to attribute the differentiation of the nervous system, and other systems, in the frog to the same type of gradient response as may exist in Tubularia or Lineus, seems to be an oversimplification of the actual facts. However, Braverman (1958a) in a short report, states that a gradient effect was noted in the inhibition of chick nervous system after treatment with extracts of adult nervous system from different levels of the brain and spinal cord. There has been no subsequent confirmation of this.

Ebert's "building block" hypothesis, derived from his experiments with chorioallantoic grafts, suggests that cells in developing embryos can either incorporate already-formed proteins into their cytoplasmic structure or can utilize these proteins in the synthesis of new proteins. According to Ebert, this accounts for the hypertrophic growth observed in spleens of the host chick embryo following grafts of adult chick spleen. Perhaps, antisera against adult tissues contain antibodies that have reactive sites complementary to tissue precursors present in the yolk. In this case, the mobilization of specific tissue substances from the yolk might be blocked.

At the present time, it is impossible to ascribe the inhibition of heart development in embryos treated with anti-heart serum to any

particular mode of action. However, due to the early stage at which the embryos had to be treated to produce heart inhibition, it seems that some very early process of differentiation is involved. If some general growth reaction only was involved in the inhibition, it would seem that the embryos could be affected over a much wider period of development than was demonstrated in the experiments described in this paper. Furthermore, a few embryos showed no signs of heart development though a normal pericardial space was present, indicating that morphological differentiation was never initiated. This would lead to the conclusion, then, that the antisera prepared against adult heart were in some way affecting the early biochemical events which ultimately result in the morphological differentiation of the heart.

C. The Effects of Antisera Prepared Against Muscle Proteins on Embryonic Development

One of the major shortcomings of the immunological approach to studying embryonic development is that most of the work has been done with highly heterogeneous antigens which are not easily analyzed. Consequently, it was believed that muscle proteins from the heart would be a source of substances which could be easily extracted in a rather pure form. Thus, the study of the effects of antisera prepared against muscle proteins on embryonic development was initiated. Unfortunately, the methods used for extraction did not produce the degree of specificity and purity desired. Therefore, the work presented in this paper failed to overcome the basic problem of heterogeneous antigens. However, it is felt that the preliminary results with antisera prepared against muscle proteins should not discourage further investigations in

this area. With procedures which have recently been worked out for the extraction of muscle proteins from the frog, it should now be possible to do a thorough analysis of the effects of antisera prepared against purified muscle protein.

SUMMARY

1. Although numerous studies have been made concerning the effects of substances from differentiated tissues, and of antisera against these substances, on the differentiation of homologous embryonic tissues, there is still considerable confusion concerning the results of these experiments. This is due in part to the fact that different organisms were employed for the study of the effects of the tissue substances and of the antisera prepared against them. The purpose of this paper is to attempt a clarification of this problem by treating the same differentiating system with substances from the homologous adult tissue as well as antisera prepared against these substances.

2. The experimental approach was to treat early embryos of Rana pipiens with tissue fractions of adult organs and with antisera prepared against homogenates of adult organs of the same species. Two methods of treatment were employed: injection of embryos with the tissue fractions and organ antisera, and culture of embryos in the presence of these substances.

3. The results of the work with fractions of adult tissues were somewhat variable but the following conclusions may be drawn: (a) It appears from these studies that the nervous system, with which most of the studies in this area have been made, is a rather poor system for demonstrating specific effects, since a variety of treatments seem to affect this system; (b) the method of injecting substances proved to be more effective than the culturing technique in producing specific effects upon the developing nervous system; and (c) a final observation from this work is that the fraction of cytoplasmic granules produced more consistent defects in the embryonic nervous system than any other centrifugal fraction employed.

4. Treatment of embryos with antisera prepared against adult heart produced the most specific inhibitory effects in the developing embryo of all the experimental procedures employed. These antisera produced a marked inhibition of heart development in 30% of all embryos injected with them. Control sera, phosphate buffer, and antisera prepared against adult brain and kidney also produced this effect but in very much lower percentages.

5. Other effects of antisera prepared against adult organs were observed in embryos in some experiments. Embryos cultured in heart antiserum were observed to have a specific defect of the central nervous system in 2 experiments. In another experiment, a large per cent of the embryos which were injected with an antiserum prepared against adult kidney showed extreme edema.

6. Analysis of the antisera prepared against adult kidney, brain, and heart showed that these organs have complementary factors in common. The antigenic properties of kidney and heart were found to be quite similar. On the other hand, when antigenic properties of heart and kidney were compared with those of brain, considerable differences could be detected. Using adsorption techniques, most of the antisera prepared against adult organs could be shown to have specific components. However, attempts to correlate heart inhibition with specific components in the antisera were unsuccessful.

7. Preliminary experiments were performed to determine the effects of antisera prepared against protein extracts of heart and skeletal muscle of frog and chick on the development of the frog heart. Unfortunately, the extracted proteins lacked the desired characteristics of homogeneity

and purity. When embryos were treated with antisera prepared against these proteins, some heart inhibition was noted, but this inhibition was not significantly greater than that observed in embryos treated with control sera.

8. Analysis of the antisera prepared against protein extracts of skeletal and heart muscle showed the following: (a) there were cross reactions between these antisera and antigens from body organs such as kidney; (b) there were no cross reactions between the antigens or antisera of frog heart muscle proteins with the antigens or antisera of frog skeletal muscle proteins; (c) the antigens and antisera of chick heart muscle proteins and chick skeletal muscle proteins did exhibit cross reactions; and (d) there were never any cross reactions of the antigens and antisera of the frog muscle proteins with the antigens and antisera of the chick material.

9. The results of these studies are discussed in connection with current theories of differentiation and growth.

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