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Gene Activation in Early Amphibian Development

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GENE ACTIVATION IN EARLY AMPHIBIAN DEVELOPMENT

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by

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ABSTRACT

The amphibian embryo has been a fascinating object for study for a century or more. It is large in size, widely available, and easy to observe throughout embryogenesis. The control of early development and the role of the genes have also been investigated for a long time. The combination of older morphological and more recent biochemical descriptions of development under normal and experimental conditions are bringing us closer to an understanding of the mechanism of early differentiation.

In the General Introduction to this thesis the role of the nucleus and cytoplasm in amphibian development are discussed. Other types of embryos are described when the evidence is clearer or more complete. The processes of cleavage and accompanying protein synthesis are independent of nuclear activity and therefore dependent on substances stored in the maternal cytoplasm. Nuclear function is first required for development beyond the late blastula stage. Gastrulation marks the beginning of rapid differentiation and diversification of cells to form the many tissues of the larval stage. The synthesis of messenger-like RNA is low during cleavage and increases from the late blastula or early gastrula stage.

The establishment of different patterns of RNA synthesis represents the first step in cell differentiation. Since the nuclei of early embryonic cells are equivalent, extra-chromosomal factors must influence chromosomal activity. Prelocalization of cytoplasmic substances has been demonstrated in non-amphibian embryos. The formation of the grey crescent in the amphibian embryo is described. Experimental analysis of tissue differentiation has revealed that cytoplasmic factors have a radially diffuse localization in three main regions of the amphibian.

The experimental section of this thesis describes the activation of RNA synthesis in the late blastula of Xenopus laevis embryos. A careful description of the onset of gene activity is fundamental to the understanding of the control of gene activity. An embryo half preparation is described which is permeable to the precursor uridine-H^3. The time course of incorporation of uridine into total acid insoluble RNA shows a marked increase between the blastula and early gastrula stages.

Autoradiographic studies demonstrated a sudden activation of nuclear RNA synthesis by the late blastula stage from an initially negligible level in the

early blastula. Presumptive endoderm and mesoderm participate in this activation. RNA synthesis per nucleus remains constant through gastrulation. The ectoderm is activated at the mid gastrula stage. Analysis of phenol extracted RNA by sucrose gradient centrifugation and gel filtration on Sephadex G-100 demonstrates that 70% of the RNA synthesized by early gastrulae is a high molecular weight heterogeneous RNA. Its base composition approaches that of DNA. 30% of the label appears in transfer RNA. At the early blastula stage only a very small amount of heterogeneous RNA is synthesized.

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GENERAL INTRODUCTION

GENE ACTIVATION AND CYTOPLASMIC LOCALIZATION IN EARLY AMPHIBIAN DEVELOPMENT

The fertilized egg is a self-contained independent unit, which, provided with adequate environmental conditions, proceeds almost inevitably to subdivide and diversify itself. The embryologist seeks to understand the control of embryogenesis, of the appearance of specific form and function in the cells descended from the original fertilized egg. The major role of the nucleus and chromosomes in heredity and in cellular activity was understood by the end of the 19th century. Also at this time the main theories of embryogenesis had been proposed. Recently, the concepts and methods developed in the field of molecular biology, especially in the control and expression of genetic activity, have provided new stimuli for the embryologist.

Many workers have suggested that differentiation of embryonic cells results from an active interaction between nucleus and cytoplasm. In an interacting system it is difficult to separate the roles of the two components; however, the importance of each will be discussed in order to understand the origin of regional differences which must provide the basis for differentiation.

The nucleus of the adult cell is necessary for maintenance of long term cellular activity (see Brachet, J., 1961, for review). Moreover, in a cell with inactivated nucleus differentiated biosynthetic functions may be inhibited immediately (Davidson et al, 1963). On the other hand natural examples are known in which a stable function of the cell exists after disintegration of its nucleus, such as hemoglobin synthesis in mammalian reticulocytes. Apparently the degree of sensitivity of a cellular function to lack of nuclear activity may vary depending on the amount and stability of substances from previous nuclear function, including messenger RNA.

I. ROLE OF THE NUCLEUS IN EMBRYOLOGICAL DEVELOPMENT

Early development may be divided into two periods; the early cleavage period is characterized by rapid cell divisions, and the later period beginning at gastrulation by rapid diversification of cell type. To what extent are these processes dependent on accompanying nuclear activity and to what extent on substances from previous nuclear function stored in the cytoplasm?

A. NUCLEAR FUNCTION IS NOT REQUIRED FOR CLEAVAGE

Several types of evidence have demonstrated that nuclear activity is relatively low during cleavage and in fact is not required for the formation of the many-celled blastula. The data suggests that cellular activities necessary for cleavage are maintained by substances and particles stored in the egg cytoplasm.

Effect of Removal of the Nucleus.

The results of enucleation experiments have clearly specified the period of insensitivity to lack of contemporary nuclear function. Harvey (1936, 1940) fragmented sea urchin eggs by centrifugation into nucleate and enucleate halves or quarters, and activated them in hypertonic sea water. The most favorable enucleate fragments formed blastulae with as many as 500 cells, but showed little evidence of differentiation or gastrulation. Parthenogenic nucleate fragments cleaved only slightly faster, but were able to form small normal plutei. This success led Harvey to suggest that under more favorable circumstances, the enucleate fragments might also be able to continue development to the pluteus stage. Although no such results were ever achieved, this was the first clear-cut demonstration of cleavage without nuclei or chromatin.

In the amphibian, Fankhauser (1934a) obtained from fragments of polyspermic urodele eggs blastulae partly composed of achromatic cells. Briggs et al (1951) showed that fertilization of enucleate anuran eggs with toluidine blue-treated or irradiated sperm, resulted in cleavage of one-half to two-thirds of the animal half to produce partial blastulae whose cells lacked 'functional' chromosomes. However, clumps of sperm chromatin were present in a small per cent of the cells. Interestingly, unlike the sea urchin, the amphibian egg completely lacking chromosomes, which had been enucleated after parthenogenic activation, could not cleave.

Maternal Control of Cleavage

The evidence suggests that the characteristics of cleavage such as rate of cell division, and position of daughter cells is dependent on maternal gene activity during oogenesis. Morphological expression of the gene activity awaits maturation and fertilization of the egg. Some of this evidence comes from crosses between parents with different cleavage patterns.

When two closely related species are crossed, succesful development of larval forms may be obtained. When two species differ in certain recognizable

characteristics, the development of the hybrid embryos may be observed and characterized as maternal, paternal or intermediate. The progeny of two species with slightly different cleavage rates develop according to the maternal pattern through blastulation in sea urchins (Driesch, 1898; Moore, A.R., 1933) and amphibians (Moore, J.A., 1941). The direction of coiling which can be foreseen at the first or second cleavage in the gastropod Limnaea is determined only by a maternal gene, and is not affected by paternal chromosomes even when they carry the gene for the opposite direction of coiling (Sturtevant, 1923).

Thus as far as can be determined, paternal genes are not translated to produce a recognizable effect on cleavage. Since nuclear activity is not required for cleavage, the cleavage pattern must be controlled by substances stored in the maternal cytoplasm.

Protein Synthesis During Cleavage

The rapid formation of spindle proteins and new cell membranes during cleavage must require the assembly of new proteins. In fact it has been demonstrated clearly in the sea urchin that a sudden activation of the incorporation of amino acids into protein occurs within minutes after fertilization (Hultin, 1961a; Giudice et al, 1962). At least some incorporated leucine-H^3 appears in the protein of the mitotic apparatus (Mangan et al, 1965). Moreover, protein synthesis is required for cleavage since the specific inhibitor of protein synthesis, puromycin, blocks development even at the first division (Hultin, 1961b). In the amphibian puromycin injected into the 2- to 4-cell Pleurodeles embryo stops cleavage after three to four divisions (Brachet, J. et al, 1964).

Ribosomes have been detected in the unfertilized egg and early embryo (Karasaki, 1959; Brown and Caston, 1962; Decroly and Cape, 1965) and bulk RNA representing largely ribosomal RNA remains constant throughout cleavage in the sea urchin (Schmidt et al, 1948) and the amphibian (Chen, 1960). The protein synthesis occurring during cleavage therefore takes place on ribosomes formed during oogenesis. Davidson et al (1964) have studied RNA synthesis during the rapid growth phase of the amphibian oocyte. They found that ribosomal RNA is rapidly and almost exclusively synthesized, and is retained as the oocyte matures. Brown and Littna (1964) have shown that RNA labeled during oogenesis can be recovered as ribosomal RNA in 2- to 4-cell and swimming embryos. The synthesis of new ribosomal RNA cannot again be detected until gastrulation in amphibia and in sea urchins (Brown and Littna, 1964; Comb et al, 1965).

Effect of Actinomycin D on Cleavage

Recently it has become possible to extend the enucleation experiments already discussed and to suppress nuclear function with actinomycin D. At low concentrations of the antibiotic, only the DNA dependent synthesis of RNA is inhibited. The chromatin is still physically present and probably the metabolic functions of the nucleus are unaffected. The effect of actinomycin D on morphological development and on RNA and protein synthesis have provided further evidence that the processes of cleavage are independent of accompanying nuclear activity.

Extensive experiments with actinomycin D have been performed on the sea urchin embryo since it is relatively permeable to this large molecule. Wolsky and Wolsky (1961) found that after fertilization continuous treatment with 25 γ/ml resulted in abnormal cleavage and death of the embryo before gastrulation. Gross and Cousineau (1963, 1964) studied development at several concentrations; at 6 γ/ml cleavage was normal but most embryos arrested at the late blastula stage. At concentrations above 20 γ/ml, cleavage was irregular and resembled that of activated enucleate fragments.

The morphological effects of actinomycin D on the development of amphibians have also been studied. High concentrations of the antibiotic or long exposures are necessary to overcome the permeability barrier. Development may be arrested at the blastula stage by exposure of Rana pipiens embryos to 100 γ/ml (Wallace and Elsdale, 1963) or by microinjection of the antibiotic into the 1- to 2-cell stage of Pleurodeles embryos (Brachet et al, 1964). Microinjection into Rana pipiens blastulae can inhibit development almost immediately (Wallace and Elsdale, 1963). At the relatively low concentration of 10 γ/ml, Xenopus laevis embryos exposed from cleavage undergo abnormal gastrulation (Brachet, J. and Denis, 1963).

Since cleavage continues in the presence of actinomycin D and protein synthesis is required for cleavage, actinomycin D-treated embryos have been carefully studied to determine whether protein synthesis can occur without accompanying RNA synthesis. In untreated embryos, low levels of RNA synthesis can be detected by incorporation of labeled precursors although bulk RNA remains constant. In the sea urchin incorporation proceeds slowly after fertilization, increases gradually for several hours, and then rises rapidly before the beginning of gastrulation (Gross and Cousineau, 1964; Wilt, 1964). Studies of phenol extracted RNA have shown that a high molecular weight RNA sedimenting heterogeneously in a sucrose gradient is labeled during cleavage of the sea urchin (Nemer, 1963; Wilt, 1964;

Gross, 1964; Gross et al, 1964) and the amphibian (Brown and Littna, 1964). Its synthesis begins between the 4-cell and 32-cell stages of the sea urchin (Glisin and Glisin, 1964) and its base composition resembles that of DNA (Comb et al, 1965; Gross et al, 1965). Synthesis of transfer and ribosomal RNA begins during gastrulation (Comb et al, 1965) and labeling of transfer RNA during cleavage is due to -C-C-A end group turnover (Glisin and Glisin, 1964; Gross et al, 1965).

The inhibition of RNA synthesis by actinomycin D is only partial during the first 3 to 4 hours of development of the sea urchin even at high concentrations of the antibiotic (Gross and Cousineau, 1964). This is probably due to the slow rate of penetration of this large molecule. However, the RNA synthesized in the presence of actinomycin D is entirely of low molecular weight unlike the control (Gross et al, 1964). By 4½ hours of development, incorporation of precursors into RNA is depressed 98% (Gross and Cousineau, 1964). On the other hand, protein synthesis as measured by the incorporation of leucine continues in the presence of actinomycin D at values slightly above the control for at least 8 hours (Gross et al, 1964). Even enucleate fragments incorporate amino acids after activation (Denny and Tyler, 1964). Thus in the early sea urchin embryo protein synthesis can continue in the absence of newly synthesized messenger RNA provided by the nucleus. Normal levels of amino acid incorporation have also been detected in activated enucleate eggs of Rana pipiens (Smith and Ecker, 1965) although no cleavage occurred.

Stable messenger RNA must be present in the unfertilized sea urchin egg and at fertilization somehow make a functional connection with the ribosomes. Polyribosomes held together by RNA strands and active in protein synthesis are demonstrable shortly after fertilization (Monroy and Tyler, 1963; Malkin et al, 1964; Stafford et al, 1964; Wilt, 1964; Burny et al, 1965; Spirin and Nemer, 1965). Maggio et al (1964) were able to extract from unfertilized eggs an RNA which stimulated amino acid incorporation by ribosomes of rat liver. Ribosomes from unfertilized eggs can respond to poly U (Nemer, 1962; Wilt and Hultin, 1962) and so are apparently normal. However, Monroy et al (1965) have recently found that trypsin treatment enhances the endogenous activity of ribosomes and their response to poly U or to sea urchin embryo RNA, suggesting that the activation of amino acid incorporation is due to some change in the relation between messenger and ribosomes.

Although the degree of inhibition of RNA synthesis by actinomycin D in amphibian embryos is unknown, the similarities in the morphological effect of

actinomycin D and in the pattern of synthesis of RNA and protein during cleavage suggest that protein synthesis during cleavage is also dependent on preexisting messenger RNA.

Conclusion

The formation of an anuclear blastula is a striking demonstration of the independence of cleavage from accompanying nuclear activity. The lack of influence of paternal genes on cleavage suggests that even when nuclei are present, their activity is without effect before gastrulation. Protein synthesis and cell division continue in the presence of actinomycin D which effectively inhibits the synthesis of high molecular weight RNA. The cleavage pattern is therefore dependent on messenger RNA and other substances inherited in the maternal cytoplasm.

B. NUCLEAR FUNCTION AT GASTRULATION

During the second period of early development beginning at gastrulation, embryos show a requirement for continuous nuclear activity. Rapid morphological differentiation commences at this stage, the development of the form and tissues characteristic of the species begins. The genes bearing the necessary information take over the direction of cellular activity. The experiments already described which demonstrate that nuclear function is not required for cleavage also indicate the time when nuclear function is first required. Anucleate and actinomycin D-treated embryos arrest at the blastula stage. In addition aneuploid embryos and the progeny of many hybrid crosses arrest at a similar stage.

The Requirements for a Complete Chromosome Set

The presence of a nucleus containing a complete haploid set of chromosomes allows development to continue beyond the blastula stage (see Fankhauser, 1955, for review). Parthenogenic amphibian eggs can reach the larval stage (Guyer, 1907; Bataillon, 1910) and Loeb and Bancroft (1913) and Loeb (1921) were able to raise parthenogenic frogs to or beyond metamorphosis. However, chromosome counts revealed that all the advanced tadpoles had transformed to the diploid condition. Hertwig (1918) obtained haploid larvae by fertilization with the sperm of another species, which usually died in the early larval stage. The typical haploid syndrome was described, whose most obvious feature is generalized oedema at hatching (Porter, 1939; Hamilton, 1963). In 1922 Baltzer (see Fankhauser, 1937) raised a haploid urodele from a fragment containing only a sperm nucleus to the end of metamorphosis.

Thus, in contrast to the anucleate embryo which can only cleave irregularly, the embryo with the haploid number of chromosomes can proceed with almost normal gastrulation, can differentiate to form the many tissues of the neurula, and very rarely can even go through metamorphosis.

The addition of a complete set of chromosomes beyond the normal diploid number has little effect on development. Fankhauser (1945) and Briggs (1947) report no difference in rate or morphology of development between triploid and diploid urodeles and anurans respectively, in spite of the large cell size of the triploid form.

On the other hand the presence of unbalanced or aneuploid chromosome numbers causes severely abnormal development (see Fankhauser, 1945, for review). Boveri (1902) was able to follow the fate of cells with different numbers of chromosomes in the sea urchin embryo. Dispermy results in a tripolar or tetrapolar mitosis at the first division, and the embryos usually develop to abnormal swimming blastulae. Since six sets of chromosomes are apparently randomly distributed to three or four poles, the daughter cells may contain a greater number of chromosomes than the haploid, but may lack a complete set of the individual chromosomes. Development of parts of the embryo did not necessarily improve with larger numbers of chromosomes and embryos with tripolar mitoses generally developed better than those with tetrapolar mitoses. From statistical analyses of these experiments, Boveri concluded that for normal development each chromosome must be represented at least once in each cell.

Fankhauser (1934b) made a similar study on pieces of polyspermic urodele eggs. In this case development proceeded only to the early gastrula stage, and throughout cleavage it was possible to identify cytologically the different chromosomes of the haploid set. Fankhauser confirmed random distribution of chromosomes in multipolar divisions by direct observation and concluded that abnormal combinations of chromosomes in the different cells accounted for the poor differentiation of these embryos. Although the cells were nucleated, development proceeded little further than in the case of the anucleate embryos. Clearly, a complete set of chromosomes in the nucleus is essential even for gastrulation.

Dalcq (1932) attempted to produce subhaploid anuran embryos by fertilizing with sperm inactivated with trypaflavin followed by partial removal of the second maturation spindle of the egg. He obtained thousands of embryos which arrested before gastrulation and believed they were subhaploid although he did not examine

them. Embryos with chromosome numbers between the haploid and the diploid showed irregularities at gastrulation and poor later development. Fankhauser (1945) also found that urodele larvae with aneuploid chromosome numbers greater than the diploid number were very abnormal.

In Drosophila omission of the entire X chromosome, which represents approximately one fifth of the chromosomal material, results in abnormal cleavage and distribution of nuclei, a very early effect.

Thus, although the haploid number of chromosomes is sufficient for complete development, aneuploidy causes severe disturbances of gastrulation and neurulation. Many genes must function in a coordinated fashion in order to direct normal differentiation.

The experiments discussed above show that loss of one or more chromosomes from the complete chromosome set is lethal. The effect of changes in or loss of a small segment of a chromosome at the level of genes has also been studied. Several mutations are known which inhibit processes of early development in Drosophila, chick, and mouse embryos (see Gluecksohn-Waelsh, 1953, for review). Deletions in the X chromosome of Drosophila of one or several bands may affect endodermal, mesodermal, or neural differentiation (Poulson, 1945). The mutation t^{12} arrests development of the mouse embryo at the morula stage before blastocyst formation (Mintz, 1964). Since only embryos which are homozygous for t^{12} die at this early stage, at least the paternal genome and probably both maternal and paternal genomes are expressed, and the effect is not entirely inherited in the cytoplasm from the mother.

The only mutation affecting development of the amphibian so far reported is a fluid imbalance first detectable at the tail bud stage, and lethal at the larval stage (Humphrey, 1948). The effect of this mutation is also observed only in embryos which are homozygous for the trait.

Development of Hybrids

The hybrid embryos of many crosses arrest at the late blastula or early gastrula stage (Pflüger, 1882; Moore, J.A., 1941, 1955) again suggesting that normal nuclear function is required for the rapid development beginning at gastrulation. In the abnormal hybrid either the genes are not activated properly or the gene products are defective.

More viable hybrids provide an opportunity to examine the onset of the

expression of gene activity. The appearance of specific paternal characteristics must be due to preceding or accompanying chromosomal activity. Boveri (1903) traced the appearance of paternal characteristics during and after formation of the primary mesenchyme of sea urchin hybrids. Moore, J.A. (1941) crossed two species of Rana with different developmental rates, and reported that the earliest detectable paternal effect occurred at the neural plate stage. He believed that this observable gene expression was due to earlier activity on the part of the paternal genes occurring at the late blastula or early gastrula stage. Porter (1941) crossed two races of Rana pipiens and found differences in the amount of epiboly and in the shape of the lateral lips of the blastopore between the hybrid and the normal diploid forms even at the gastrula stage.

Appearance of new proteins at a certain stage suggests that preceding gene activity has occurred. Clayton (1953) demonstrated immunologically the appearance of a new antigen at the crescent blastopore stage of gastrulation in the newt. Harding et al (1954) were able to detect paternal antigens as early as the late blastula stage in hybrid sea urchins.

Synthesis of RNA

The expression of gene activity as detected by the appearance of morphologically observable paternal characteristics or paternal antigens begins in the early gastrula stage of amphibian development. The production of specific proteins and enzymes, and the acquisition of differentiated cell form and membrane structure all more or less clearly depend on gene activity. Gene activity itself can be studied by measurement of RNA synthesis.

The time of onset of RNA synthesis in the amphibian was first analyzed by measuring the level of total acid insoluble RNA. Although the synthesis of messenger RNA's which must control the differentiation of cells cannot be detected by measurement of the level of bulk ribosomal RNA, increases in ribosomal RNA are also due to greatly increased gene activity. Bulk RNA remains constant during cleavage, but the results on the time of increase of bulk RNA are somewhat contradictory. Steinert (1951) using the method for differential extraction of RNA and DNA developed by Ogur and Rosen (1950) obtained different results in two experiments on Axolotl. In one experiment the RNA content rose only after gastrulation; in another, it rose gradually from the blastula stage through gastrulation. In Rana esculenta the increase in RNA followed the former pattern. According to Krugelis et al (1952) RNA increases steadily in Ambystoma from the early gastrula

stage. Using the method of Ogur and Rosen on three species of Triton, Chen (1960) found that net synthesis of RNA began at gastrulation, and subsequently rose steadily to triple the RNA content by the neurula stage. However, his data was quite scattered and significant differences appeared between measurements on two different batches of eggs from the same species. The results reported by Bristow and Deuchar (1964) recall one of the experiments of Steinert. They used a modified method of Ogur and Rosen for the extraction of RNA from Xenopus laevis embryos and found a steady rise from 4.4γ per fertilized egg to 5.7γ per late neurula. However, the differences were signifcant only from the blastula stage on. These workers suggested that the early synthesis of RNA is related to the early movements in this species which precede external signs of gastrulation.

The discrepancies in these results as to the stage of initiation of net RNA synthesis may be due to interference by DNA, variations between batches of eggs, or differences among species. However, there can be no doubt that net synthesis of bulk RNA begins around the time of gastrulation.

Several workers have investigated the incorporation of various labeled compounds into RNA. This method allows detection of the synthesis of small amounts of RNA including messenger RNA. An ever present problem in these experiments is the lack of permeability of the outer coat of the embryo to precursors. For example, Kutsky (1950) found that labeled phosphate failed to penetrate Rana pipiens embryos exposed continuously from fertilization. Hyla regilla gastrulae incorporated only three times more glycine-C^{14} into acid insoluble material than boiled gastrulae, and neurulae showed the same incorporation as boiled neurulae (Friedberg and Eakin, 1949).

As already mentioned low levels of RNA synthesis can be detected during cleavage (Flickinger, 1954; Brown and Littna, 1964). Incorporation studies have shown that after cleavage the rate of synthesis rises dramatically. Flickinger (1954) incubated Rana temporaria embryos of a given stage for 5 hours at 25°C in the presence of $C^{14}O_2$. Amphibian embryos are permeable to this precursor. He extracted RNA from the cold acid insoluble precipitate according to the method of Ogur and Rosen (1950). His results reported as CPM per mg carbon showed that embryos incubated from the 2-cell to the mid cleavage stage incorporated a small amount of label into RNA while the blastulae (presumably they remained in the blastula stage for the 5 hours of the incubation) and gastrulae showed successive large increases.

In agreement with Flickinger, Grant (1955, 1958) found increasing RNA synthesis at gastrulation after injection of P^{32} into the female or injection of glycine-C^{14} into embryos of different stages. Decroly et al (1964) exposed Xenopus laevis embryos to P^{32} for periods of 3 minutes, 10 minutes, and 2.5 hours. Apparently under their conditions the embryos were at least partially permeable to the labeled phosphate, since counts were obtained in the alkaline hydrolysate of cold acid insoluble material. Their results indicate a significant incorporation during cleavage, and a large increase at the gastrula and again at the neurula stage.

The experiments of Flickinger, Grant, and Decroly et al may be summarized as follows: a small but slowly increasing amount of label appears in RNA during cleavage, at the late blastula or early gastrula stage the rate of incorporation rises, and continues to rise steadily throughout neurulation. Protein synthesis also increases at the late blastula or early gastrula stage (Flickinger, 1954; Cohen, 1954; Grant, 1958).

A few experiments have been performed to compare the synthetic activity in different parts of the embryo attempting to relate nuclear activity to the morphogenic movements of gastrulation and the beginning of differentiation of axial structures on the dorsal side. In the paper described above Friedberg and Eakin (1949) found no significant difference in the incorporation of glycine-C^{14} into total acid insoluble material per mg dry weight between dorsal and ventral halves separated at the gastrula stage. At the neurula stage the dorsal half was significantly more active. Flickinger (1954) measured the incorporation of C^{14} of $C^{14}O_2$ into nucleic acids and protein in several parts of the Rana temporaria gastrula. He found that the order of decreasing activity in CPM per mg carbon for both nucleic acids and protein was: ectoderm, dorsal lip, ventral lip, and yolk. However, the difference between dorsal and ventral lips was not large. By the neurula stage the dorsal axial structures incorporated five time more C^{14} into nucleic acids.

Even though none of these workers measured specific RNA synthesis, we may conclude that at the early gastrula stage there is an animal-vegetal gradient of synthetic activity when measured as CPM per mg dry weight or per mg carbon. As gastrulation proceeds, the dorsal side begins to become more active than the ventral side, and by the neurula stage the dorsal ectoderm and mesoderm are the center of synthesis. However, none of these results measure the activity per cell or per nucleus. Since the presumptive endoderm in the region of the vegetal pole is composed of much larger cells, and contains much more dry weight or

carbon in the form of inert yolk, the activity of an endodermal nucleus could be as great as that of the nuclei of other regions. Perhaps the higher incorporation by the dorsal half when measured per mg dry weight is due to slight differences in cell size and yolk content between the dorsal and ventral lips of the young gastrula.

More accurate information on the synthetic activity of nuclei in various regions of the embryo can be obtained from autoradiographic studies. Tencer (1958) incubated Axolotl embryos for 5 hours in $C^{14}O_2$ during early, middle and late gastrula stages. Gastrulation lasts for 12-15 hours in this form. In the early gastrula stage, the density of nuclear grains was similar in four regions of the embryos: dorsal ectoderm, ventral ectoderm, dorsal lip, and presumptive endoderm. Since endodermal nuclei are larger than the nuclei of other regions, the total activity of one nucleus must be more than that of nuclei of other regions. The density of labeling in the cytoplasm was much less, and showed an activity decreasing in the order of the regions given above. When nuclei and cytoplasm were counted together, the density of grains also decreased markedly in the four regions, thus confirming that nuclear activity may be similar even though there are large differences in activity per total area or per mg dry weight. RNase removed one half to three quarters of the grains from the combined cytoplasmic and nuclear grain counts. By the late gastrula stage nuclear differences were seen in the four regions with the activity decreasing in the dorsal ectoderm, ventral ectoderm, dorsal lip, and presumptive endoderm.

Denis (1964) studied the incorporation of uridine-H^3 over a period of 6 hours into nuclei of different regions explanted from the Pleurodeles embryo. His results differ somewhat from those of Tencer. In the early to mid gastrula he found a similar activity in grains per nucleus in the presumptive neural and mesodermal tissue, but much less in the presumptive endoderm. By the late gastrula stage the presumptive neural tissue was more active than the presumptive mesoderm. Tencer's results may be more reliable since they were performed on whole embryos.

The isolation and characterization of newly synthesized RNA yields information complementary to that obtained from autoradiographic studies. Autoradiography reveals nothing of the type of RNA labeled while isolation of labeled RNA reveals nothing of its site of synthesis.

A careful analysis of RNA synthesis during anuran development has been carried out by Brown and his coworkers. Brown and Littna (1964) have analyzed

RNA extracted from Xenopus laevis embryos by sucrose gradient centrifugation after injection of P^{32} into the female. A small amount of labeled heterogeneous material appeared in both ovulated unfertilized eggs and in 2- to 8-cell embryos. This material accumulated or turned over more rapidly during cleavage and blastulation, but a high per cent of the counts were apparently in transfer RNA. At gastrulation the level of incorporation increased ten fold. Newly synthesized ribosomal RNA was detected for the first time and by the neurula stage composed about half the labeled material. The base composition of labeled RNA of the gastrula stage and of RNA heavier than ribosomal RNA from the neurula to the heart beat stage was found to approach that of DNA. Brown and Littna have identified the labeled non-ribosomal RNA as messenger RNA because of its base composition and sedimentation properties. Synthesis of a mixture of messenger-like RNA and ribosomal RNA during gastrula and neurula stages of Xenopus laevis has also been observed by Mariano and Schram-Doumont (1965).

In a recent report Denis (1965) has demonstrated in a different way the synthesis of messenger-like RNA during early development. Xenopus laevis embryos were labeled at various stages with $C^{14}O_2$ for one hour. The rapidly labeled RNA was concentrated by hybridization with DNA, and it was found that at the gastrula stage 2% of the DNA could be saturated with RNA and 8 to 14% of the RNA counts could hybridize with DNA. With increasing age differences between two groups of embryos, there was decreasing competition between the labeled RNA's, indicating synthesis of new species of messenger RNA.

The pattern of RNA synthesis is similar in the early development of sea urchins (Comb et al, 1965). In fish (see Spirin et al, 1965) the synthesis of increasing amount of non-ribosomal RNA chromatographed on methylated albumen kieselgur columns was observed from the mid blastula to mid gastrula stage.

The experiments on synthesis of total RNA, incorporation of label into RNA studied by extraction of acid insoluble counts or by autoradiography, and on isolation of whole labeled RNA, all support the conclusion reached earlier, that important changes in nuclear activity occur around the time of gastrulation. The most sensitive methods show that synthesis of a small amount of RNA, probably messenger RNA, occurs during cleavage. This synthesis increases in the late blastula stage, apparently in preparation for the period when the requirement for and morphogenic expression of nuclear activity begins. During gastrulation, messenger RNA synthesis continues to increase, and ribosomal RNA synthesis begins. Nuclear RNA synthesis has been observed in the presumptive ectoderm, mesoderm, and endo-

derm in the mid or late gastrula.

Effect of Actinomycin D on Gastrulation

Sea urchin or amphibian embryos exposed continuously to actinomycin D from fertilization arrest at the late blastula stage (Gross and Cousineau, 1964; Wallace and Elsdale, 1963; Brachet, J. et al, 1964). The synthesis of high molecular weight RNA is inhibited by actinomycin D during cleavage of sea urchins (Gross et al, 1964). These facts suggest that the high molecular weight RNA synthesized during cleavage is stored for participation in the initiation of gastrulation. The process of gastrulation also requires accompanying RNA synthesis since gastrulae exposed to 20 to 100 γ/ml of actinomycin D soon cease to develop (Flickinger, 1963; Brachet et al, 1964). That actinomycin D does in fact inhibit RNA synthesis in amphibian gastrulae has been shown by Denis (1964) in his autoradiographic study of the incorporation of tritiated uridine.

Conclusion

A major change in the processes of development occurs at the beginning of gastrulation. Cell activities increase, diversify, and become subject to nuclear control. Morphological expression of genetic traits appears for the first time. Incorporation of precursors into RNA increases rapidly in the late blastula and gastrula, and a significant fraction of the newly synthesized RNA is messenger-like. A complete chromosome set is required for successful gastrulation and RNA synthesis must remain undisturbed both before and during gastrulation.

C. THE GENETIC EQUIVALENCE OF NUCLEI OF DIFFERENTIATED CELLS

The nucleus is only one component of the machinery which controls cellular activity. The nucleus is embedded in cytoplasm and must be in continuous and reciprocal communication with it. In the developing embryo relatively fixed patterns of genetic activity are in the process of becoming established in nuclei of identical genetic capacity.

The identity of early cleavage nuclei was established quite early which disproved Weismann's theory of qualitative nuclear division. When moderate pressure is applied to the fertilized sea urchin egg, the third cleavage plane is vertical rather than horizontal and produces an abnormal distribution of nuclei within the cytoplasm. When pressure is released, development proceeds to produce a normal embryo (Driesch, 1892). Similar experiments have been performed on the early frog embryo (Born, 1893) and many other embryos. Since nuclei can change their position within the cytoplasm and thus the position of their daughter nuclei

in the developing organs without adverse effect, the nuclei must be identical with regard to their developmental capacity up to this stage. Spemann (1928) in a classic experiment demonstrated this equivalence up to the 16 cell stage in urodeles. He partially constricted the freshly fertilized egg in a meridional plane so that both pronuclei lay to one side of the constriction. After one or more cleavages of the nucleate half, one daughter nucleus crossed through the cytoplasmic connection left by the partial constriction. This single nucleus was able to promote the development of a whole embryo of half size, when derived from the first to the third cleavage. The nuclei of this stage are therefore not only equivalent but possess full developmental capacity. They are true replicas of the original fusion nucleus.

Testing of the developmental capacity of nuclei from much later stages became possible when Briggs and King (1952) developed the nuclear transplantation technique for Rana pipiens. In brief, a nucleus was taken from a somatic cell of a blastula and transplanted to an activated enucleate egg. The subsequent development of the egg constituted a test of the developmental capacity of the donor nucleus. It was found that blastula nuclei were capable of supporting complete development. In later experiments (Briggs and King, 1957, 1960) endodermal nuclei from various stages up to tailbud tadpoles were tested, and with increasing age of the donor nucleus, a decreasing per cent of the hosts developed to complete blastulae. Of these a decreasing per cent became feeding tadpoles until essentially no tadpoles were produced. However, Gurdon (1960) showed that more complete development with nuclei from advanced stages could be attained if another species, Xenopus laevis, was used. In fact, if all experimental sources of damage to the donor nuclei were considered, 24% of the nuclei of differentiated intestinal epithelial cells from feeding tadpoles could in turn produce feeding tadpoles when transplanted (Gurdon, 1962). This strikingly demonstrated the full potential of nuclei from differentiated cells of at least one tissue. Recently, development of metamorphosed frogs using nuclei transplanted from the nervous system at the closed neural fold stage has been obtained by Simnett (1964).

Indirect evidence suggests that nuclei of most adult tissues retain their full genetic capacity, although it may be more or less repressed by secondary factors. The constancy of chromosome number and of chromosome form throughout the tissues of the body has long been known. Similarly the constancy of the DNA content in isolated nuclei from different tissues (Boivin et al, 1948; Mirsky and Ris, 1949) suggests that essentially all the genes are retained. In sciarid

flies the structure of interphase chromosomes can be visualized as polytene chromosomes in several tissues. It is found that the number, distance, and arrangement of the intensely staining bands are similar although the pattern of chromosomal activity is different (Beermann, 1963). Since particular bands can be correlated with particular genes, apparently all the genes are present in the nuclei of differentiated tissues.

II. ROLE OF THE CYTOPLASM: LOCALIZATION OF CYTOPLASMIC SUBSTANCES

Since the nuclei of differentiated cells of the amphibian larva can retain their full genetic potential, and yet nuclear activity is required for differentiation and development at least from the gastrula stage on, different patterns of of genes must become active in different embryonic cells while the inactive genes remain undamaged. The cytoplasm of the recipient egg in nuclear transplantation experiments clearly influences the activity of the genes in the donor nucleus. This has been demonstrated biochemically by Gurdon and Brown (1965). The donor endodermal cells shortly before transplantation synthesized mostly ribosomal RNA while the recipient in the mid-blastula stage synthesized mostly heterogeneous and low molecular weight RNA. Logically the appearance of differences among identical nuclei must arise from preexisting cytoplasmic differences. "It is hard to conceive of the several chromatic determinants coming into operation, each at the right place and time, save in a heterogeneous medium." (Jenkinson, 1914).

The problem of cytoplasmic prelocalization has been discussed and studied since the end of the last century. Evidence began to accumulate in support of the theory that differentiation of nuclei of various regions of the egg was due to the effect of the cytoplasm in which a particular nucleus happened to find itself. The cytoplasm was conceived as affecting reversibly the initial patterns of gene activity, and the process of continuing differentiation presumably involved interaction between nucleus and cytoplasm. Cytoplasmic prelocalization may be defined as the localization in a region of the egg of substances or qualities which influence or determine the subsequent differentiation of the cells of the region. Substances may also become localized after fertilization. "Simple cytoplasmic differentiation serves to start the machine whose essential and probably most complicated mechanism is located in the nuclei." (Boveri, 1902).

The cytoplasm of an egg in which factors are prelocalized cannot be homogeneous, but specific factors must be more or less localized in certain regions

of the egg. Inhomogeneities may or may not be visible in the whole or sectioned egg, and visible inhomogeneities may or may not be correlated with the distribution of factors.

Examples of Cytoplasmic Prelocalization

Cytoplasmic prelocalization can most easily be demonstrated in those embryos in which particular factors necessary for development of a particular organ system have a definite and restricted localization in the embryo from the one-cell or early cleavage stages. Such embryos are called 'mosaic' because they may be considered as being made up of a mosaic of regions containing the different factors necessary for the development of the various organs. In the annelids and molluscs cell lineage studies can be performed such as that of Conklin (1897) on a marine gastropod which traced the origin of all the major organ systems to particular cells of the cleavage stage.

One means of demonstrating a restricted localization of cytoplasmic factors is to remove a piece of cytoplasm in a given location and to observe a specific effect on later differentiation. In a few annelids and molluscs a 'polar lobe' is formed during the first few cleavages. It is a cytoplasmic enucleate body which retains a narrow connection to one of the cleavage cells. After the division is completed, it is resorbed into that cell to reappear again at the next division. Here is a perfect opportunity to remove a specific type and amount of cytoplasm contained in the polar lobe. Such experiments have been performed and the development of specific structures, for example the mesoderm bands, apical tuft, shell, and heart, has been shown to be dependent on cytoplasm released to specific cells of the embryo from the first, second, or third polar lobe in the mollusc Ilyanassa by Clement (1952, 1962), in the mollusc Dentalium by Wilson (1904) and in the annelid Sabellaria by Novikoff (1938). Recently a biochemical study by Davidson et al. (1965) has shown that removal of the first polar lobe from the snail Ilyanassa causes a decline in the rate of increase of RNA synthesis several hours later, although the number of nuclei remains normal. Apparently cytoplasmic substances necessary for later gene activation were removed with the polar lobe.

The development of isolated parts is another means of testing whether the factors necessary for development of a particular structure are localized only in the cells which normally give rise to that structure, or whether the factors have at first a broader localization. These experiments compare the differentiation potential of a cell or group of cells with its prospective fate in normal develop-

ment. In the annelids and molluscs, isolated cells or groups of cells from the two-cell to the 16-cell stage produce essentially the same structures they would produce in normal development of the whole, and certainly do not produce more (Crampton, 1896; Clement, 1956; Wilson, 1904; Novikoff, 1938).

The ascidian embryo is interesting in that it is an extreme case of visible cytoplasmic inhomogeneities. Up to five different types of cytoplasm can be observed to localize themselves after fertilization and can be traced to specific tissues in later development (Conklin, 1905). Isolated parts up to the 16-cell stage also develop according to their prospective fate (Conklin, 1905). Conklin (1931) was able to show by centrifugation that the visible cytoplasmic inclusions could be disturbed without affecting subsequent development so that they themselves could not represent the actual determining components. However, more prolonged centrifugation did disrupt normal development, and Conklin believed that derangement of the colorless ground substance constituted derangement of the 'organ-forming substances'.

The sea urchin shows a more diffuse type of localization which has led several workers (Runnström, 1928; Horstadius, 1939) to suggest that two opposing gradients of determining factors overlap each other, one gradient having its maximum at the animal pole, the other at the vegetal pole. The diffuse localization of all factors necessary for normal development is demonstrated by the normal development of the equatorial half of a 32-cell embryo as well as of the two combined polar quarters from the same embryo (Horstadius, 1939). When one embryo is divided in half equatorially, the two halves develop more or less as parts although the vegetal half can in rare cases produce a normal embryo. However, the factors necessary for development of vegetal structures must be present in the animal half in a latent form since treatment with lithium chloride results in a normal pluteus (Horstadius, 1939). Progressive localization occurs up to the gastrula stage when the four quarters develop as parts (Horstadius, 1947-1949).

A clear case of a direct effect of the cytoplasm on the chromosomes is provided by a study of the origin of the germ cells in some species of the nematode Ascaris and of some insects. In these cases only the germ cell line retains the full chromosome complement of the fertilized egg, while all the nuclei of cells which give rise to somatic tissues undergo chromatin diminution. For example, at the second division of Ascaris megalocephala univalens, one of the cells divides normally, while in the other, the chromosomes break up into small pieces and certain parts are eliminated from both daughter nuclei. This process repeats itself three

more times: one of the nuclei containing the complete chromosome set divides normally and the other produces two cells with a reduced chromosome complement (Boveri, 1887). Boveri (1910) was able to show that the surrounding cytoplasm controlled the type of division of a particular nucleus. The relation between cleavage planes and cytoplasm could be altered by centrifugation or by dispermy so that two cells rather than one contained vegetal cytoplasm. In this case both cells retained their full chromosome complement during the next division.

The origin of germ cells in other animals also suggests an early localization of factors determining the germ cell line even though no direct effect on the nucleus can be observed. In beetles, the posterior pole contains a special granular cytoplasm. Hegner (1911) showed that removal of the cytoplasm by pricking or damage caused by cauterization reduced or completely eliminated the germ cells at later stages.

In anurans islets of special cytoplasm which can be revealed by stains have been traced from their formation in the vegetal region during the 1-cell stage into the definitive germ cells of the larval stage (Bounoure, 1934; Blackler, 1958). These islets have been observed in Rana temporaria, Bufo bufo, Xenopus laevis (Bounoure, 1934; Blackler, 1958) and in Rana pipiens (Di Berardino, 1961). They also contain RNA as revealed by methyl-green pyronine (Blackler, 1958). It is interesting to note that between the blastula and gastrula stages, the islet of a cell moves from a peripheral to a juxtanuclear position, approximately at the time when nuclear activity is first expressed.

The Grey Crescent in Amphibian Embryos

The study of cytoplasmic localization in the amphibian embryo has occupied many workers. This embryo has been considered to be of the regulative type (parts can develop to more than their prospective fate), and therefore appears to have a diffuse localization of cytoplasmic factors. However, the various factors are not spread evenly throughout the early embryo, but are localized in rather broad areas.

The spherical amphibian embryo shortly after fertilization has two axes with respect to which any region of the embryo can be described and related to the future body plan of the neurula and larva stages. The animal-vegetal axis normally runs parallel to the direction of gravity and is characterized by the following: the cortex of the animal hemisphere is more darkly pigmented, in many cases appearing almost black, and the vegetal hemisphere is lighter, often white or yellow;

when cleavage begins, the vegetal hemisphere is composed of distinctly larger cells; and sections show that a gradient in the concentration and size of yolk platelets increases toward the vegetal pole.

The second axis is the dorsal-ventral which runs perpendicular to the animal-vegetal axis. It defines the bilateral symmetry of the egg and corresponds approximately to the dorsal-ventral axis of the larval stage. At the 1-cell stage it can be discerned more or less distinctly in various species of anurans and urodeles. In the clearest cases, soon after fertilization a crescent shaped area on the dorsal side between the animal and vegetal hemispheres develops a special pattern of pigmentation intermediate between the dark color of the animal hemisphere and the light color of the vegetal. In anurans it is commonly called the grey crescent. In some species this grey crescent is observable up to the blastula stage, in others it becomes less distinct and the dorsal side can be located by its generally slightly lighter color, and by the smaller size of its cells. In some species the dorsal and ventral sides can be distinguished in sectioned material by differences in the thickness of the cortex, and in the distribution of yolk platelets and cytoplasm (Dalcq and Dollander, 1948; Ancel and Vintemberger, 1948; Nieuwkoop and Faber, 1956).

The origin and cytology of the grey crescent has been thoroughly studied in Rana fusca by Ancel and Vintemberger (1948). (See Diagram 1). The grey crescent is definitely formed by about two hours after fertilization; it is 30^{o} wide at its center with its highest point near the equator, and stretches from end to end approximately 180^{o} around the egg. Under normal conditions the side which will develop the grey crescent is determined by the sperm; the path of the sperm entrance is found in the center of the ventral side opposite the middle of the grey crescent (Roux, 1903; Brachet, A., 1911; Ancel and Vintemberger, 1948).

Ancel and Vintemberger found that the grey crescent is formed by a rotation of 30^{o} of the whole outer pigmented cortex of the egg, the ventral cortex moving down and the dorsal up. This was observed by electrolytic marking of the cortex; surface marks in the median plane moved 30^{o} while deeper marks showed that the inner cytoplasm of the egg was stationary. Lateral marks moved less and the ends of the axis of rotation were apparently in the equatorial lateral region. This rotation proceeded slowly for one hour before the definitive appearance of the grey crescent. Its peculiar color is due to the sliding off of a thin layer of outer pgiment leaving the deeper less dense pigment exposed. The authors suggested that the sperm alters the balance of the cortex on the underlying cytoplasm,

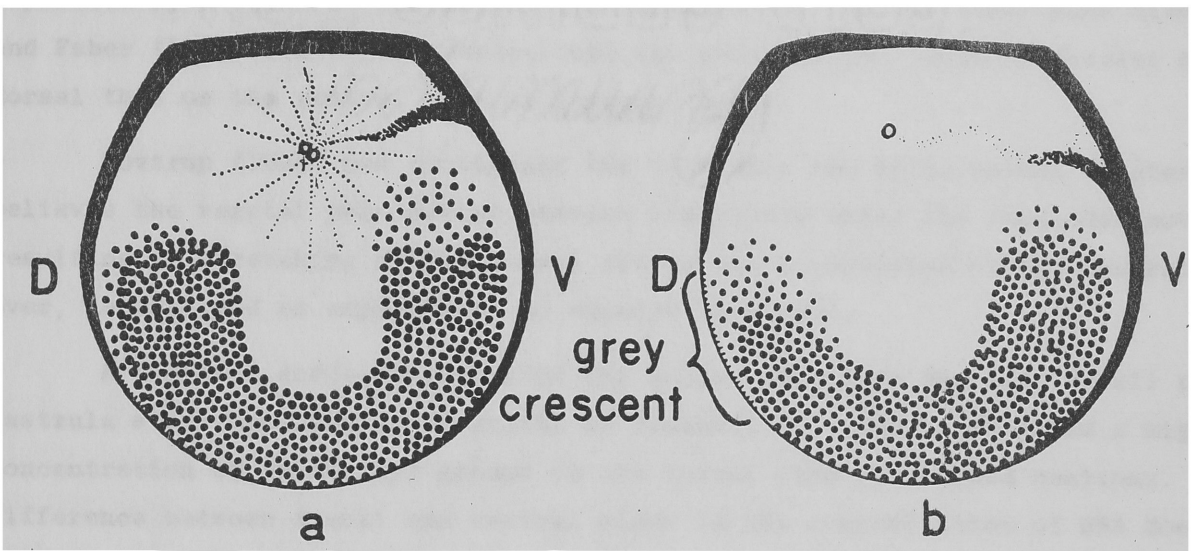


Diagram 1. Schematic vertical sections through eggs of Rana fusca. (a) Copulation of pronuclei just before formation of the grey crescent. (b) Immediately after formation of the grey crescent. The cortical pigment in black, the vegetal yolk heavily dotted. D = dorsal side; V = ventral side. (After Ancel and Vintemberger, 1948)

pulling the cortex down on the side of the sperm entrance. That the sperm does not play a necessary role in the establishment of bilateral symmetry is shown by the fact that during parthenogenic development after prick (Brachet, A., 1911) or electric shock (Ancel and Vintemberger, 1948) a typical grey crescent is formed, but its position is without relation to the position of the original stimulus. As a result of the rotation, the contact between the vegetal yolk mass and the cortex is more intimate on the dorsal side, while on the ventral side they are separated by a layer of protoplasm (See Diagram 1). On the other hand Nieuwkoop and Faber (1956) state that the subcortical protoplasmic layer is thicker on the dorsal than on the ventral side of Xenopus laevis.

Lovtrup (1958) has criticized the view that the whole cortex rotates. He believes the vegetal pole cortex remains stationary while the remainder moves, resulting in stretching of the dorsal cortex and compression of the ventral. However, he reported no experiments to support this idea.

A chemical differentiation of the dorsal side from the late 1-cell to the gastrula stage has been demonstrated by Brachet, J. (1940). He found a higher concentration of sulfhydryl groups on the dorsal side in stained sections. A difference between dorsal and ventral sides in the concentration of RNA does not appear until after gastrulation is under way, as already discussed above.

Prospective Fate Maps

The significance of the grey crescent has long been recognized as marking the location of the beginning of invagination in the gastrula and the future dorsal side of the neurula (Roux, 1903; Brachet, A., 1911). More detailed studies of the prospective fate of various regions have been performed by vital staining with neutral red or nile blue sulfate. A localized region is colored at an early stage and the fate of the stained cells in later stages traced by dissection or sectioning of fixed material. In this way complete prospective fate maps of the embryo surface have been constructed. However, a map of the 1-cell stage has not been completed and the extent of rearrangement of cytoplasmic material during cleavage is unknown. Certainly some cortical material comes to occupy an internal position by moving in along the first few cleavage planes (Schechtman, 1935; Ballard, 1955). Also the special staining cytoplasmic islets which have been correlated with the origin of the germ cells (see above) have been observed to migrate inward from the vegetal pole along the cleavage planes.

Complete maps of the early blastula of Triton and Bombinator and the very

early gastrula of Triton were published in the classic work of Vogt in 1929 (See Diagram 2a). A few marking experiments applied to the morula stage showed that the major relationships between areas were already established at this time. The material of the grey crescent lying in the dorsal equatorial region above the dorsal lip of the early gastrula becomes the notochord and part of the neural tissue, the main axial structures. The lateral equatorial region becomes somite material. The future epidermis occupies approximately the ventral animal quarter of the surface, and the future intestinal tract and related organs approximately the lower third of the vegetal region. Further details are not necessary for the present discussion. For a description of the stages of gastrulation of Xenopus laevis see Section IV of the experimental part of this thesis. For more detailed information on amphibian gastrulation see Vogt (1929), Rugh (1951) and Nelsen (1953). Unfortunately no internal material can be vitally stained so that many of the mesodermal and endodermal derivatives were not located.

Besides a beautiful description of the movement of cells during gastrulation, Vogt's results provide the bases for the study of the powers of regulation of the amphibian embryo. Is the localization of the cytoplasmic factors necessary for development of axial structures correlated with the location of the grey crescent or do such factors have a more diffuse localization?

Determination of the Presumptive Tissue Regions: Explants, Transplants, and Defects

Holtfreter in 1938(b) published maps of the differentiation potential of isolated parts of the early gastrula of urodeles and anurans (See Diagram 2b). He isolated small pieces of the surface or of internal material in physiological saline. An equatorial region, narrow on the ventral side and wider on the dorsal side, possessed wide powers of regulation. In the dorsal region the presumptive notochord area of the fate map when isolated could form notochord, neural tube, somite, and epidermal tissue. The regulatory power decreased as more lateral areas were tested, and isolated parts from the ventro-lateral region formed muscle and sometimes neural tissue. The area which could produce notochord was about twice as large as the area which does form notochord in normal development. Yamada (1950) has shown that ventral explants can be 'dorsalized' by treatment with ammonia. 75% of the treated explants produced notochord and neural tissue.

Holtfreter found that the area below the equatorial ring produced endodermal tissue and pieces taken from the median internal region below the blastocoel floor differentiated specifically into digestive tube, liver, lungs, gonads,

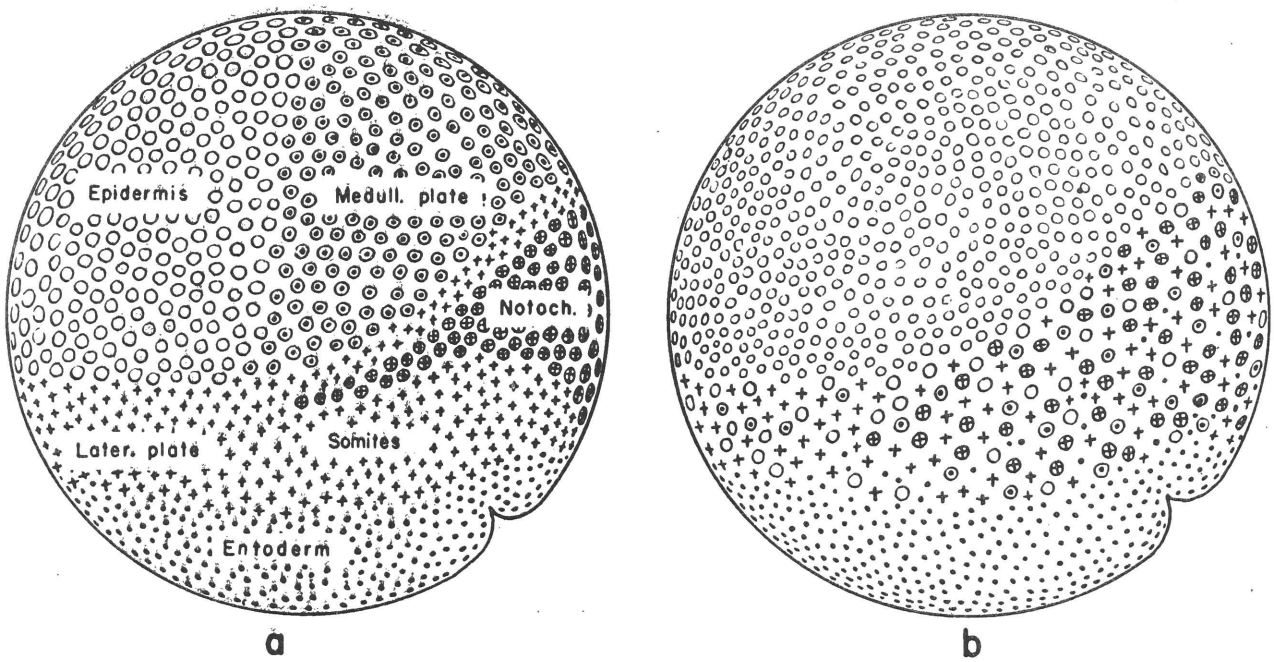


Diagram 2. (a) Differentiation in normal development (prospective significance; (b) differentiations obtained when small pieces of the prospective areas are cultured as explants. The results are projected back upon the sideview of an early gastrula. (After Holtfreter, 1938b)

blood cells, and heart tissue, depending on their location. Thus the endodermal products appear to be highly determined by the early gastrula stage. The region above the equatorial ring including the epidermal and medullary plate regions of the fate map produced only epidermis in culture. As mentioned, neural tissue in isolation was not formed from the presumptive medullary plate region, but only in conjunction with notochord and muscle tissue in the equatorial region. This finding supports the conclusion of Spemann and Mangold (1924) that during normal development neural tissue is induced by underlying notochord and mesoderm.

The lability of the determination of the ectoderm of the early gastrula has been demonstrated in various ways. Since the discovery of neural induction of the presumptive epidermis by the dorsal lip region (Spemann and Mangold, 1924) inducers have been found in many live and dead tissues, and in various pure chemicals (see Holtfreter and Hamburger, 1955, for review). Barth (1941) found that isolated pieces of Ambystoma ectoderm from the early gastrula could form neural tubes, and when two pieces were combined to form a larger piece, good neural tubes were obtained in 50% of the cases. Holtfreter (1944) repeated this and found that indeed Ambystoma ectoderm could form neural tissue unlike Triturus which he had used in his earlier experiments, and he suggested that this neural induction was due to the presence of more cellular decomposition in the Ambystoma explants. In 1947 he proposed that neural induction involved a sublethal effect on the induced cells themselves since even Triturus ectoderm could produce neural tissue in the absence of cytolysis when treated with mild acid or alkali. Recently Barth and Barth (1963, 1964) have shown that lithium chloride can sequentially induce small explants of Rana pipiens presumptive epidermis to form various types of nerve, pigment, and neuroglia cells. Ectodermal transplants can even form mesodermal tissue (but not endodermal) when transplanted to an area which normally produces mesoderm (Mangold, 1925; Holtfreter, 1938a; Spofford, 1948).

Centrifugation can also have a triggering or inductive effect on presumptive epidermis resulting in at least the partial development of axial structures. The maximum effect results from centrifugation of the late blastula and early gastrula. The original dorsal side differentiates normally, and the secondary axial structures appear at the neurula stage as outgrowths resembling tails containing notochord and neural tube. They may occur anywhere from the head, to the ventral and lateral belly, to the tail (Banta and Gortner, 1915; Beams et al, 1934). The development of these structures has been described by Pasteels (1948, 1953). During centrifugation at 460g for a few minutes the embryos orient with the vegetal pole toward

the centrifugal end of the tube. The blastocoel roof collapses, and a mass of ectodermal cells from the roof persists on the lateral or ventral side. At the neurula stage this mass divides into neural and mesodermal layers, attracts some of the neighboring mesodermal tissue, and later produces the secondary outgrowth. That the effect is not due to contact between the blastocoel roof and the floor is shown by the isolation of the ectodermal cells right after centrifugation. These isolated pieces can produce notochord, somites, and neural tissue. Apparently centrifugation has a direct effect on the ectoderm resembling the effect of Holtfreter's sublethal cytolysis (1947).

Thus the ectoderm of the early gastrula, be it presumptive epidermis or presumptive neural tissue, usually forms epidermis when isolated, but a slight change in the medium can cause neuralization. Apparently, the factors necessary for neuralization are distributed throughout the animal hemisphere and the equatorial region, but they are more easily activated in some areas than others.

The cells of the dorsal lip region normally begin the process of gastrulation by invaginating and rolling forward under the ectoderm. Later they produce notochord, while more lateral cells form the adjacent somites. The ectoderm overlying these mesodermal structures is induced to form neural tissue.

Transplantation experiments have shown that the dorsal lip region, unlike the ectoderm, differentiates in a new host not according to its new surroundings, but develops the same tissues as in isolated explants. However, these tissues are more organized due to the influence and participation of the host. The classical experiment of Spemann and Mangold (1924) demonstrated that a median piece from the dorsal lip of a Triton early gastrula when transplanted to the ventral or lateral ectoderm of another early gastrula could induce a secondary embryo with neural tube, notochord, somites, and in later experiments even normal brains. Transplantation between species showed that the secondary structures were all composed of cells derived from both the host and from the transplant. The various factors responsible for induction have been analyzed by many workers (see Spemann, 1938). A very refined experiment of this type has been performed by Curtis (1960, 1962). Small grafts (150μx150μ) of grey crescent cortex either from the late 1-cell or the 8-cell stage of Xenopus laevis when transplanted to the ventral side of a late 1-cell embryo usually induced a secondary dorsal lip and axis with neural tube, notochord, and somites. However, when the same grafts were transplanted to the ventral side of the 8-cell stage there was no sign of a secondary axis. Apparently, the 8-cell stage has become less responsive than

the 1-cell stage. By the early gastrula, either the embryo has again become more responsive or the transplantation of a larger piece results in successful induction of a secondary axis.

The effect of defects in the grey crescent area indicate to what extent the necessary cytoplasmic factors are localized within the grey crescent, and whether other regions of the equatorial ring may take over the normal function of the median dorsal lip area. Removal of a 150μx150μ piece of cortex from the center of the grey crescent at the 1-cell stage in Xenopus laevis inhibits development beyond the blastula stage (Curtis, 1962). The wound heals nicely so that there is little danger of mechanical inhibition. At this stage there is remarkable localization of the factors necessary for the development of cells which will initiate gastrulation and ultimately form axial structures. Earlier workers had produced defects in later stages by pricking the 1-cell stage (Brachet, A., 1905; Pasteels, 1932). However, no such complete inhibition was obtained, perhaps due to lack of actual removal of cortical material. By the 8-cell stage removal of a similar piece of cortex allows normal development (Curtis, 1962).

Defect experiments carried out at later stages necessarily involve removal of nuclei. However, the genetic equivalence of nuclei throughout early development makes it unlikely that deficiencies appearing later are due to the absence of particular nuclei. Rather it is the effect of removal of specific regions from an unhomogeneous cytoplasm that is being tested.

Destruction without removal of one dorsal animal cell of the 8-cell stage of Rana fusca containing part of the grey crescent (clearly a different type and much larger defect than removal of a small piece of cortex) often inhibited gastrulation, perhaps due to mechanical difficulties. However, even in this case several relatively normal and two perfect larvae were obtained (Votquenne, 1933). When two cells of the 8-cell stage of Rana pipiens containing most of the grey crescent were destroyed without removal by injection of trypan blue, no neurulae were obtained, while killing of the two corresponding ventral cells had little adverse effect (George, 1918). Although these experiments are relatively crude, apparently the factors necessary for axial development are still localized within the grey crescent area in one half of the embryo at the 8-cell stage.

George (1918) also destroyed the entire grey crescent area in the early blastula, presumably an area including almost half the circumference of the egg, and found no neuralization although there was some attempt at gastrulation. A

similar wound on the ventral side did not impair gastrulation. Excision of the cells of the entire grey crescent region from the late blastula of the urodele did not prevent gastrulation, but the embryos lacked notochords and were of the spina bifida type due to failure of closure of the neural tube (Goerttler, 1926). Partial removal of the grey crescent area in the blastula or gastrula of Rana pipiens may result in spina bifida and partial doubling of the notochord (Dalcq, 1940).

Thus by the blastula and early gastrula stages, the factors necessary for development of the notochord are predominantly localized within the grey crescent area of the dorsal half. The experiments on explants have defined three main regions, the animal region of labile ectoderm, the endodermal region capable of self differentiation, and the equatorial region wider on the dorsal side with broad powers of differentiation of dorsal axial structures (see Diagram 2). Transplantation experiments have confirmed that the ability to form axial structures is concentrated in the dorsal marginal zone. However, even when the entire presumptive notochord is removed more ventrally located cells may invaginate, form mesoderm and somites, and permit neural differentiation of the overlying ectoderm. When the center of the presumptive notochord region is removed, the two adjoining parts of the equatorial ring are able to regulate, and to form notochord tissue..

Development of Embryo Halves

To further test whether the notochordal factors are located entirely within the dorsal half, the development of isolated ventral halves has been studied. Soon after Driesch (1892) showed that a normal pluteus could be obtained from one cell of the 2-cell stage of the sea urchin, several workers obtained the same result with amphibian embryos (Hertlizka, 1897; Spemann, 1901). However, it was believed if the first cleavage plane lay in the frontal rather than the median sagittal plane, separation of the first two cells resulted in a dorsal half with complete powers of regulation and a ventral half which was able to gastrulate and to form three germ layers, but never neurulated (Spemann, 1938). In these experiments, however, the relation between the division plane and the grey crescent was not determined, and it was only inferred that the constriction had been frontal when one half failed to neurulate. Fankhauser (1930) and Streett (1940) even claimed that since 15-20% of the halves isolated at the one cell stage were unable to neurulate, these must represent the ventral halves determined before the appearance of the grey crescent.

However, Dalcq and Huang (1948), Dollander (1950), and Brice (1958), studied the development of separated halves at the 2-cell, blastula, and gastrula stages of Triton, and in each case marked the position of the original grey crescent. At the 2-cell and blastula stages, the dorsal and ventral halves were found to be almost equally able to neurulate, although smaller pieces of the dorsal side than of the ventral side could develop successfully. By the gastrula stage, however, the ventral half formed mesoderm in relatively few cases while the dorsal half could still regulate to form a whole as previously reported by Ruud and Spemann (see Spemann, 1938). Thus it was dramatically shown that at least after the two-cell stage the grey crescent does not represent a specific cytoplasmic localization of notochordal factors. Rather it represents a region which preferentially initiates gastrulation and the formation of axial structures during normal development, but all the factors necessary for neuralization actually extend into the ventral half through the blastula stage. Once invagination and differentiation of axial structures begins on one side, the factors in other regions are inactivated.

Experimental Location of the Grey Crescent

The evidence presented so far has shown that the dorsal lip region has broad powers of differentiation when isolated or transplanted. Also the capacity to form axial structures is not localized in a distinct median dorsal region, but tapers off gradually in more ventral regions of the equatorial ring. Evidence that the position of the grey crescent can be located at the will of the experimenter will now be discussed. Even after the appearance of the grey crescent, the position of the dorsal lip can be shifted to another point on the surface of the embryo. These experiments give some suggestions as to the mechanism of formation of the grey crescent, the relation between the grey crescent and the dorsal lip, and the location of cytoplasmic substances.

Rotation of the fertilized egg before the appearance of the grey crescent can determine the position of its appearance. Pflüger (1883) was the first to show that by maintaining the fertilized egg in an inverted position, normal development can occur, but with the position of the original animal and vegetal cortex inverted relative to the larval structures. Born (1885) found that during such inversion a major rearrangement of the cytoplasm took place with the heavy vegetal yolk mass moving down under the influence of gravity.

Ancel and Vintemberger (1948) have thoroughly studied the relation between the direction of rotation and the position of appearance of the grey crescent and

dorsal lip. The rotation was usually performed slightly differently than in the case of Pflüger and Born. The freshly laid egg of Rana fusca was placed on a slide out of water with the vegetal pole on the upper side, but with the animal-vegetal axis forming an angle of 45° to the perpendicular. The egg was then fertilized or activated and the slide with the egg adhering by its jelly coat was returned to water. Within 15 minutes the vegetal pole and cortex began to rotate downward under the influence of gravity. Two hours later 96% of the eggs developed the grey crescent in the normal position between the animal and the vegetal hemispheres, but on the side which had originally been placed facing upward (see Diagram 3). Thus the dorsal-ventral axis ran perpendicular to the axis of rotation. Such a rotation if performed up to one hour after fertilization could determine the location of the grey crescent, but after this time the grey crescent appeared in a position random with respect to the plane of rotation. The effect of the sperm in determining the dorsal-ventral axis could be overcome but the rotation was more effective if the egg was allowed to rotate on a slide in a humid chamber rather than in water. These workers concluded that the grey crescent is formed in the region of the cortex which undergoes the most pressure from the heavy body of the yolk attempting to rotate within the cortex. When the rotation occurs in water only slight resistance is offered to the rotation of the cortex while in the humid chamber there is more resistance due to the tighter contact between cortx, jelly and slide. The cortex rotates more slowly than the inner cytoplasm and yolk and finally is displaced by about 30° from its original position relative to the inner mass. Again as in the normal case, a close contact between yolk and cortex is established on the dorsal side, whereas they are separated by cytoplasm on the ventral side. The result is similar to that which occurs during normal development of the grey crescent which Ancel and Vintemberger have shown to be a result of a rotation of the cortex on the stationary cytoplasm.

More drastic rotations can have an effect for a longer time after fertilization, even after the appearance of the grey crescent. Schultze (1894) rotated the embryo of Rana fusca at the 2-cell stage. The animal pole was placed downward and the embryo compressed between slides so that the cortex was unable to rotate. In some cases double embryos were obtained, one from each of the first two cells. Apparently, again, the rearrangement of the relation between yolk, cytoplasm, and cortex due to the downward movement of the yolk mass in each cell resulted in the establishment of new centers for the development of future dorsal lip regions. This experiment was repeated on the 1-cell and 2-cell stages of

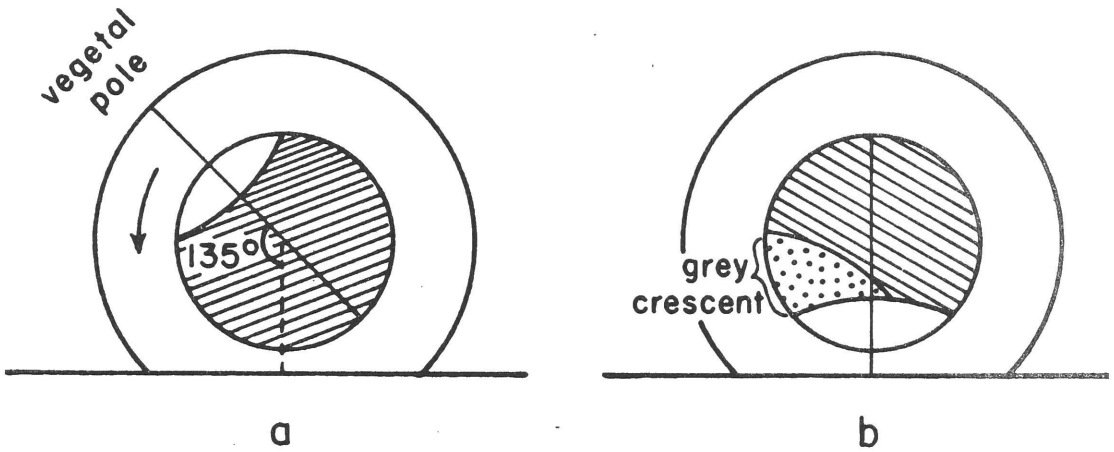


Diagram 3. (a) The egg is placed on a slide with its vegetal pole up and with the egg axis at an angle of 45° to the perpendicular. The egg is fertilized or activated and returned to water. (b) After 35 minutes the vegetal pole revolves to the lowest position and after two hours the grey crescent appears on the side which rotated downward. (After Ancel and Vintemberger, 1948)

Rana fusca by Penners and Schleip (1928) and by Pasteels (1938) who studied the relation between the position of the original grey crescent and the position of the new dorsal lip(s). Both double and single embryos could be obtained from the 1- and 2-cell stages. The new dorsal lip(s) appeared always at the edge of the yolk mass, and preferably near the original grey crescent. When two areas of cortex met these conditions depending on the new shape and position of the yolk mass, two dorsal lips were formed. These experiments show the long lasting influence of the original grey crescent, but clearly it is not sufficient or necessary for development of a dorsal lip.

If the 1- or 2-cell embryo is compressed with the vegetal pole up but the animal-vegetal axis at an angle of 45° to the perpendicular, then an interaction of the position of the original grey crescent, and of the axis of rotation can be observed in the determination of the new dorsal lip (Pasteels, 1940, 1964). When the axis of rotation is parallel to the axis of symmetry of the embryo, the dorsal lip appears most commonly at the original grey crescent, less frequently in the position found by Ancel and Vintemberger for rotations before the appearance of the grey crescent, and even less frequently at an intermediate position. Thus the competition between the original grey crescent and the rotation is usually decided in favor of the former. In this case it is unlikely that a change in position of the dorsal lip is due to a relocation of a specifically located substance.

Two reports have appeared which suggest that the position of the dorsal lip can be altered by localized chemical treatment. Lovtrup and Pigon (1958) placed the 2-cell stage of Ambystoma embryos in a tube and exposed them to air on one side only. Since in this species the grey crescent cannot be seen, random orientation of the original dorsal side with respect to the side exposed to air would be expected. However, 94% of the embryos gastrulated on the side exposed to air. Lovtrup (1958) has proposed an attractive hypothesis of the mechanism of the determination of bilateral symmetry in the amphibian embryo. As mentioned earlier, he believes the grey crescent represents an area of cortex which has undergone some stretch. This could result in an increased permeability to many substances including oxygen. The resulting increased metabolism in interaction with radially distributed cytoplasmic factors necessary for the development of axial structures would determine the dorsal side. Barth (1964) was able to shift the position of the dorsal lip 45° from the center of the grey crescent by exposure of the ventral side of the 1-cell stage to various test compounds such as

ATP, other nucleotides, and sulfhydryl-containing compounds. More work is required along these lines.

Conclusion

The grey crescent of the amphibian appearing on one side of the egg is a visibly differentiated region of the cytoplasm which can be correlated with the development of the dorsal side. However, the cytoplasmic factors necessary for differentiation of axial structures are actually distributed over a larger area. From the 8-cell to the late blastula stages the factors are present in an equatorial band wider on the dorsal side corresponding roughly to the presumptive mesoderm. The development of embryos with the grey crescent area removed, and of ventral halves, demonstrates that axial structures may be formed by ventral regions. However, the grey crescent represents the preferential site of differentiation of the dorsal lip and notochord. It is initially formed by a mechanical effect resulting from a special relation between cortex, cytoplasm, and yolk platelets. Perhaps the most immediate cause is a change in the cortex itself. Less work has been done on the determination of the endoderm but the differentiation of explants according to their prospective fate (Holtfreter, 1938b) suggests an early and relatively precise localization of cytoplasmic factors.

The vital role of both nucleus and cytoplasm in embryogenesis has been discussed. The late blastula appears to be the critical stage of interation between differentiated cytoplasm and the fine machinery of the genetically identical nuclei. Nuclear activity is relatively quiescent up to this stage, so that it may be expected that new genes are activated at this time by or under the influence of cytoplasmic substances. Molecules from one tissue can influence the differentiation of another tissue as shown by the transmission of inductive influences through fine filters (Saxen, 1963).

A useful comparison can be made with the recently discovered nuclear action of several hormones in mammals, chickens, and insects (Karlson, 1963; Williams-Ashman et al, 1964; Davidson, 1965). In these systems new chromosomal loci or the synthesis of new types of messenger RNA are activated by the hormone (the mechanism of the reaction between hormone and gene is not understood). However, the specificity of the reaction resides within the target nucleus; nuclei of different tissues react differently or not at all. The ability of the genes to respond to hormones is built into the chromosomal or nuclear structure during development of tissues.

In embryogenesis also, perhaps relatively small molecules activate certain genes. However, is is the initiation of the very process of differentiation of nuclei that we are trying to understand, and the cause of the differential response of nuclei must be sought in the activator itself. The most likely characteristic of the activators that could explain their differential effect is their location, discussed throughout the second section of this introduction as localization of cytoplasmic factors. The mechanism of the laying down and maintenance of a pattern of localized cytoplasmic factors, of the release of these substances to the nucleus at the proper time, and of the actual direct or indirect interaction with DNA remain the most challenging problems of embryology today.

EXPERIMENTAL

ACTIVATION OF RNA SYNTHESIS IN *XENOPUS LAEVIS* EMBRYOS

I. INTRODUCTION

In the amphibian embryo rapid morphological differentiation of cells begins at the onset of gastrulation. Within a few hours the embryo is converted from a spherical blastula to an elongate organism in which the main axial structures of nervous system, notochord, and digestive tract are delineated. This process is under the control of the complex nuclear machinery as shown by experiments on enucleated and actinomycin D-treated embryos reviewed above. Cellular differentiation can be observed already in the early gastrula in specific morphogenic cell movements and can be demonstrated more indirectly by explanting small pieces of tissue. The three main tissue regions, ectoderm, mesoderm, and endoderm, not only have a different fate in normal development, but also react quite differently in new or indifferent environments. For example, Holtfreter (1938b) found that endodermal explants differentiate approximately according to their normal fate, explants from the equatorial ring corresponding roughly to the presumptive mesoderm can produce nervous system, notochord, and somites, and presumptive epidermis and neural tissue explants yield only indifferent epidermis.

The late blastula represents a phase of preparation for the intense activity of gastrulation. We propose that the progressive determination of cell type involves interaction between initially identical nuclei and the regionally differentiated cytoplasm starting before the beginning of gastrulation. Although cytoplasmic substances are still elusive, nuclear activity itself can be studied. The initiation of specific patterns of nuclear RNA synthesis represents the first step in active determination of cells. Earlier workers have shown a large increase in the rate of incorporation of labeled precursors into RNA in the late blastula stage (Flickinger, 1954; Grant, 1958).

This work was undertaken to define the period of genetic activation and to analyze the product of the activation process. The time course of total RNA synthesis was measured from the morula to the late gastrula stage, the time of activation of nuclei of different cell types was studied by autoradiography, and the type of RNA synthesized in the newly activated cells was characterized by analysis of phenol-extracted RNA. These experiments provide the basis for subsequent work on the mechanism of cytoplasmic control of nuclear activation.

II. GENERAL METHODS

Choice of Animal, Xenopus laevis

The South African clawed toad, Xenopus laevis, was first described by Daudin in 1803. The native anuran, Rana pipiens, is commonly used for embryological studies throughout the United States. However, X. laevis was chosen for this work in preference to other amphibians primarily because of the ease with which its embryos can be obtained all year round. The adults go into amplexus and provide fertilized eggs in response to gonadotropic hormone from a variety of sources (Aronson, 1944). The female is widely used for pregnancy tests (Weisman and Coates, 1944). Although oogenesis is seasonal in nature (Berk, 1938), the animal kept under constant laboratory conditions is equally responsive at all times of the year. The male can be stimulated every 10 days and the female every six weeks to two months.

Other advantages of the animal include the ease of care of the adults. They do not require fresh running water at all times nor live moving food. The relatively fast development of the embryo allows several stages to be collected in one day, and the speed with which sexual maturity is reached decreases the cost of raising the adults and is of advantage for genetic experiments. RNA can be extracted more readily from X. laevis embryos than from R. pipiens (Brown and Littna, 1964).

A few disadvantages of X. laevis should be mentioned. The relatively small size of the embryo, about one mm. in diameter, makes observation and dissection more difficult. With X. laevis it is not possible to fertilize a large number of eggs simultaneously. The R. pipiens female stores her eggs in the uterus following pituitary injection. The experimenter can then strip the female, that is simply squeeze the ripe eggs out of the uterus. A large number of eggs can be fertilized with sperm from minced testes. In X. laevis the eggs pass directly down the oviduct and are extruded. Fertilization is most easily accomplished with a stimulated male who fertilizes each egg as it passes out of the female. If many embryos of a particular stage are required, either the fertilized eggs of several pairs of adults laid over a period of a short time are collected, or embryos of the desired stage are picked out singly from a mixture of embryos of various stages. Finally, since X. laevis is not native to the United States, adults must be imported from South Africa or obtained from special breeders.

Care and Breeding of Adults

Healthy medium sized adults were obtained from Jay E. Cook, Cockeysville, Md. The successful care and breeding of X. laevis has been described by several workers (Bles, 1906; Aronson, 1944; Weisman and Coates, 1944; Cameron, 1947; Nieuwkoop and Faber, 1956; Vanable, 1962). Our method resembles most closely that of Vanable. When not in use males and females were kept separately in sinks in slowly moving shallow cool water. One day prior to injection they were placed in a room maintained at 18°C in stainless steel pans containing tap water. One or two were kept in each pan. The chorionic gonadotropin, Antuitrin-'S' (Parke-Davis) was injected into the dorsal lymph sac in two steps. The males and females each received 100 units in 0.2 ml of saline at the first injection; 6 to 12 hours later the male received 150 units in 0.3 ml and the female 300 units in 0.6 ml. After the first or second injection, each female with one or two males was placed on a stainless steel gauze platform suspended a few inches from the bottom of a plastic or glass breeding chamber containing spring water from the Crystal Spring Water Co., N.Y. Amplexus and ovulation occurred three to ten hours after the second injection. The eggs when laid and fertilized fell through the stainless steel gauze, and thus escaped being damaged or eaten by the adults. Larger ovulations occurred at a temperature of 25°C. The chambers were covered with a dark cloth and disturbed as little as possible during ovulation. The second injection was usually given in the evening and the embryos of all stages from 1-cell to the beginning of gastrulation were collected the next morning. The adults were removed and kept in pans in the 18°C room until ready for another breeding. The water was changed once or twice a week, and all adults were fed chopped calves liver once a week.

Collection of Embryos and Preparation of Labeled Embryo Halves

The embryos were transferred from the mating chamber to glass bowls or petri dishes with a large wide-mouthed pipette and rubber bulb (meat-baster). Embryos of the desired stages without signs of damage or cytolysis were moved to small petri dishes with watch makers' forceps gently pinching only the jelly coat. Embryos were dejeled in crystalline papain (Mann Research Labs, N.Y.), 0.1 mg/ml in 10% Barth's Solution X (Barth and Barth, 1959) containing 0.08 M cysteine as activator. The pH was adjusted to 6.7 to 6.8 with NaOH. This solution is approximately that of Spiegel (1951). The embryos remained for approximately 30 minutes in papain with occasional gentle shaking until the jelly coat was dissolved and removed. If the vitelline membrane was still present, it was gently pulled off

during the dissection of halves. The dejeled embryos were transferred to 10% Solution X in distilled water with a pipette whose bore was slightly larger than the embryos, and rinsed three times with fresh 10% Solution X. Dejeled embryos are sensitive to handling and difficult to turn over for observation of the progress of gastrulation most readily seen in the vegetal half. A mirror placed under the petri dish on the microscope stand according to the method of Pflüger (1883) allows both animal and vegetal sides to be seen simultaneously. The stages were characterized according to Nieuwkoop and Faber (1956). Stages 8 and $8\frac{1}{2}$ were usually chosen according to the approximate cell diameter of animal pole cells, measured at a magnification of 400 times, 0.1 mm for stage 8 and 0.05 mm for stage $8\frac{1}{2}$.

The outer cortex of the amphibian embryo is relatively impermeable to precursors other than $C^{14}O_2$. To allow penetration of uridine-H^3, embryos were cut in half, a method used by Friedberg and Eakin (1959). Groups of ten embryos were transferred to a petri dish containing a layer of hardened paraffin covered by full strength Solution X (Barth and Barth, 1959). Dissection was performed with a very sharp fine grade micro-scalpel with little distortion of the shape of the embryo. The embryos were divided into dorsal and ventral halves; dorsal halves were saved for incubation, and the ventral halves usually discarded. Damaged cells were gently scraped away. A small piece of animal ectoderm was removed to reduce the tendency of the ectoderm to curl up and cover the remainder of the embryo. In some of the later stages the entire yolk plug was included in the dorsal half.

Some difficulty was at first encountered in distinguishing the dorsal and ventral sides at the blastula and circular blastopore stages. At the blastula stage, the dorsal side can be identified by its lighter color, the smaller size of its cells, and the sharper line between the dark animal half and the light vegetal half. At the circular blastopore stage, the dorsal side retains its lighter color, and in addition the pigment line of the ventral lip is thicker than that of the dorsal lip. These characteristics vary in the embryos from different pairs of adults.

Embryo halves were gently transferred in groups of ten to small tubes or beakers and were incubated for one hour in 0.6 to 0.8 ml of Solution X containing uridine-5-H^3 (New England Nuclear Corp. or Nuclear-Chicago), specific activity 1.24 to 22.2 mC/μM, 0.012 to 0.20 μM/ml. The embryo halves were shaken gently, several times during the incubation. The initiation, broadening, and deepening

of the dorsal lip as well as cell division, continued normally in the incubated halves. At the end of the incubation, the embryos were rinsed three times with Solution X and either frozen for subsequent extraction of RNA or fixed for autoradiography.

In all experiments except where otherwise mentioned, radioactivity was analyzed by direct transfer of solutions to scintillation vials containing 15 ml of Bray's solution (Bray, 1960) and counted in a Nuclear Chicago liquid scintillation spectrometer. Analysis showed that the volumes of the various solutions used, 0.1 to 0.5 ml, did not significantly quench tritium. Background was automatically subtracted by the spectrometer.

III. SYNTHESIS OF TOTAL ACID INSOLUBLE RNA IN XENOPUS LAEVIS EMBRYOS

Introduction

Experiments on the incorporation of uridine-H^3 into total acid insoluble RNA of X. laevis served as a relatively simple method of measuring RNA synthesis in our preparation of embryo halves, and of defining the characteristics of uridine-H^3 incorporation around the time of gastrulation. The permeability of the embryo halves to the precursor was established, and the time course of incorporation was described from the morula to the late gastrula. Use of uridine-H^3 of high specific activity made it possible to study several stages with a relatively short incubation period of one hour. These experiments were considered preliminary to a more detailed analysis of gene activation by autoradiography and phenol extraction of RNA.

Methods

Embryo halves were prepared and incubated in triplicate groups of five or ten as described in General Methods. At the end of the incubation, the labeled halves were frozen in one ml conical tubes at -20° C. The next day the halves were thawed at 0° to 4° C, homogenized in a small volume of cold 0.2 N perchloric acid (PCA), and centrifuged in a clinical centrifuge. The precipitates were washed twice with cold 0.2 N PCA, once with 3:1 ethanol:ether, once with 3:1 ethanol:ether at 60° C for five minutes, once with 100% ethanol at room temperature, and dried under a gentle stream of air.

In early experiments, RNA was extracted by a modified method of Ogur and Rosen (1950). The precipitates were carefully suspended in 0.8 N PCA and allowed to stand at 25° C for four hours to extract RNA. After completion of

centrifugation, aliquots of the supernatant were taken for analysis of tritium and RNA. The precipitates were then suspended in 0.5 N PCA and heated at 70°C for 20 minutes, centrifuged, and the supernatant analyzed for hot acid soluble tritium counts, presumably due to incorporation of the precursor into DNA. Later it was found that more complete extraction of RNA counts could be obtained with RNase. The precipitates were suspensed in three times crystallized beef pancreas RNase (Worthington Co.), 1 mg/ml in 0.01 M Tris pH 7.6 containing 0.1 M NaCl, for one hour at 37°C. 4 N PCA was added to a final concentration of 0.2 N, the suspensions were chilled, centrifuged, and the supernatants taken for analysis of tritium and RNA. Hot acid extraction was performed as described above. Incorporation into acid insoluble RNA after 30 seconds incubation was subtracted as a zero time value.

The data of Table II demonstrate that 90-95% of the counts incorporated at various stages are extractable with RNase. The use of uridine-5-H^3 as precursor precludes the possibility of incorporation of label into DNA as thymidine. In a similar experiment, RNA was extracted with 0.8 N PCA at 25°C for four hours and about 24% of the counts remained unextracted at three different stages. Clearly this method does not solubilize all the labeled RNA.

For study of acid soluble counts, the embryo halves at the end of incubation for various periods of time in the presence of uridine-H^3 were washed once with Solution X, then homogeneized in 0.2 N PCA, and the first cold 0.2 N PCA supernatant saved for analysis of radioactivity.

In some of the experiments, the results are presented as CPM/γRNA in order to eliminate variability due to differences in size of the embryo halves. It was felt that the measurement of RNA content was more meaningful than that of protein or nitrogen which depend significantly on the content of yolk platelets. RNA was analyzed by the phloroglucinol method of Dische and Borenfreund (1957). Corrections were made in the data for a 6% rise in the total RNA per X. laevis embryo between stages 8 and 11 (Bristow and Deuchar, 1964). Our analyses showed that there is no significant difference between the RNA content of dorsal and ventral halves at the stages studied.

Results

The outer coat of the amphibian embryo is relatively impermeable to uridine. The first experiment was designed to test the availability of this precursor to the cells of the embryo halves. As can be seen in Table I, embryos with a small

TABLE I

URIDINE-H^3 INCORPORATION INTO ACID INSOLUBLE RNA BY DIFFERENT PREPARATIONS OF MID-GASTRULA EMBRYOS

Preparation	CPM/γRNA per 10 embryos
Embryos with hole	5.
Embryo halves	99.
Dissociated embryos	90.

In the first group a small opening was made into the blastocoel with a micro-scalpel. Embryo halves were prepared as described in Methods except the two halves were not completely separated. Embryos were dissociated according to the Methods of Section IV. The acid insoluble RNA counts are those extracted in 0.5 N PCA at 25°C for 4 hours.

opening made into the blastocoel incorporated only 5% as much as the embryo halves. Probably the opening was partially healed during the incubation. Table I also shows that there was little difference between the incorporation by the cells of the embryo halves and by dissociated single cells. Diffusion between the cell layers of the embryo halves therefore did not limit the availability of the precursor. The slight decrease in the activity of the dissociated cells was probably due to damage incurred during dissociation.

Another experiment designed to test the permeability of the embryo halves compared the uptake of uridine-H^3 into acid soluble and acid insoluble material. The intracellular acid soluble material contains the pool of precursors for RNA synthesis. As is shown in Fig. 1 label appears rapidly in the acid soluble fraction while incorporation into acid insoluble material rises slowly and steadily. Clearly the availability of acid soluble precursor does not limit the rate of incorporation of label into high molecular weight RNA.

The reliability of the method is demonstrated in Fig. 2. The counts incorporated are seen to be proportional to the number of dorsal halves incubated.

The effect of varying the concentration of uridine-H^3 in the incubation medium was studied (Fig. 3). Concentrations of 0.012 to 0.20μM/ml were used throughout the experiments. These low concentrations preclude any inhibitory effect of the precursor. No differences were seen in the results with different concentrations of uridine.

Our primary aim was to describe the initiation and time course of RNA synthesis preceding and during gastrulation. Two experiments on the rate of synthesis or incorporation per hour in various stages are plotted in Figs. 4 and 5. The actual data obtained in the experiment of Fig. 4 are presented in Table II. Unfortunately, it was impossible to separate the morula stage cleanly into dorsal and ventral halves since it is composed of relatively few cells. However, it is clear that the total RNA synthesis is at a low level in the early blastula and undergoes a marked activation during gastrulation. The activity of dorsal and ventral halves is similar during blastulation, but by the gastrula stage the dorsal half is slightly more active.

Discussion

In this section some characteristics of the incubation system and the reliability of the method have been established. The permeability of the embryo halves to uridine-H^3 was greatly increased over that of embryos with a small hole.

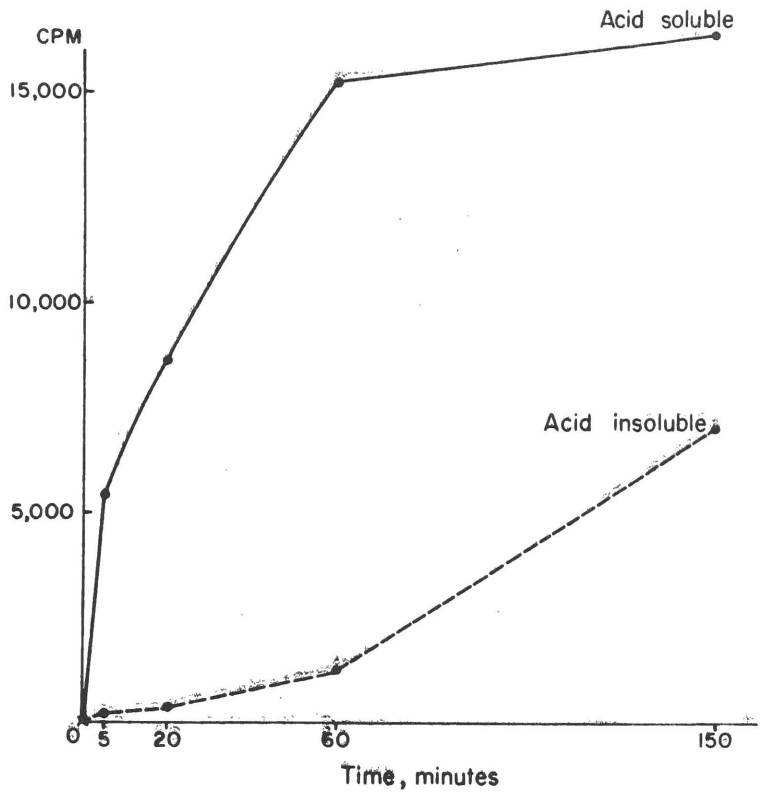


Fig. 1. Time course of incorporation of uridine-H^3 into acid insoluble and acid soluble material. Triplicate groups of 5 mid-gastrula dorsal halves were incubated as described in Methods for various periods of time. The radioactivity in the first cold acid supernatant was measured. The total acid insoluble counts were extracted from the dried alcohol precipitates in 0.5 N PCA at 70°C for 20 minutes. The value at zero time was subtracted from both curves. The ordinate represents counts per 5 dorsal halves.

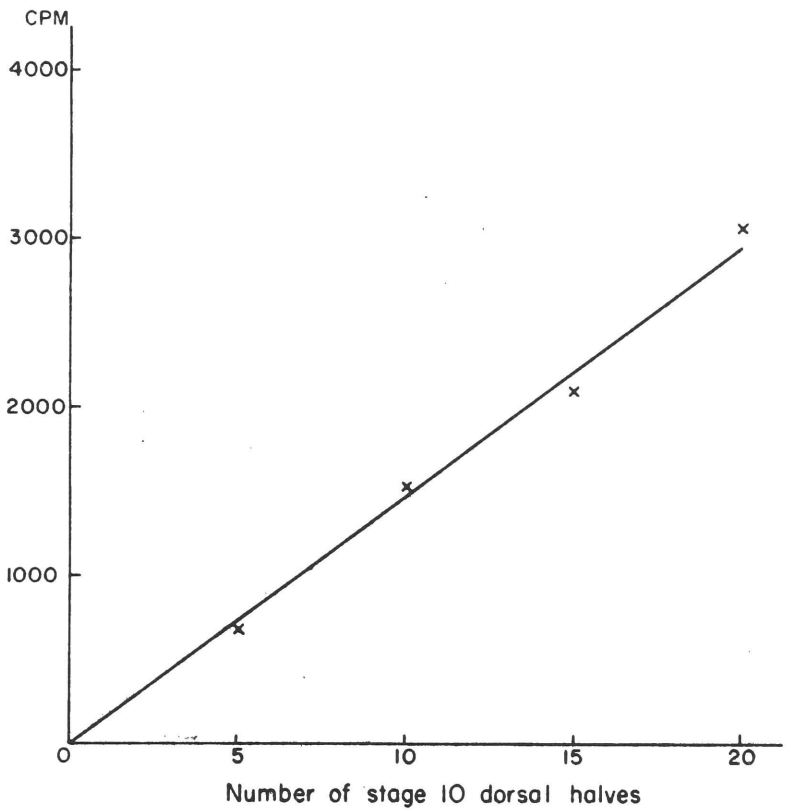


Fig. 2. Uridine-H^3 incorporation into acid insoluble RNA proportional to number of embryo dorsal halves. The counts were extracted with RNase.

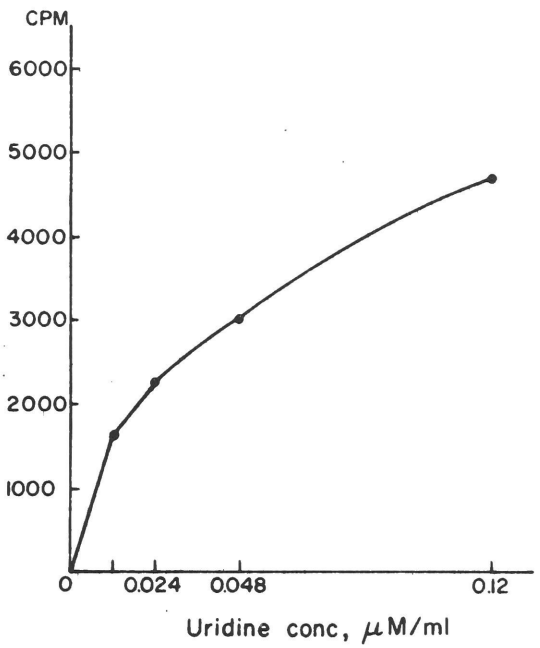


Fig. 3. Incorporation of uridine-H^3 into acid insoluble RNA; effect of varying uridine concentration. Counts were extracted from stage 10 dorsal halves with RNase.

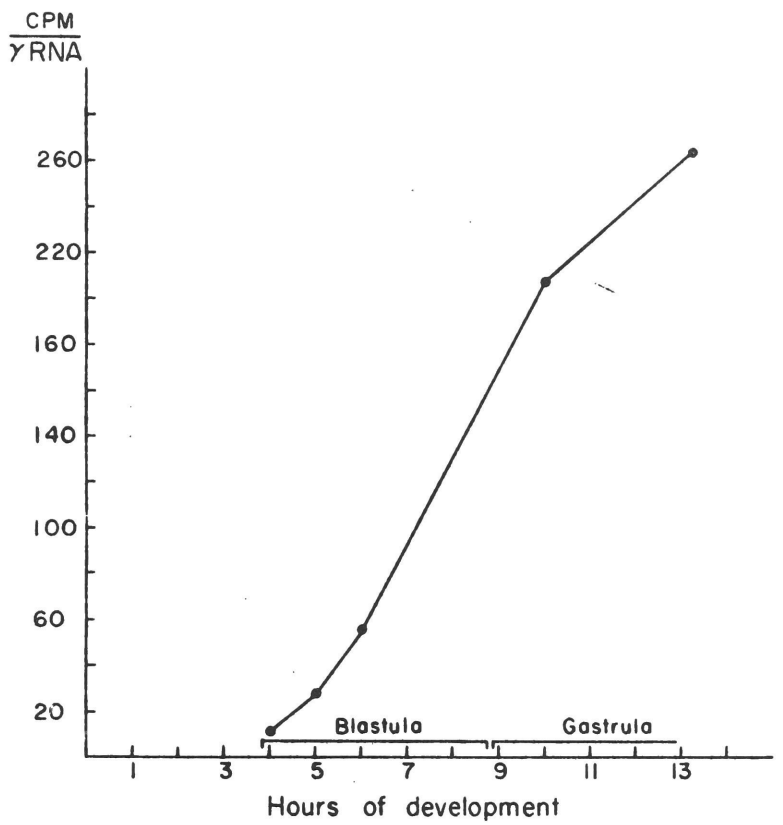


Fig. 4. Incorporation of uridine-H^3 into acid insoluble RNA of dorsal halves at different stages of development. Counts were extracted with RNase.

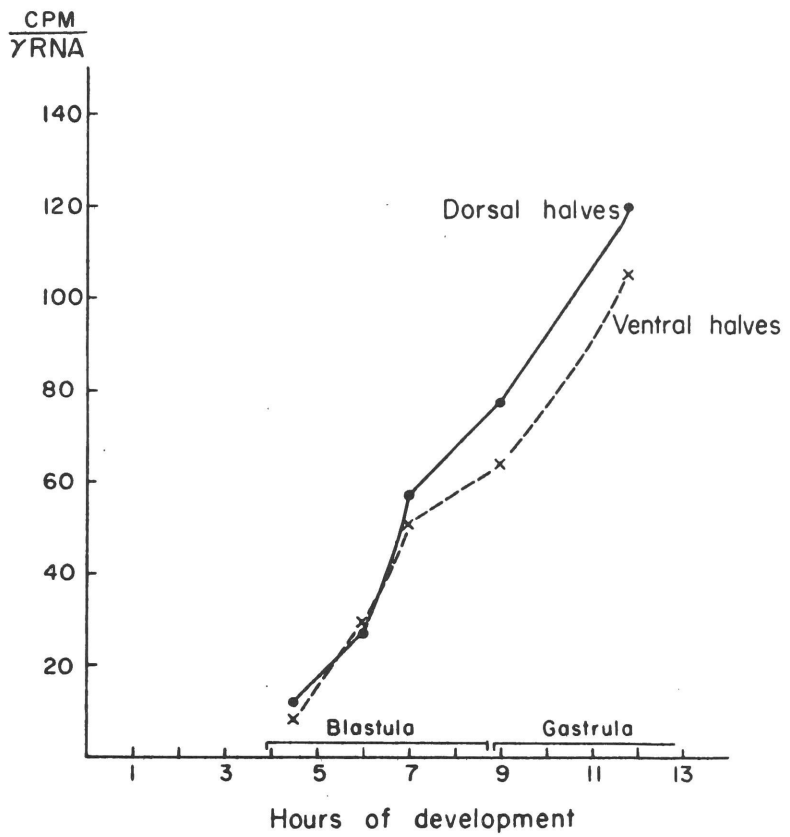


Fig. 5. Incorporation of uridine-H^3 into acid insoluble RNA at different stages of development. Triplicate samples of 10 dorsal and 10 ventral halves of various stages were incubated as described in Methods. Counts were extracted with RNase.

TABLE II

INCORPORATION OF URIDINE-H^3 INTO ACID INSOLUBLE RNA AT DIFFERENT STAGES OF DEVELOPMENT OF DORSAL HALVES

Stage	Counts extracted with RNase	Counts extracted by subsequent 0.5 N PCA at 70°C	% counts in hot acid extract
7	211	0	0
8	579	33	5.3
$8\frac{1}{2}$	1190	66	5.3
$10\frac{1}{4}$	3790	248	6.1
12	5089	519	9.3

This table presents the actual data from which Fig. 4 was calculated.

Little difference could be observed in the incorporation by embryo halves or by dissociated cells, and the rate of incorporation was shown not to be limited by the availability of acid soluble precursors.

The specificity of the precursor, uridine-5-H^3, precludes significant incorporation into DNA. The high specific activity of the precursor permitted relatively short incubation times of embryos at several stages, and the estimation of RNA synthesis during periods of low activity.

The most significant result is the detailed description of the time course of RNA synthesis in X. laevis embryos from the early blastula to the late gastrula. The results obtained are consistent with those of Bristow and Deuchar (1964) who found a slow but steady rise in the total RNA content of X. laevis embryos from the blastula stage on. Similarly, Flickinger (1954), Grant (1958), and Decroly et al (1964) observed a steady rise in the incorporation of precursors into RNA in various anurans starting at the blastula stage. However the present results show a much more dramatic increase in the rate of synthesis starting from an initial low value at the early blastula stage.

The slightly higher activity of the dorsal half of the gastrula as compared to the ventral half was also found by Flickinger (1954). More rapid synthesis as well as the dorsal convergence of cells of high RNA content during gastrulation must explain the increase in RNA in the dorsal lip of the gastrula described by Brachet (1940, 1942).

Summary

The permeability of the embryo halves to the precursor uridine-H^3 was established, and the dependence of incorporation of the precursor on its concentration was described. RNA synthesis per embryo half increases from an initially low value in the early stage and the rate continues to rise dramatically throughout gastrulation. Dorsal halves become more active than ventral halves during gastrulation.

IV. RNA SYNTHESIS IN XENOPUS LAEVIS EMBRYOS AS STUDIED BY AUTORADIOGRAPHY

Introduction

This work was originally undertaken to explore the relation between activation of genes and cell differentiation around the time of gastrulation in Xenopus laevis embryos. Differences among the cells in size, and RNA and yolk platelet

content exist from early cleavage and are derived from the structure of the fertilized egg. New cell differentiation occurring at gastrulation can most readily be observed as specific morphogenic cell movements. The most striking movement is that of the presumptive mesoderm down to and around the blastopore lip, and up under the ectoderm. These processes of gastrulation are under nuclear control as demonstrated by the results of enucleation or treatment with actinomycin D already reviewed.

If the inititation of specific patterns of nuclear RNA synthesis represents the first step in active differentiation of cells, RNA synthesis should precede observable differentiation. Our problem was to study when nuclear activity commences in the presumptive tissue regions, and to relate this activity to cytological differentiation and experimentally analyzed tissue determination. Clearly autoradiography is the method of choice for this purpose. The results presented in Section III suggested that it would be possible to study the incorporation of the specific RNA precursor, uridine-H^3, by autoradiography.

A brief descriptive list of the developmental stages of X. laevis is also provided in this section as a guide for the reader and as a means of describing the areas of the embryo analyzed in the autoradiographic studies.

Methods

A preliminary study of the cytology of X. laevis blastulae and gastrulae was undertaken. Whole embryos were dejeled as described in General Methods, and fixed in Smith's fixative consisting of: 45 ml of distilled water saturated with picric acid, 5 ml of concentrated formaldehyde, 5 ml of glacial acetic acid, and 45 ml of 95% ethanol. Embryos were dehydrated in ethanols, cleared in amyl acetate, oriented and embedded in 53-55° Bioloid paraffin, and sectioned sagitally at 10μ. Sections were stained with Ehrlich's haematoxylin and eosin, or with azure B (Conn et al, 1960). Azure B is a metachromatic stain for nucleic acids (Flax and Himes, 1952), and in amphibian embryos is essentially a stain for RNA. Stained sections were photographed in a Zeiss photomicroscope on Kodachrome II film for artificial light.

Labeled dorsal and ventral halves to be used for autoradiography were prepared as in General Methods. They were fixed and embedded as just described for whole embryos, and sectioned sagitally at 5 to 7μ. A group of three to five median sections was mounted on a separate slide for treatment with RNase, and sometimes an additional slide was prepared for staining with azure B. The slides were hydra-

ted to water, and the sections to be treated with RNase were placed flat in large petri dishes and covered with a solution of three times crystallized beef pancreas RNase (Worthington Co.), 1 mg/ml in 0.01 M Tris, pH 7.6 containing 0.1 M NaCl. The petri dishes were placed in a humid chamber for one hour at 37°C. Control slides treated with buffer only did not lose significant activity as compared to untreated slides. After RNase treatment, all slides were rinsed with water, and placed in two 5 minute changes of cold 5% trichloroacetic acid (TCA) to remove acid soluble radioactivity. PCA washes were not used at this stage since this acid tends to inactivate the photographic emulsions used for autoradiography (Prescott, 1964). The slides were rinsed in running distilled water for 30 minutes, air-dried, and covered with AR 10 stripping film from Kodak, Ltd., London, according to the method of Fitzgerald (1959). After 2 to 7 days, the autoradiographs were developed in Kodak D 19, stained lightly with haemotoxylin to color the nuclei without extensive staining of the gelatin film, dehydrated, and mounted. Photographs were taken in a Zeiss photomicroscope on Adox KB 14 black and white film.

The results were analyzed in the following way. The median sagittal sections of each embryo were located, and one section divided into the areas described in Results. Grains could be distinguished from pigment granules by their smaller size and their position slightly above the section. Nuclei were located under phase contrast. The grains in each of ten nuclei were counted in each area and the figures averaged. A nearby section treated with RNase was counted in the same way. The RNase figures were subtracted from the control figures for each region. Four embryos were analyzed in a given experiment for a given stage, and the data averaged. The standard deviation was calculated for each group of embryos, the percent standard errors averaged, and the percent standard error of the mean calculated.

Embryos were dissociated in Barth and Barth's (1959) dissociation medium consisting of Solution X without calcium or magnesium and with 1.3 mM disodium salt of ethylenediaminetetraacetic acid. After 30 to 60 minutes embryos were dissociated into single cells by gentle pipetting. Cell counts were performed in a haemacytometer on duplicate cell suspensions of 10 to 50 embryos, depending on the stage.

Description of Stages of Development of Xenopus laevis

For the convenience of the reader, a brief descriptive list of the stages of development of *X. laevis* from morula to late gastrula is provided here. For

more detailed descriptions of amphibian gastrulation see Rugh (1951) and Nelsen (1953). The list of stages and times of development at 22-24°C are taken from Nieuwkoop and Faber (1956). The description and interpretation of the movements of presumptive tissue areas are based on the work of Vogt (1929), Pasteels (1942, 1949), Weisz (1945), Nieuwkoop and Florschutz (1950), and Nieuwkoop and Faber (1956). The stages are illustrated in Fig. 6 in the photographs of median sections of whole X. laevis embryos stained with azure B. Complete removal of the azure B stain by RNase is demonstrated in one of the photographs of the stage 10 embryo. The (presumptive) neural tissue, mesoderm, and endoderm are indicated in the overlay of Fig. 6 in green, red and stippling respectively. The border between neural and mesodermal tissue is not supposed to be exact.

Stage 6½: Age 3½ hr. Morula stage. The embryo is composed of about 48 blastomeres and resembles the stage 7 embryo.

Stage 7: Age 4 hr. Large-cell blastula stage. The animal hemisphere is composed of smaller cells staining more darkly for RNA. The blastocoel is situated acentrically toward the animal pole.

Stage 8: Age 5 hr. Medium-cell blastula stage. The blastocoel roof has thinned, and the smaller animal cells have moved down slightly.

Stage 8½: Age 6 hr. Medium-fine-cell blastula stage.

Stage 9: Age 7 hr. Fine-cell blastula stage. The processes of thinning of the blastocoel roof and of downward movement of the smaller cells has continued.

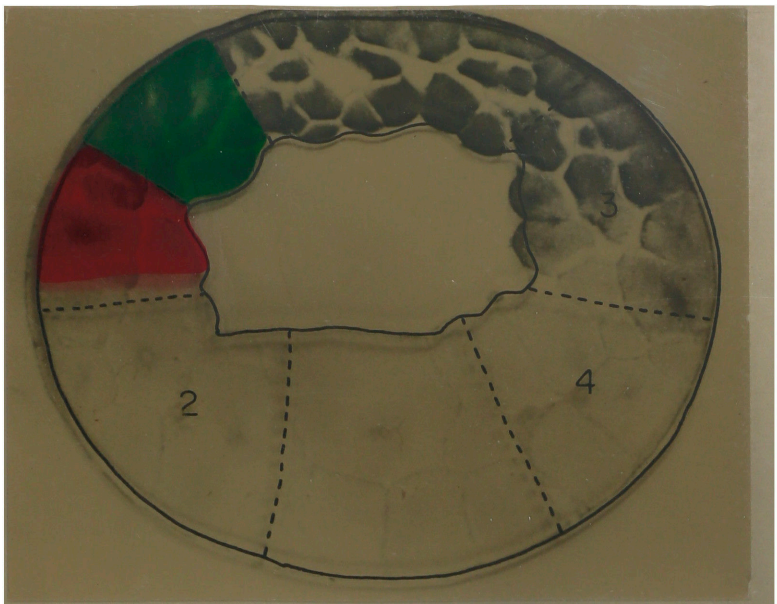
Stage 10: Age 9 hr. Initial gastrula stage. The blastopore is indicated for the first time by the concentration of pigment on the dorsal surface just inside the vegetal field. The inward movement of the cells of the equatorial region has begun on both dorsal and ventral sides.

Stage 10½: Age 11 hr. Crescent-shaped blastopore stage. The pigmented line of the blastopore has extended laterally to form a crescent shape and is cut in section at its median point. Presumptive mesoderm cells have continued to move inward in the region above the dorsal lip, and then upward under the overlying layer of cells. The leading cells in this process represent prechordal and anterior mesoderm.

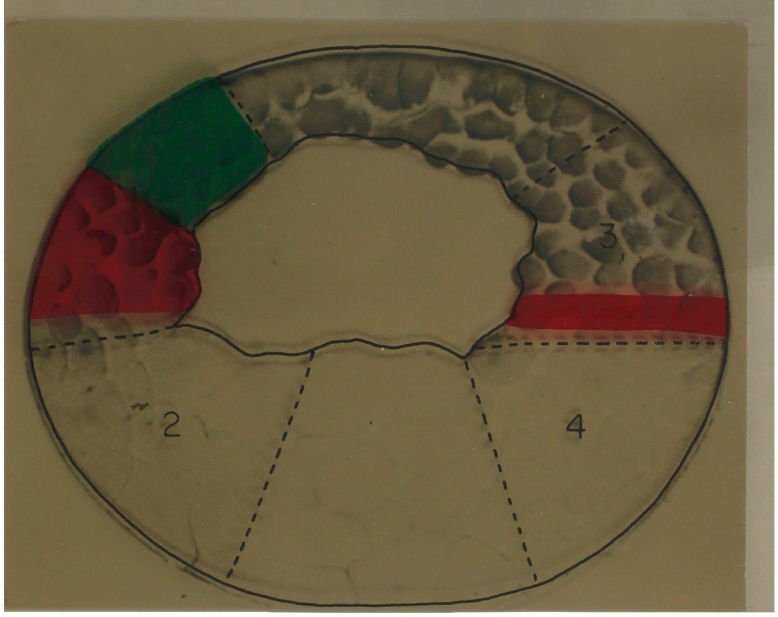
Stage 11: Age 11-3/4 hr. Horse-shoe shaped blastopore stage. A definite layer of mesodermal cells is forming under the ectoderm. The blastocoel floor has moved up on the dorsal side. A fine pigment line indicates the beginning of

Fig. 6. Photographs and diagrams of azure B stained median sections of whole embryos. Stages were determined according to Nieuwkoop and Faber (1956). The dorsal side is to the left in every photograph. The second photograph of stage 10 in Fig. 6 is of a section adjacent to that of the first, but was treated with RNase. The overlay diagrams the movements of the (presumptive) mesoderm and neural tissue, colored red and green respectively. (Presumptive) endoderm is indicated by stippling. The numbered areas outlined with a heavy dashed line were analyzed in the autoradiographic studies, and correspond to the numbered areas in Tables III and IV. Magnification 66x.

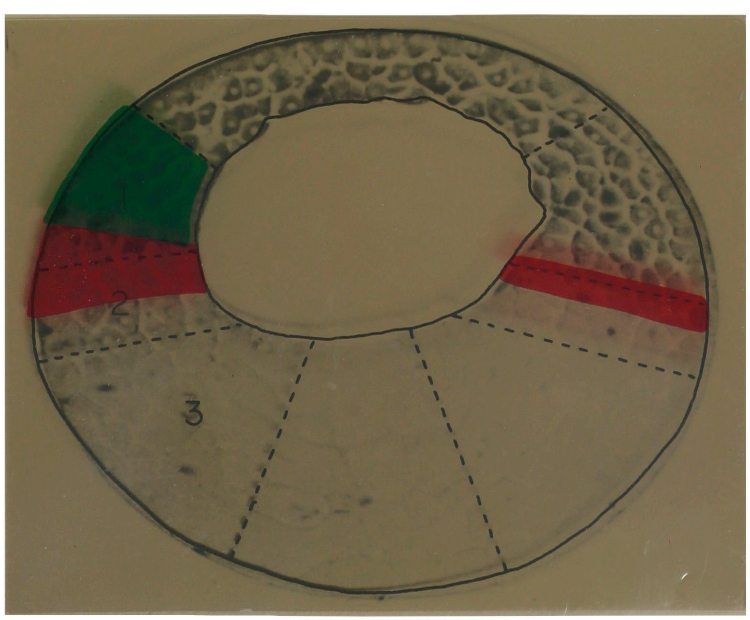
Median Sections of Xenopus laevis Embryos Stained with Azure B



Stage 7 (4 hrs)



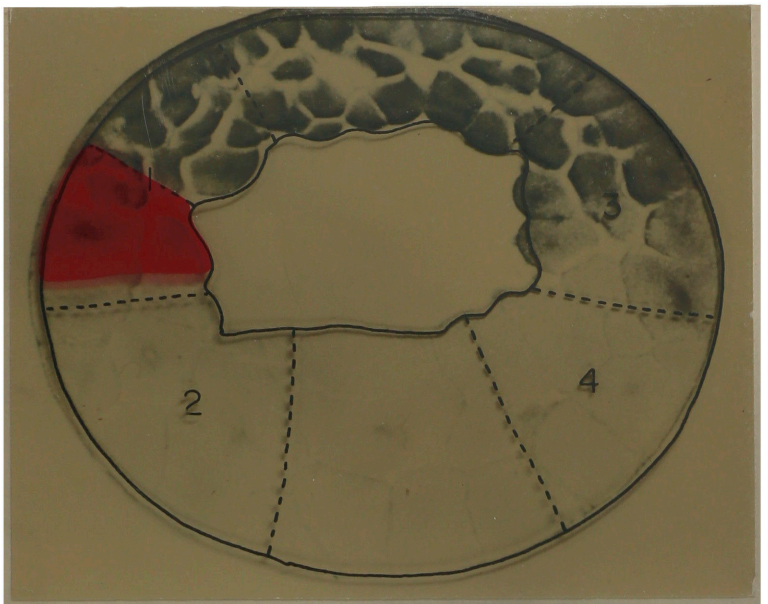
Stage 8 (5 hrs)



Stage $8\frac{1}{2}$ (6 hrs)

Fig. 6. Photographs and diagrams of azure B stained median sections of whole embryos. Stages were determined according to Nieuwkoop and Faber (1956). The dorsal side is to the left in every photograph. The second photograph of stage 10 in Fig. 6 is of a section adjacent to that of the first, but was treated with RNase. The overlay diagrams the movements of the (presumptive) mesoderm and neural tissue, colored red and green respectively. (Presumptive) endoderm is indicated by stippling. The numbered areas outlined with a heavy dashed line were analyzed in the autoradiographic studies, and correspond to the numbered areas in Tables III and IV. Magnification 66x.

Median Sections of Xenopus laevis Embryos Stained with Azure B



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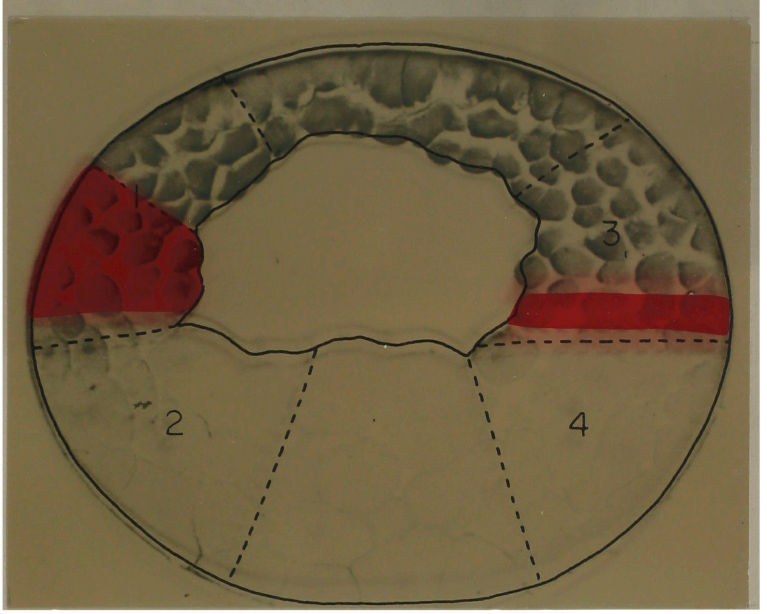

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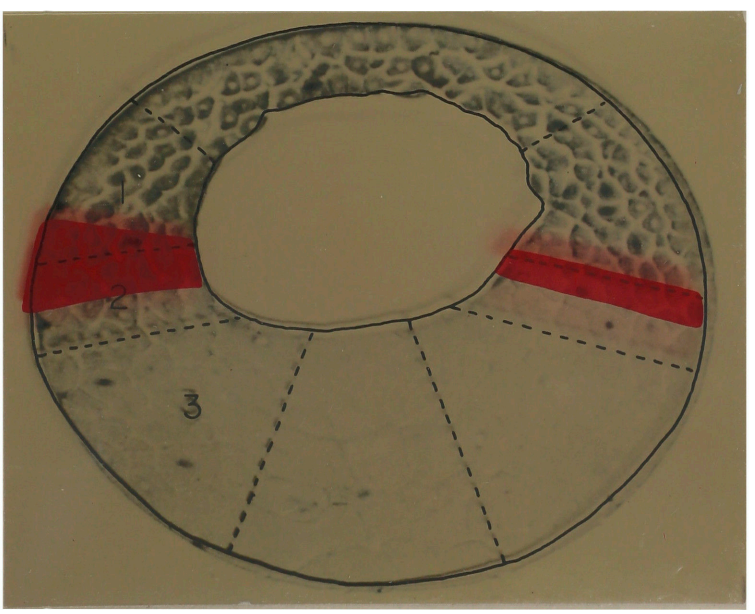

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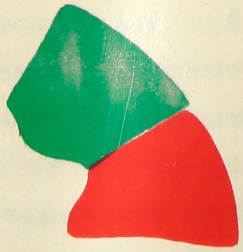
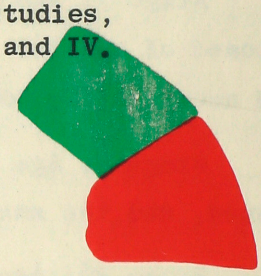
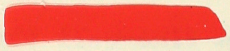
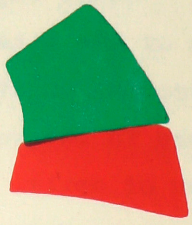
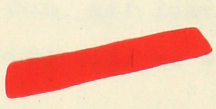


Fig. 6. Photographs and diagrams of azure B stained median sections of whole embryos. Stages were determined according to Nieuwkoop and Faber (1956). The dorsal side is to the left in every photograph. The second photograph of stage 10 in Fig. 6 is of a section adjacent to that of the first, but was treated with RNase. The overlay diagrams the movements of the (presumptive) mesoderm and neural tissue, colored red and green respectively. (Presumptive) endoderm is indicated by stippling. The numbered areas outlined with a heavy dashed line were analyzed in the autoradiographic studies, and correspond to the numbered areas in Tables III and IV. Magnification 66x.

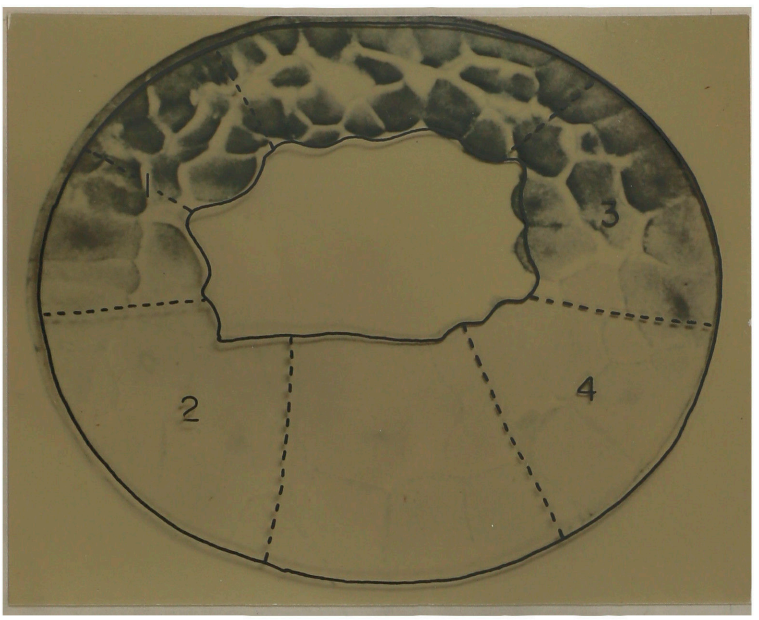




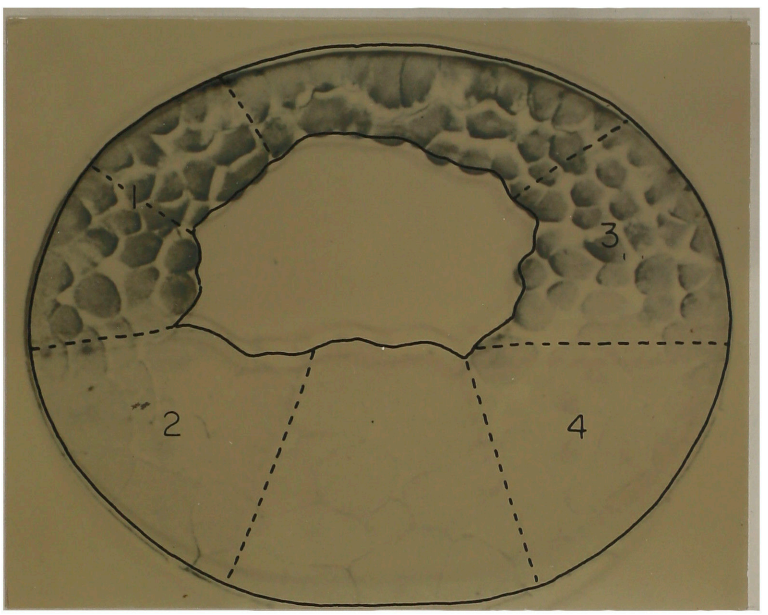




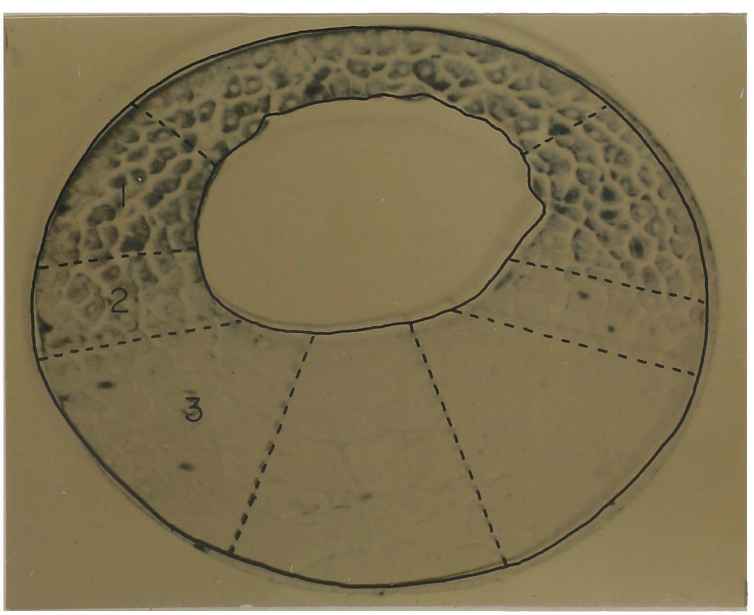
tions of Xenopus laevis Embryos
Stained with Azure B



Stage 7 (4 hrs)



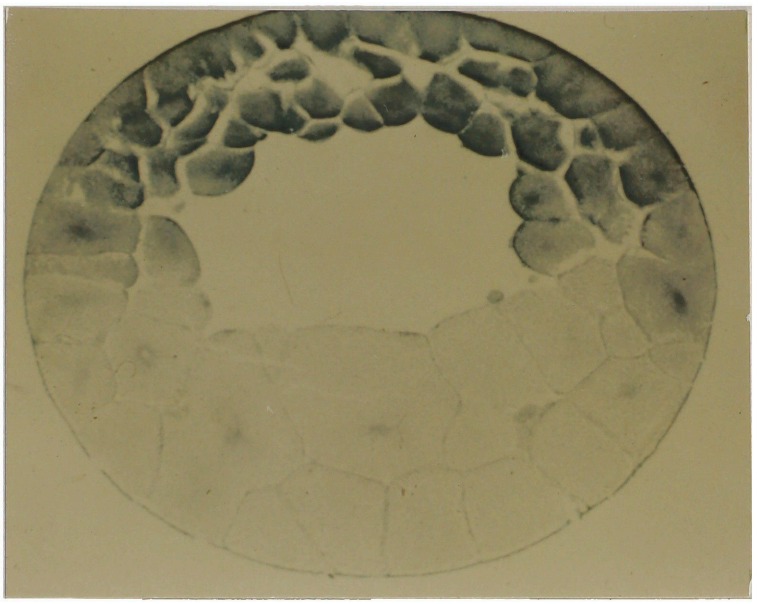
Stage 8 (5 hrs)

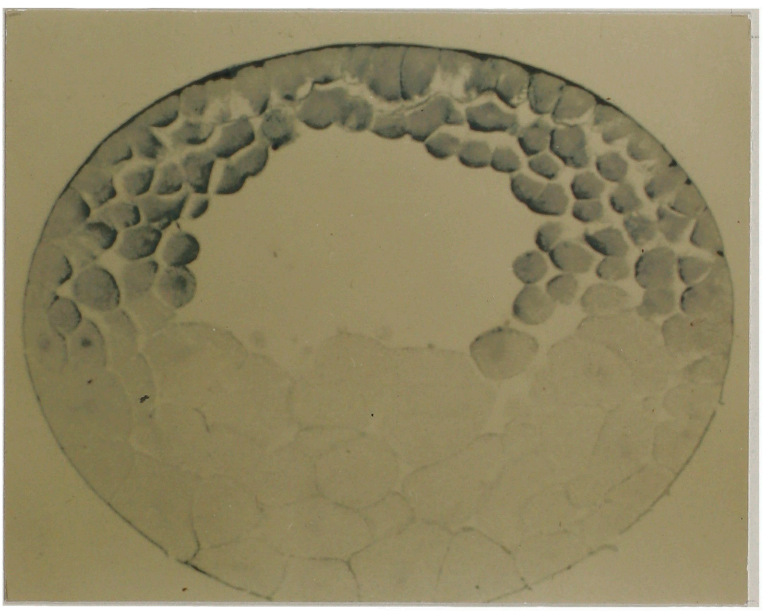


Stage $8\frac{1}{2}$ (6 hrs)

Median Sections of Xenopus laevis Embryos
Stained with Azure B

Fig. 6. Photographs and diagrams of azure B stained median sections of whole embryos. Stages were determined according to Nieuwkoop and Faber (1956). The dorsal side is to the left in every photograph. The second photograph of stage 10 in Fig. 6 is of a section adjacent to that of the first, but was treated with RNase. The overlay diagrams the movements of the (presumptive) mesoderm and neural tissue, colored red and green respectively. (Presumptive) endoderm is indicated by stippling. The numbered areas outlined with a heavy dashed line were analyzed in the autoradiographic studies, and correspond to the numbered areas in Tables III and IV. Magnification 66x.





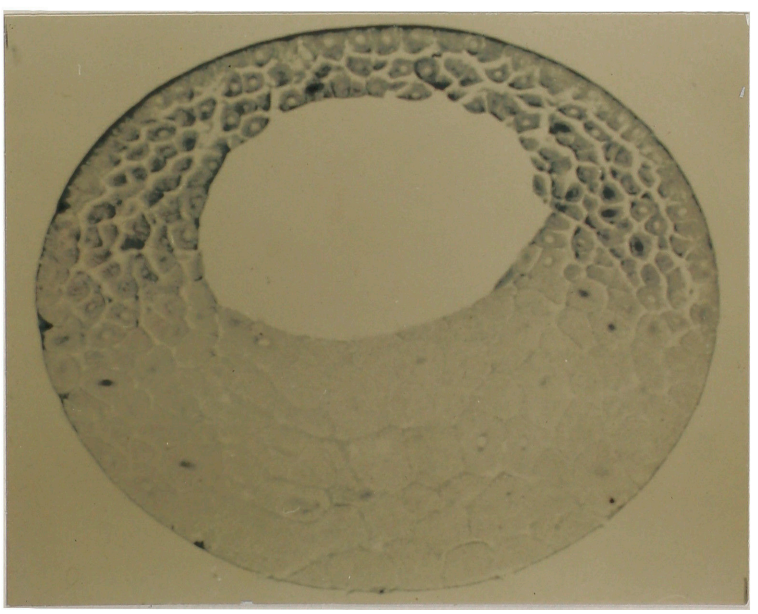


Fig. 6
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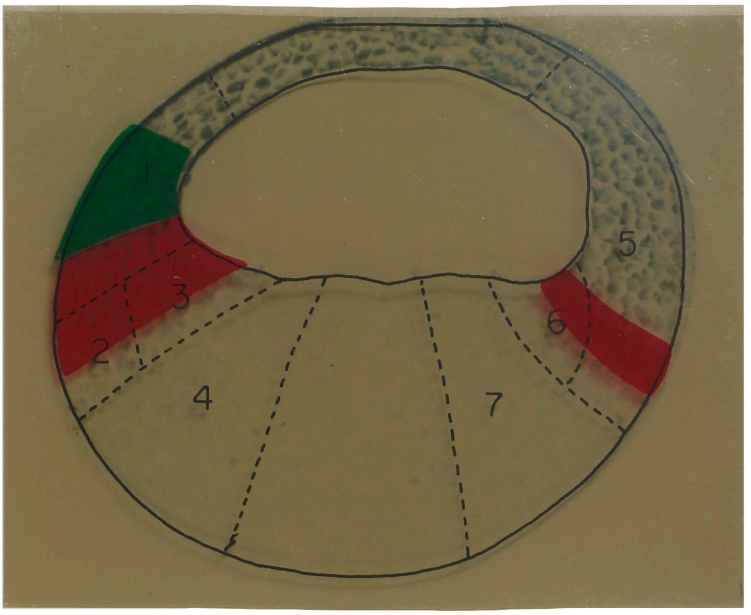
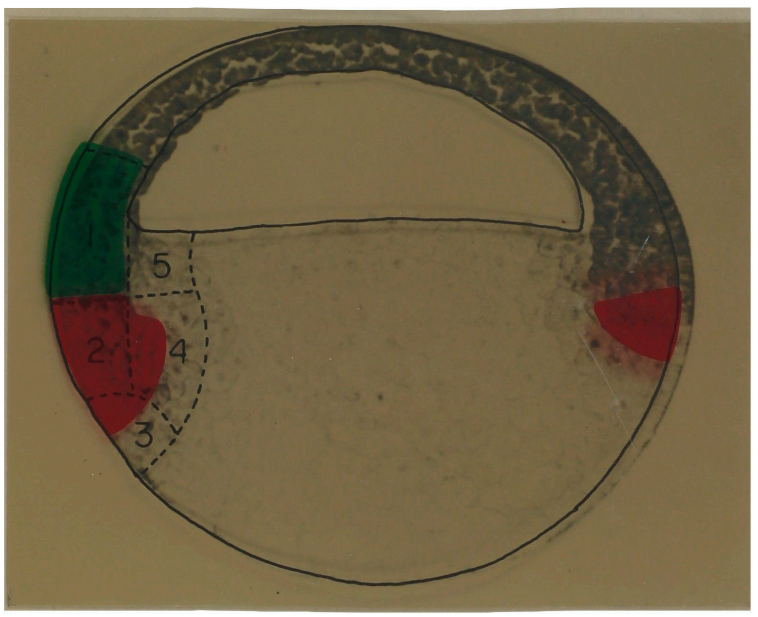
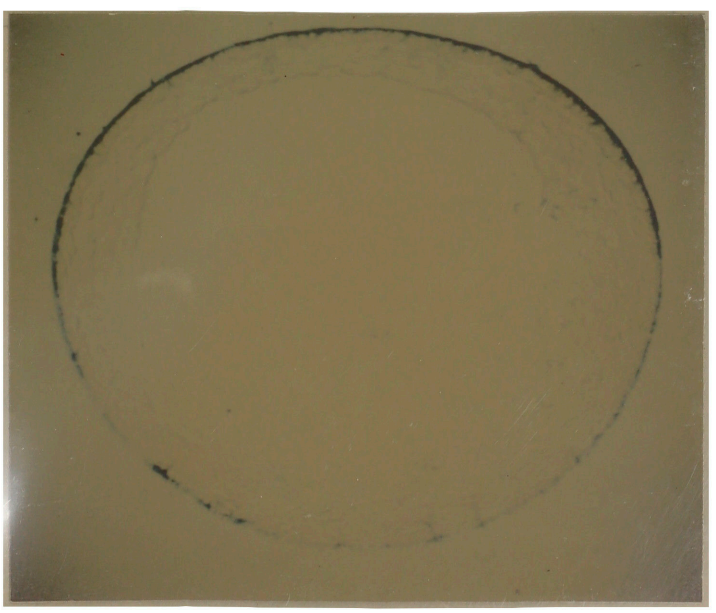


Stage 9 (7 hrs)



Stage 10 (9 hrs)



Stage 10 (9 hrs)

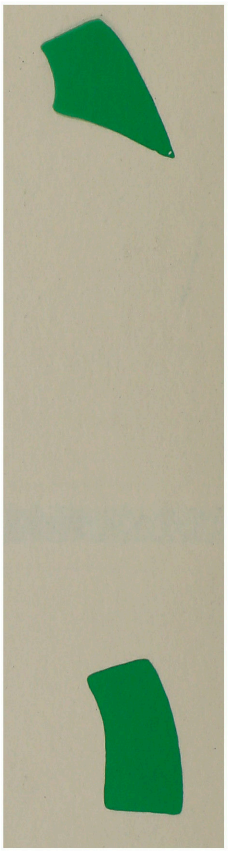


Fig. 6
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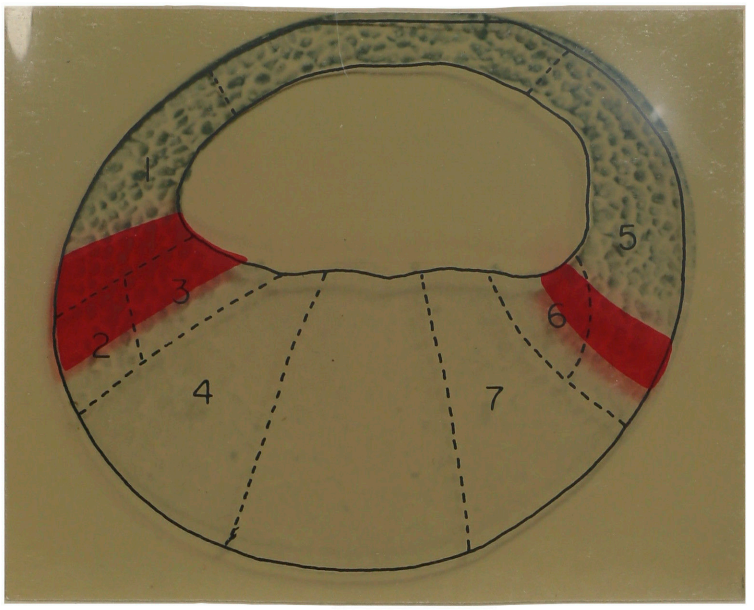

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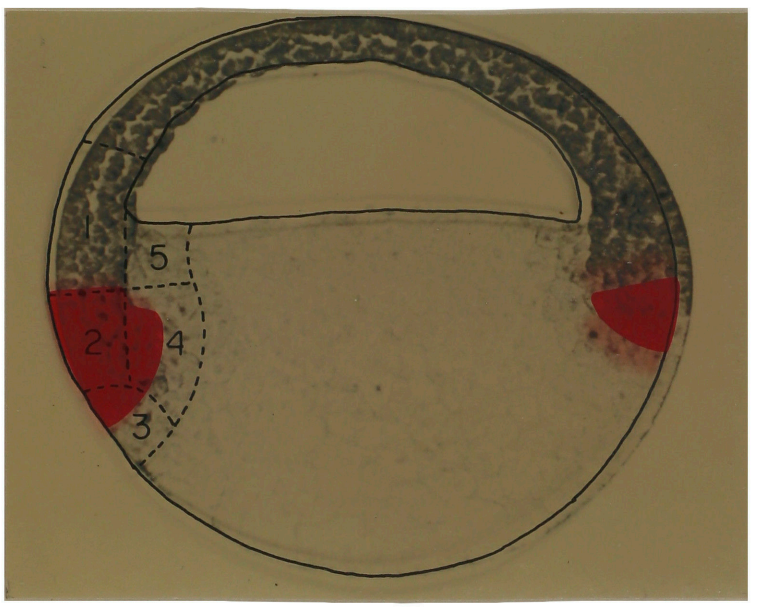
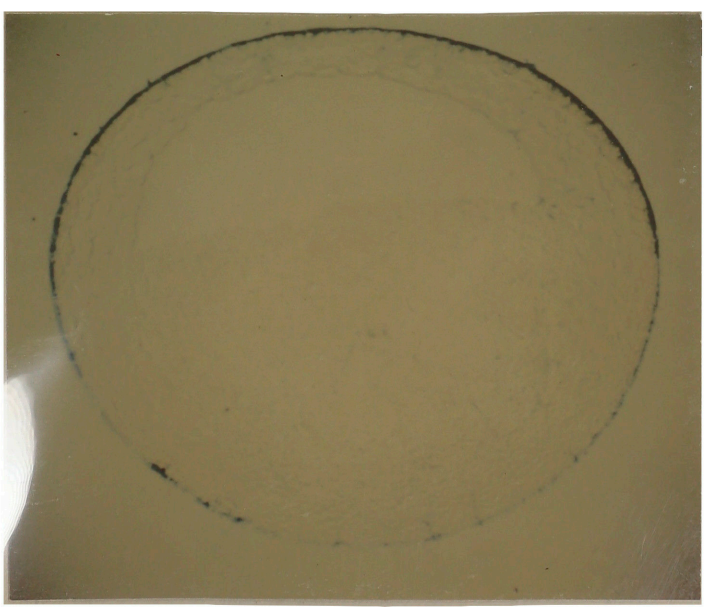


Stage 10 (9 hrs)



Stage 10 (9 hrs).

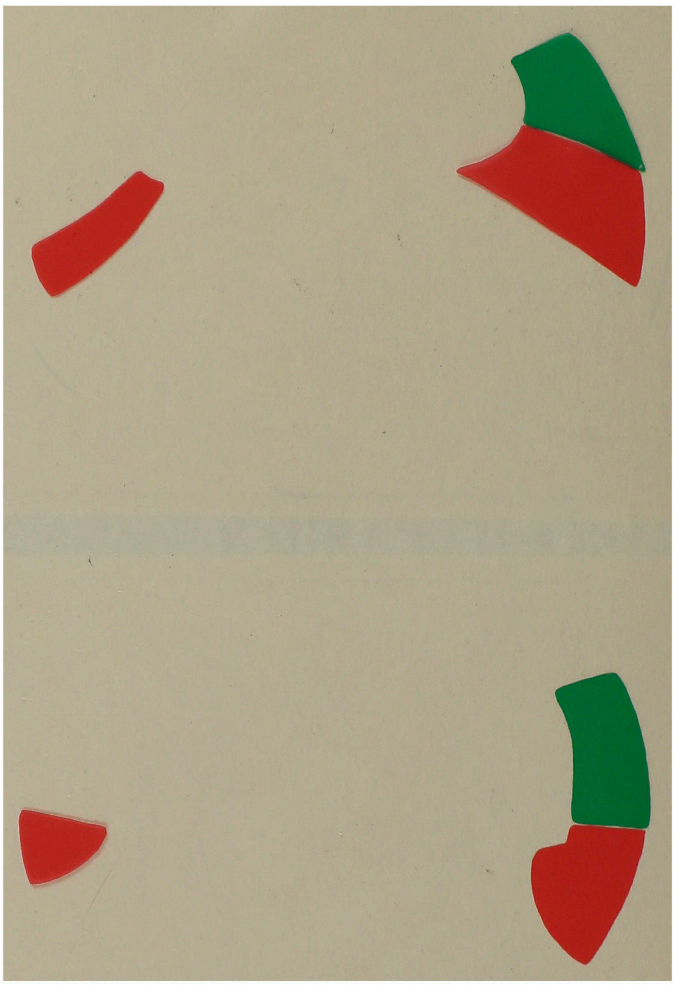


Fig. 6
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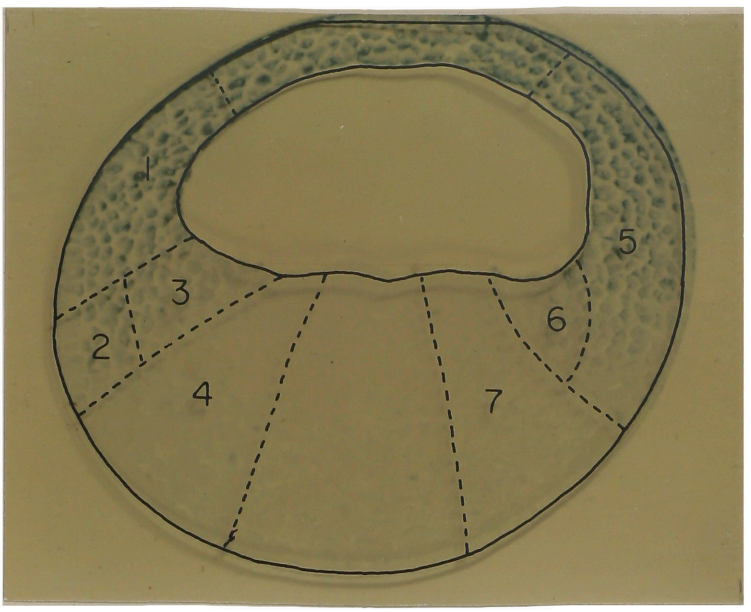

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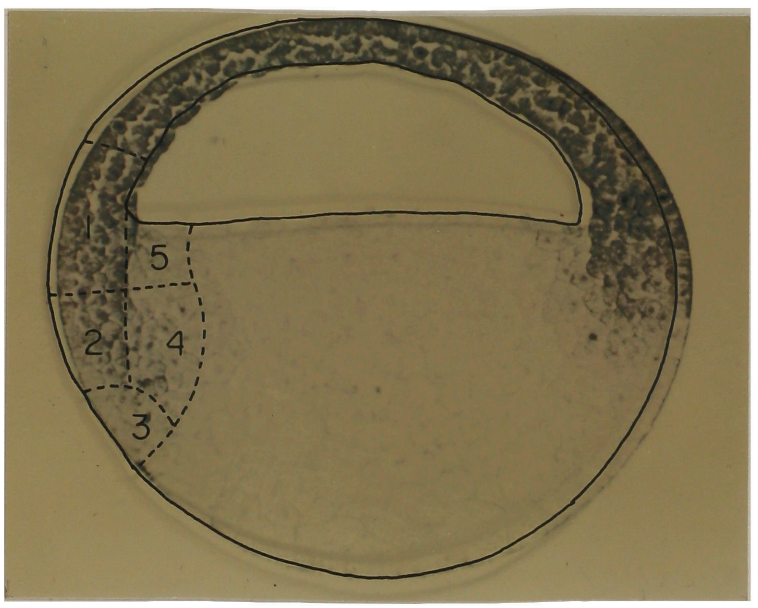


Stage 10 (9 hrs)

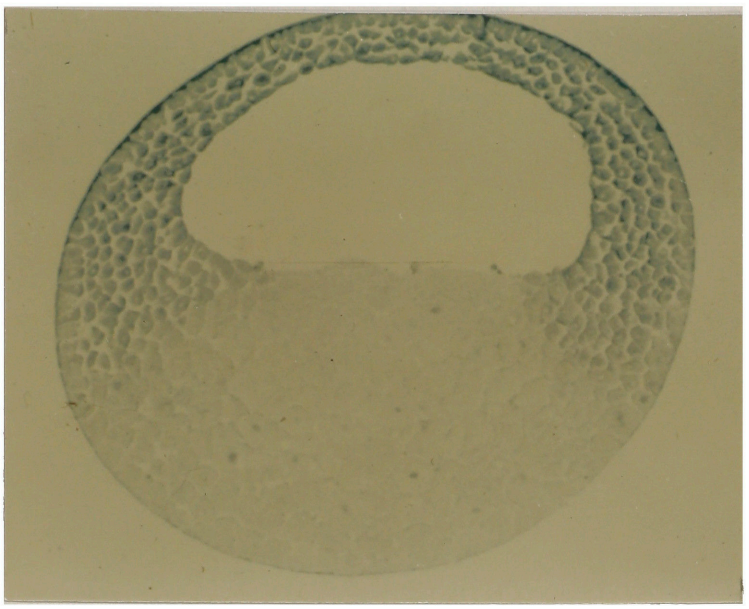
Stage 10 (9 hrs).

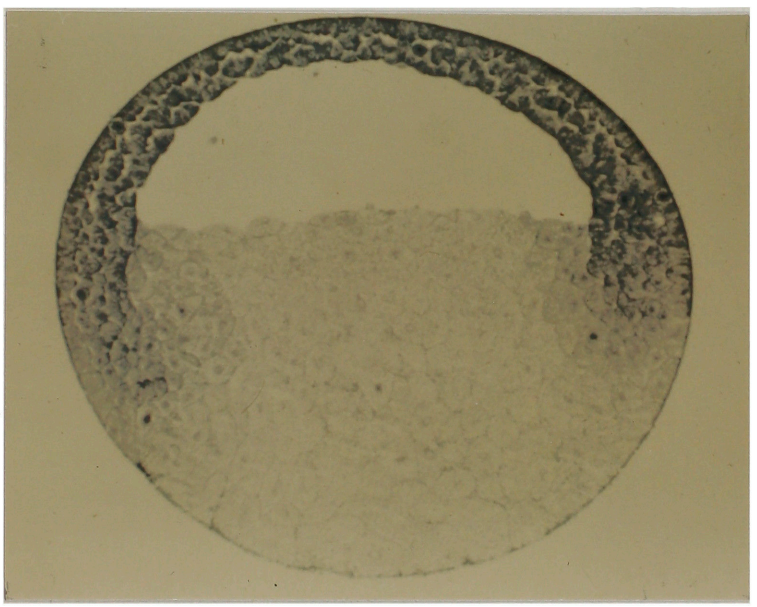
Fig. 6
"continued"

Stage 9 (7 hrs)

Stage 10 (9 hrs)

Stage 10 (9 hrs)





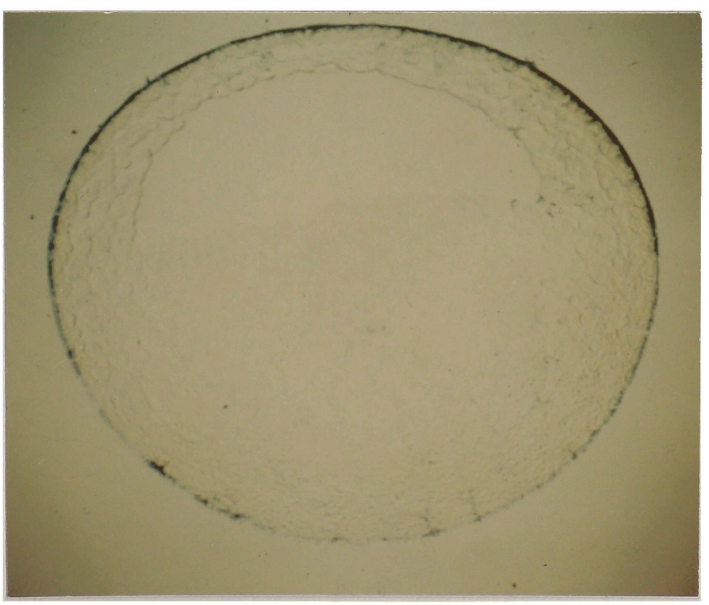


Fig. 6
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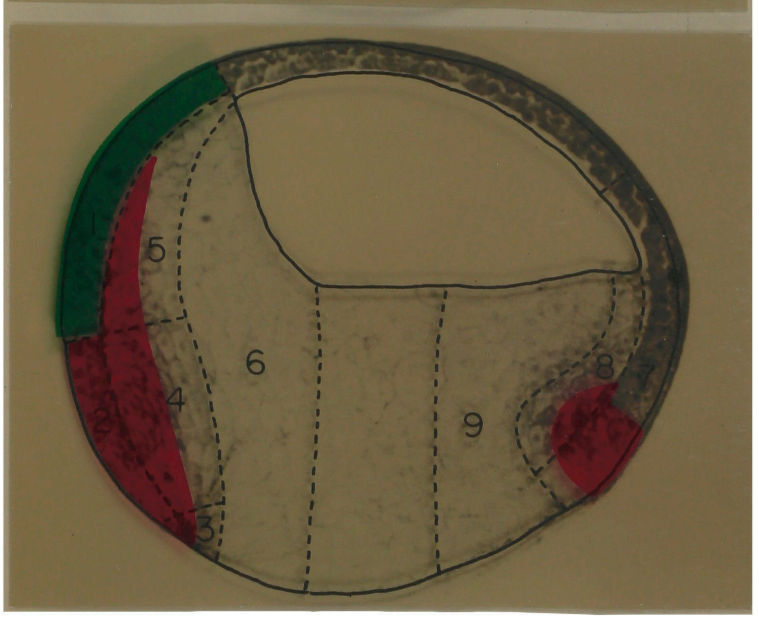
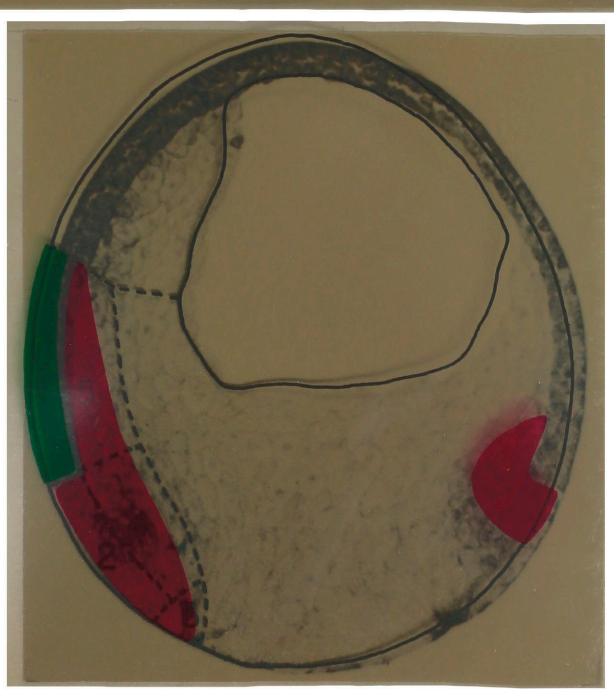
Stage $10\frac{1}{2}$ (11 hrs)



Stage 11 ($11\frac{3}{4}$ hrs)



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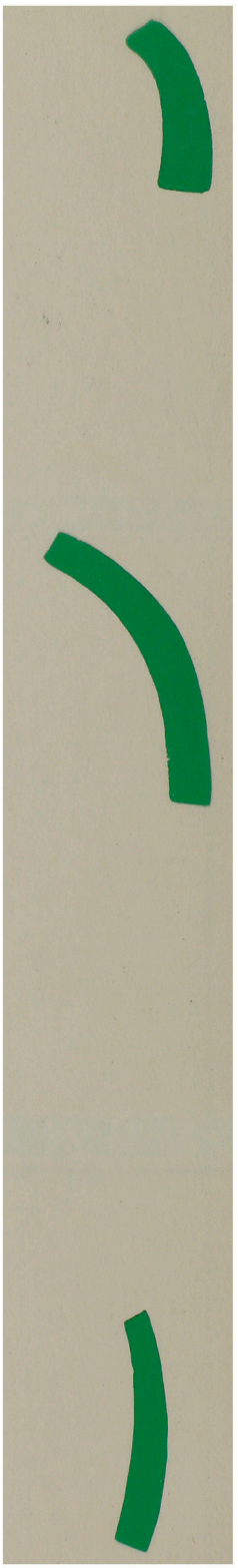

Fig. 6
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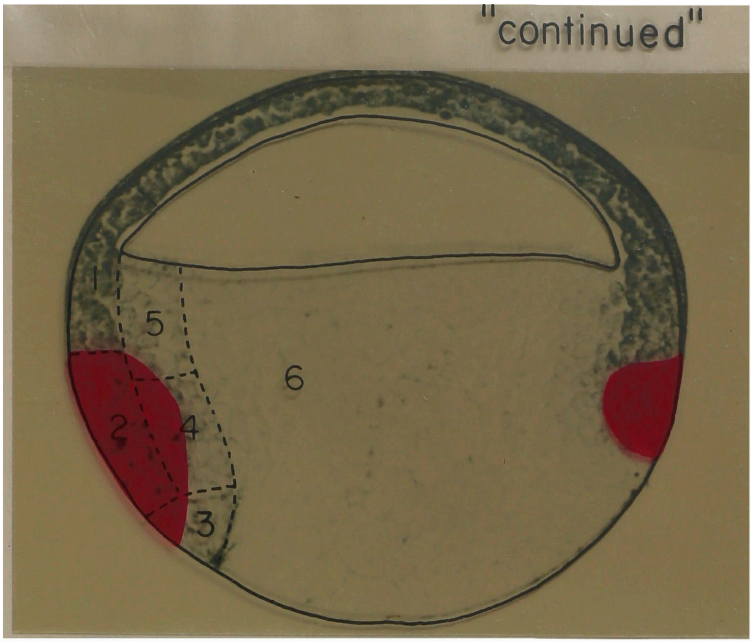
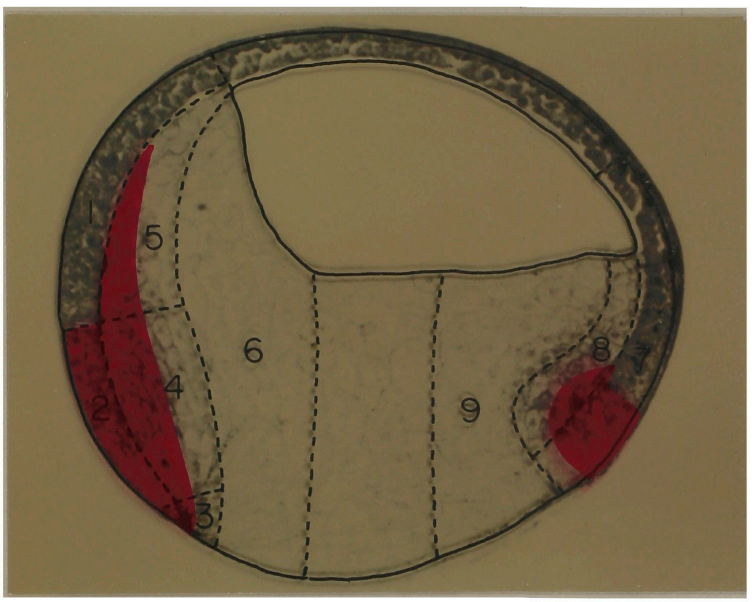
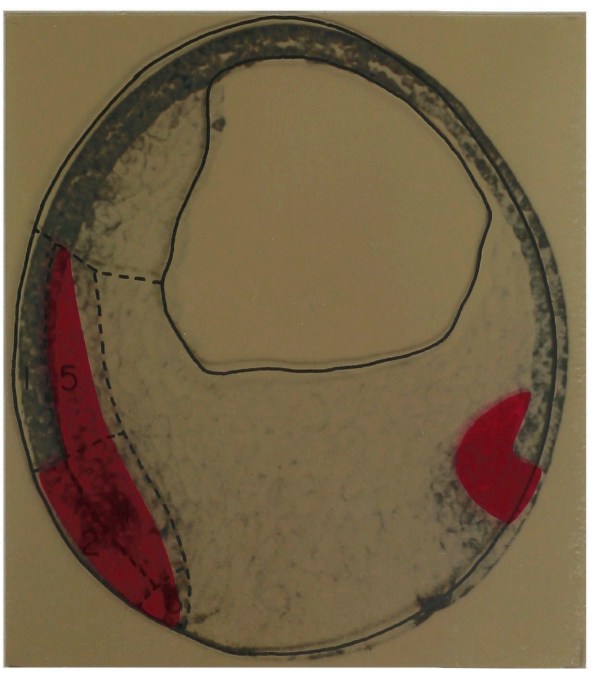


Stage $10\frac{1}{2}$ (11 hrs)



Stage 11 ($11\frac{3}{4}$ hrs)



Stage $11\frac{1}{2}$ ($12\frac{1}{2}$ hrs)

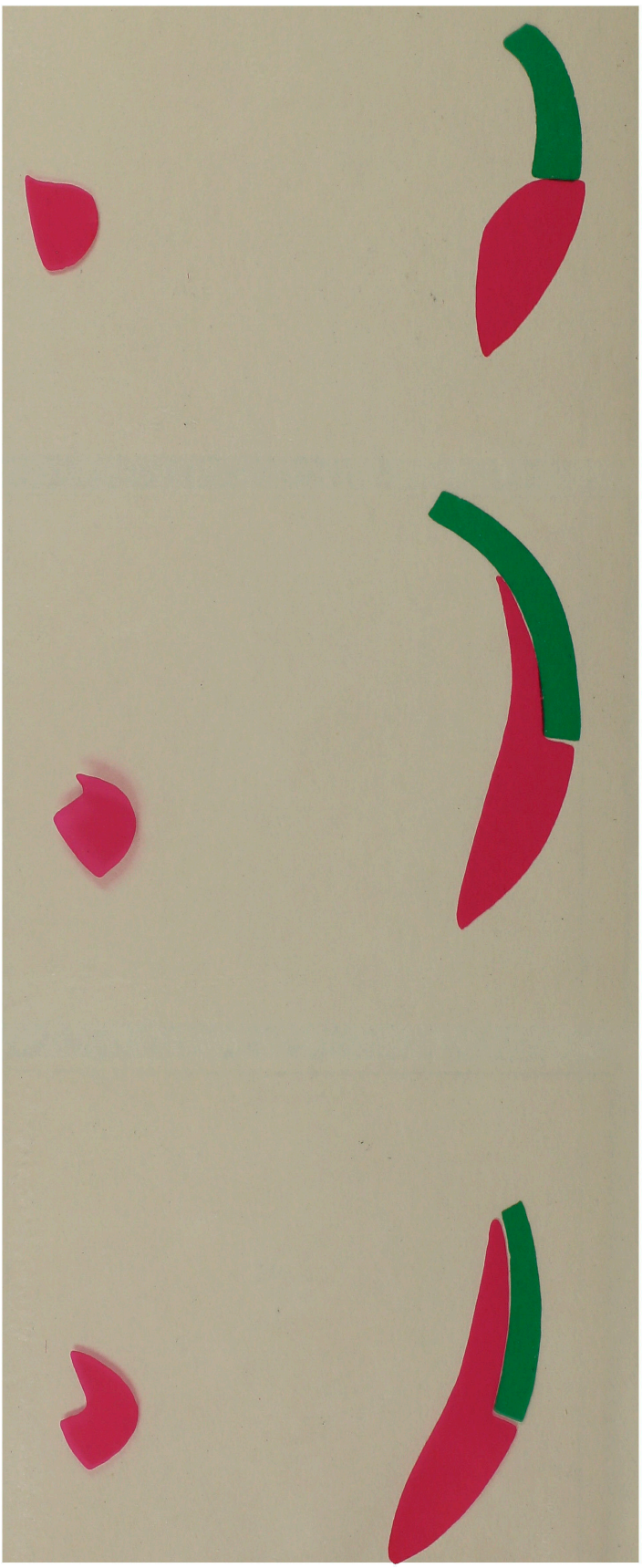


Fig. 6
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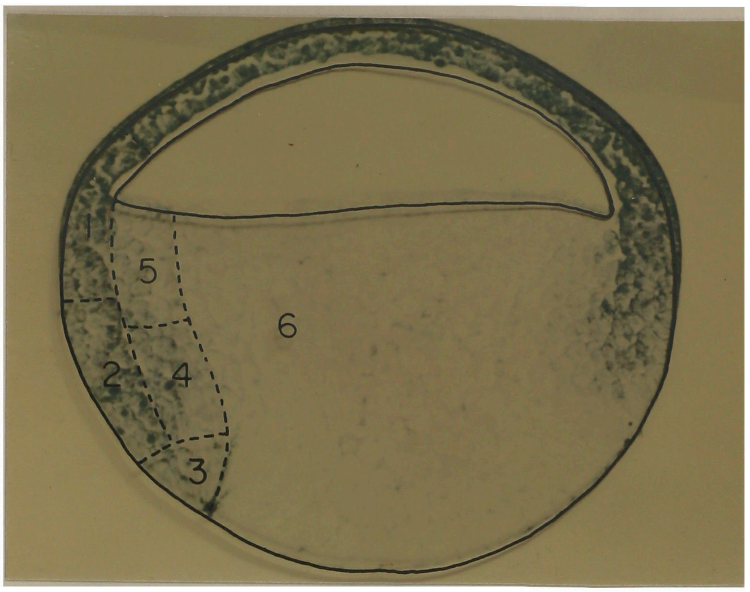
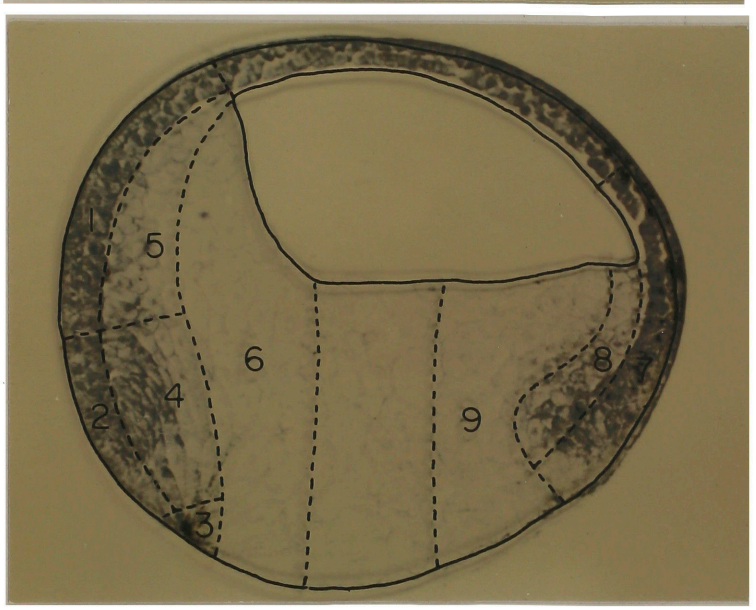
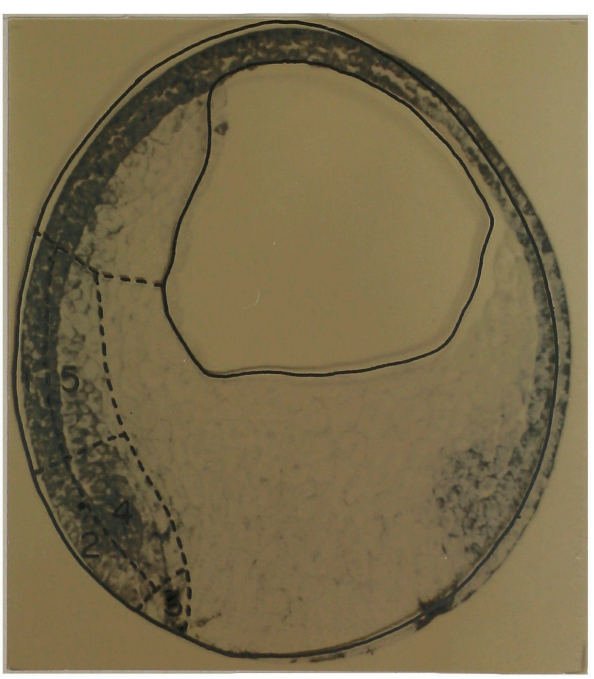


Stage $10\frac{1}{2}$ (11 hrs)



Stage 11 ($11\frac{3}{4}$ hrs)



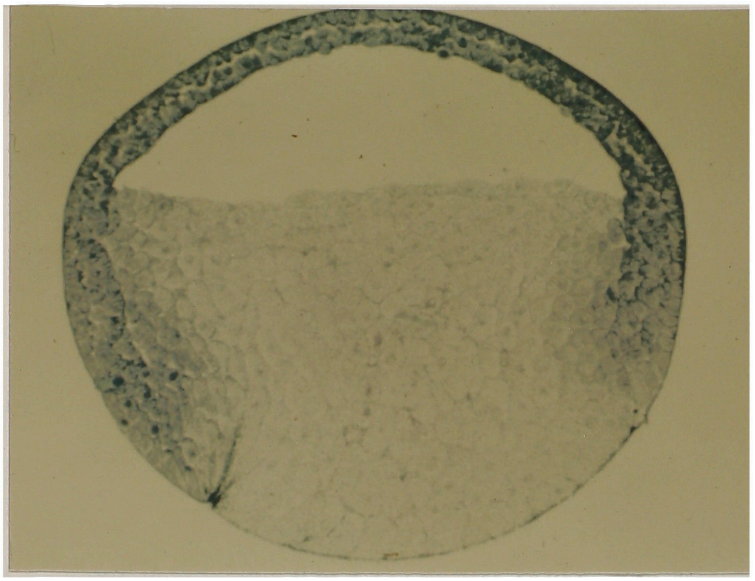
Stage $11\frac{1}{2}$ ($12\frac{1}{2}$ hrs)

Fig. 6
"continued"

Stage $10\frac{1}{2}$ (11 hrs)

Stage 11 ($11\frac{3}{4}$ hrs)

Stage $11\frac{1}{2}$ ($12\frac{1}{2}$ hrs)



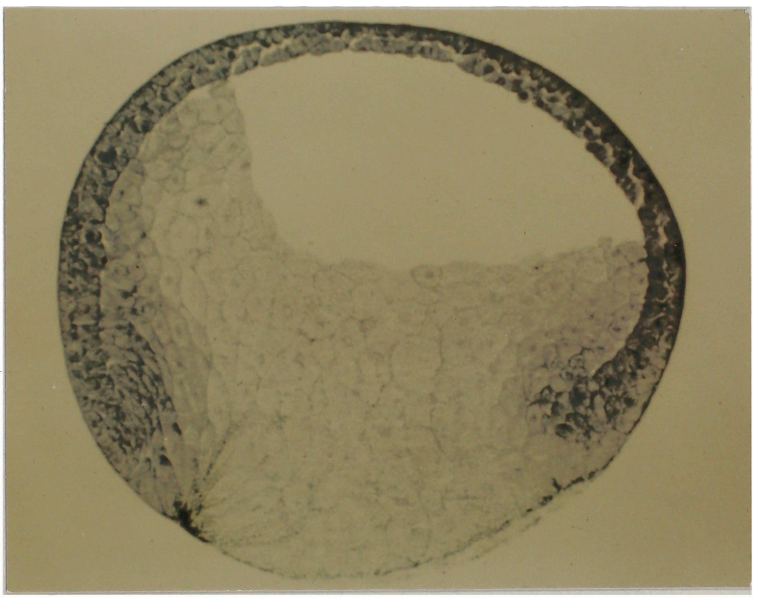
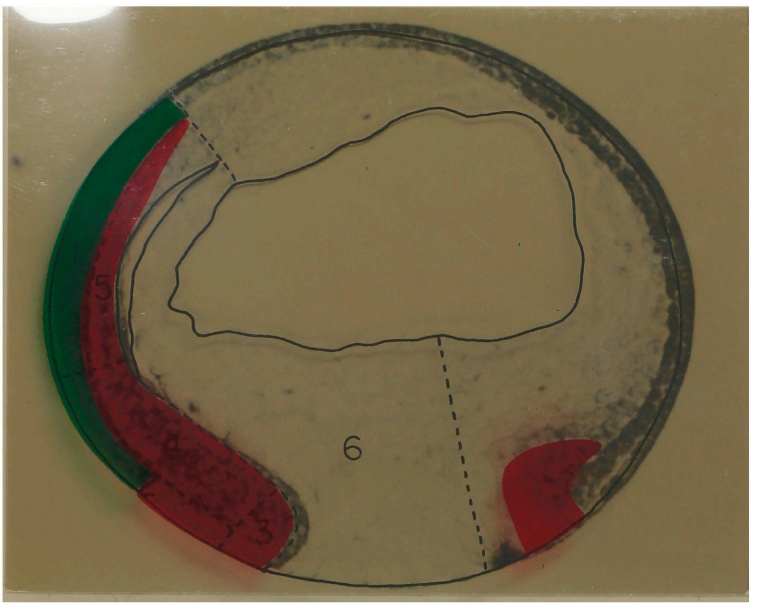
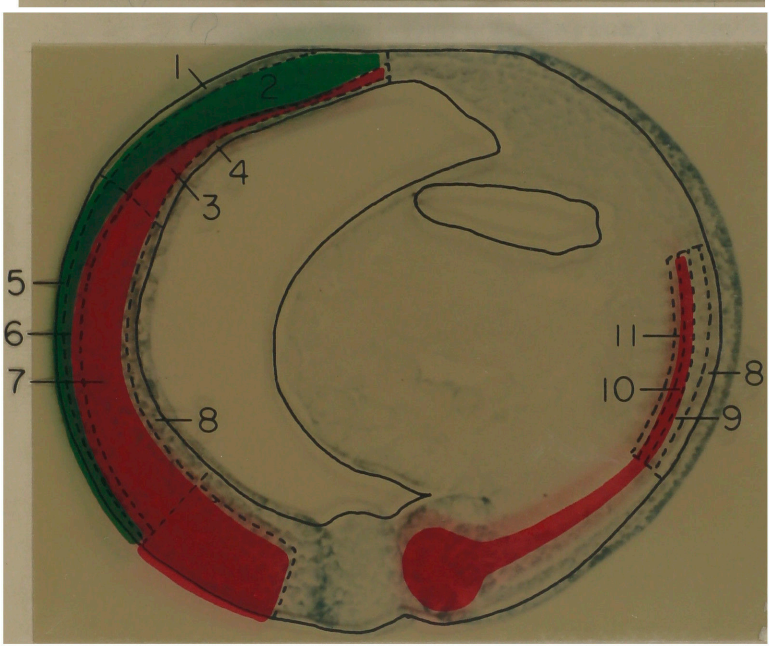


Fig. 6
"continued"



Stage 12 ($13\frac{3}{4}$ hrs)



Stage 13 ($14\frac{3}{4}$ hrs)

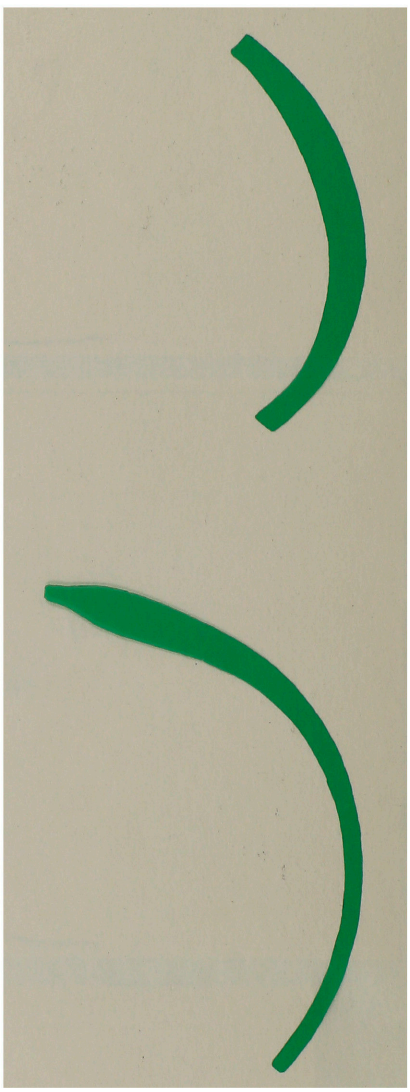
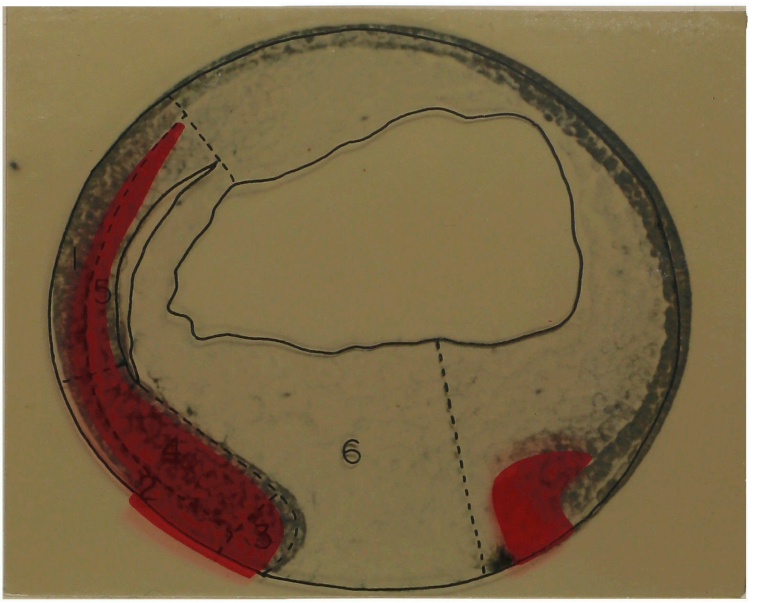
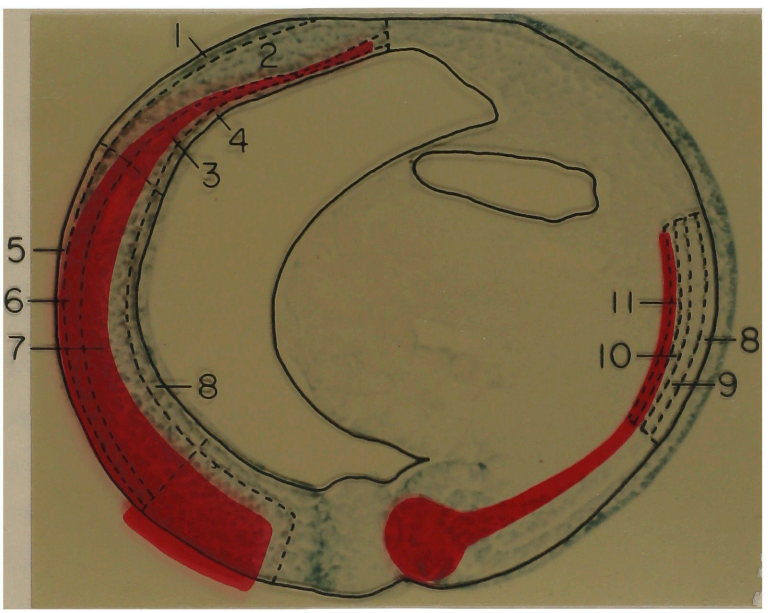


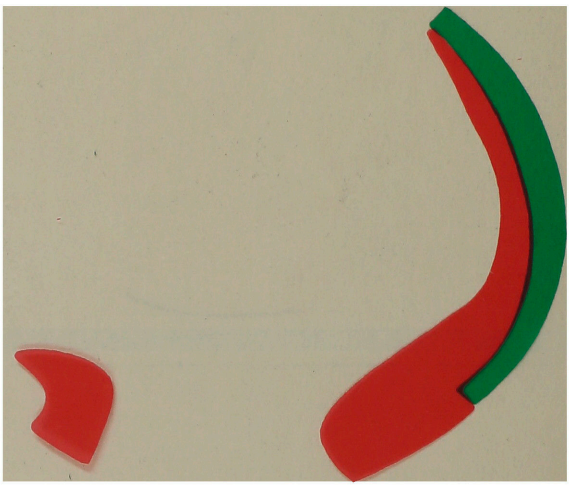
Fig. 6
"continued"



Stage 12 ($13\frac{3}{4}$ hrs)



Stage 13 ($14\frac{3}{4}$ hrs)



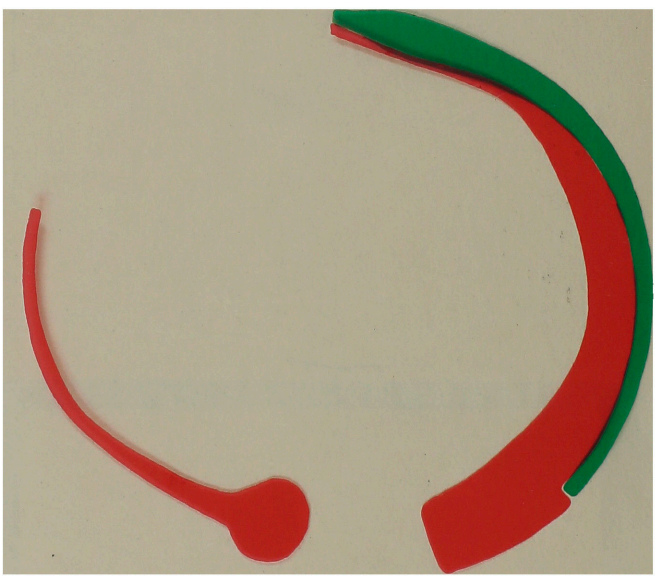
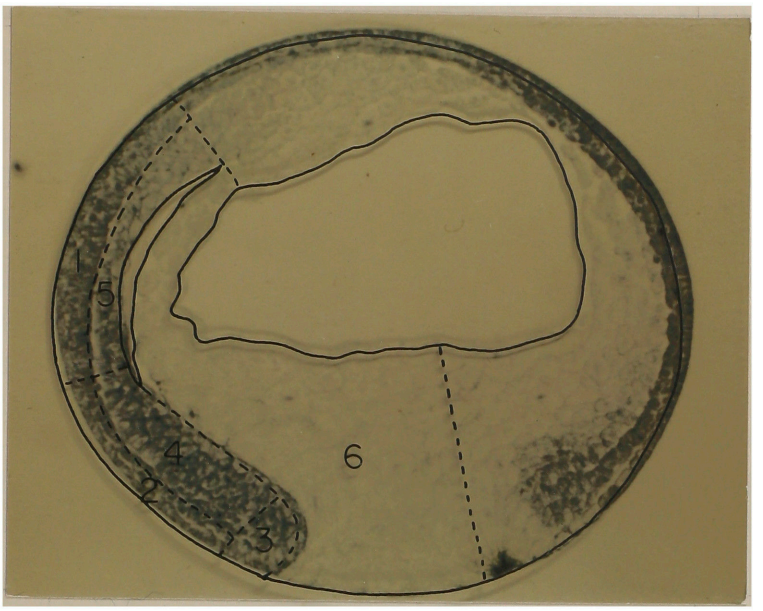
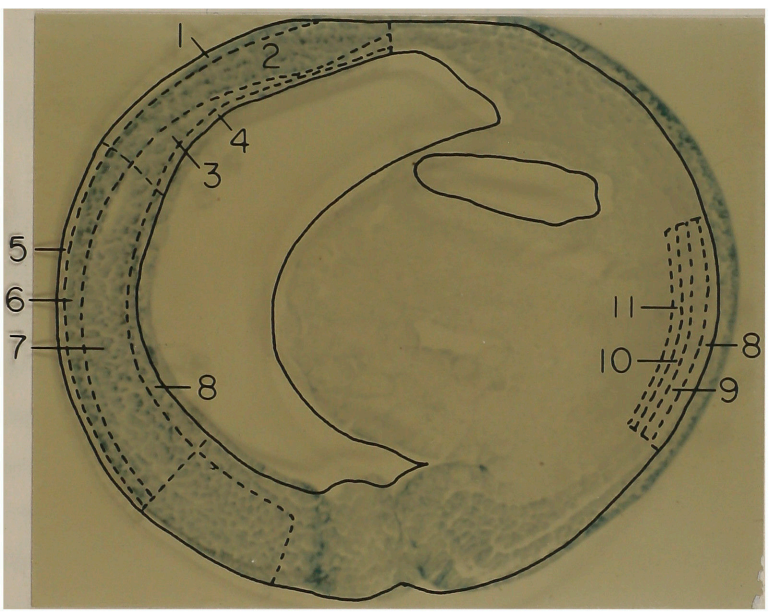


Fig. 6
"continued"



Stage 12 ($13\frac{3}{4}$ hrs)

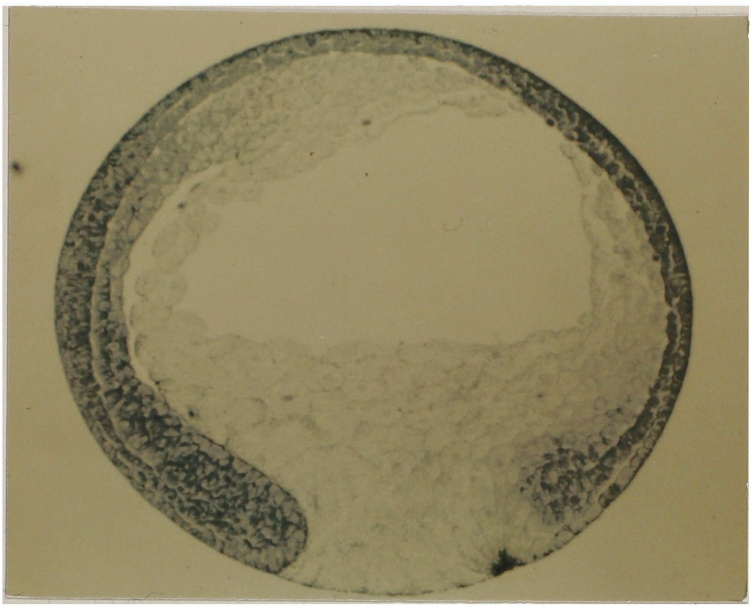


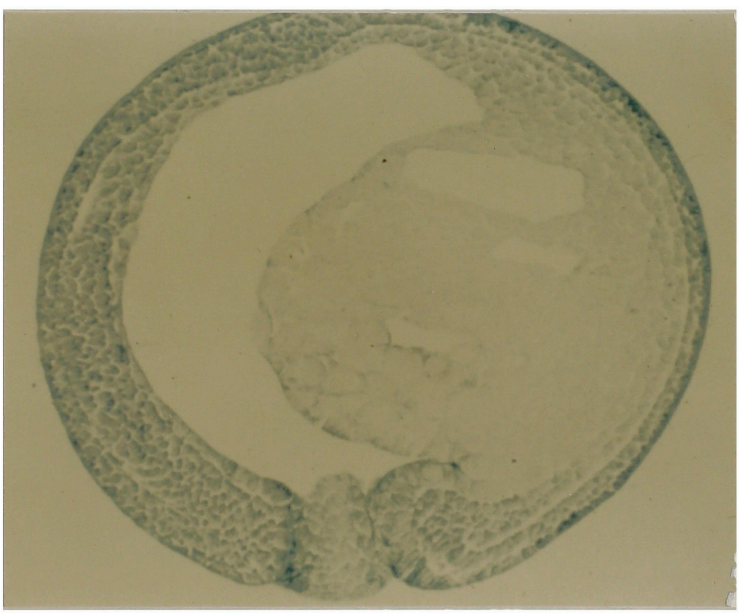
Stage 13 ($14\frac{3}{4}$ hrs)

Fig. 6
"continued"

Stage 12 (13¾ hrs)

Stage 13 (14¾ hrs)





the formation of the archenteron.

Stage 11½: Age 12½ hr. Large yolk plug stage. The blastopore groove appears almost circular around the vegetal pole and is seen in section cut at two points. The formation of the mesodermal layer continues on dorsal and ventral sides.

Stage 12: Age 13¼ hr. Medium yolk plug stage. A small archenteron has formed, a process clearly separated from the preceding formation of the mesoderm layer. It is a slit-like groove extending from the dorsal lip of the blastopore up under the mesoderm where it widens into a shallow hollow. In this region a definite thickening of the ectoderm can be observed, the beginning of neural differentiation.

Stage 13: Age 14-3/4 hr. Slit-blastopore stage. The yolk plug has become small and slit-shaped. The archenteron has expanded greatly and the blastocoel is almost obliterated. The future cranial part of the nervous system is indicated as a definite thickening in the inner sensorial layer of the ectoderm almost opposite the yolk plug. More posteriorly the mesoderm forms a thick layer, the notochord. A thin layer of endoderm lines the dorsal surface of the archenteron.

Results of Autoradiographic Studies

The possible hydrolysis of labeled RNA by the acid fixative must be considered. Davidson (1964) compared the amount of RNA label extracted by the method of Ogur and Rosen (see Methods of Section III) from unfixed oocytes of X. laevis or oocytes fixed in Smith's fixative, and found no difference. Thus the fixative does not remove label which is insoluble in 0.2 N PCA. Also isolated labeled embryo RNA was precipitated in Smith's fixative, 0.2 N PCA, or 5% TCA for one hour at 0° to 4°C. The fixative precipitated about twice as many counts as the two pure acids, again suggesting that it does not hydrolyze high molecular weight RNA in fixed embryos. 5% TCA precipitated 10% less counts than 0.2 N PCA, so that probably more 'acid soluble' RNA was removed from the autoradiographs than from embryos homogenized in 0.2 N PCA described in Section III.

The results of the autoradiographic studies are presented in Tables III to V as grains per nucleus in a given region of a given stage. Cytoplasmic labeling was not significantly above background. Resolution with tritium and AR 10 stripping film is about 2μ and nuclear diameters ranged from 10 to 25μ, so grains could be definitely localized as intranuclear. The grains per nucleus in RNase treated sections are mostly due to background activity and reflect changes in the

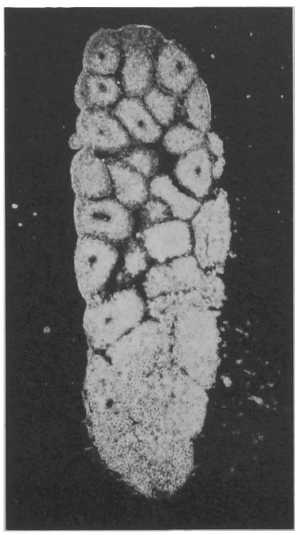
size of the nuclei. The values were in stage 7, 12 for presumptive mesoderm and ectoderm, and 17 for presumptive endoderm; in stage 8, 6 and 5 respectively; and they decreased steadily to approximately 1 in all regions of stage 13. These values were subtracted from the grains per nucleus of untreated sections. DNA synthesis was not detectable under the conditions used. The regions listed in the tables are numbered and diagrammed in the overlay of Fig. 6.

Studies of azure B stained sections of embryo halves revealed any cellular rearrangements, which tended to occur in early stages. Dark field photomicrographs of typical dorsal half autoradiographs are shown in Fig. 7 to demonstrate their morphology. High magnification photomicrographs of nuclear labeling in various regions of embryos of various stages are presented in Fig. 8. Nuclear labeling is also observable in the low magnification photomicrographs of embryos exposed to uridine-H^3 of high specific activity in Fig. 9. Grain counts were not possible on such densely labeled nuclei. The larger endodermal nuclei appear less densely labeled.

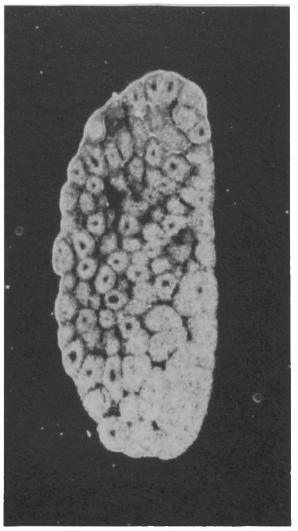
Table IIIa includes data from several experiments on all stages of dorsal halves studied. A supplementary experiment using uridine-H^3 of a different specific activity is presented in Table IIIb. Clearly a marked activation of nuclear RNA synthesis occurs in presumptive mesoderm and endoderm between stages 8 and 9, mid to late blastula. Within a one hour period between stages 8 and $8\frac{1}{2}$ nuclear grain counts jumped from a value of approximately 0.5 to a value of 10 to 15 indicating at least a twenty- to thirty-fold increase in activity per nucleus. A further increase occurs in the next one hour period between stages $8\frac{1}{2}$ and 9. From stage 9 to 13, the activity per nucleus in inner mesoderm and endoderm remains relatively constant, and is highest in the prechordal and anterior mesoderm, the first material to invaginate and move upward. During gastrulation the mesoderm cells remaining on the outer surface are quiescent and are progressively activated as they move inward.

The presumptive neural tissue shows a small but significant period of activity around the beginning of gastrulation, and becomes definitively activated in stage $11\frac{1}{2}$, the mid-gastrula. By stage 13 when the beginnings of neural differentiation are visible on the surface of the embryo and in sections, RNA synthesis per nucleus is highest in the anterior neural tissue. The ectoderm at all stages after stage 8 showed a marked activity at the surface of the cut, no matter where in the animal region the cut was made. This activation could be due to substances

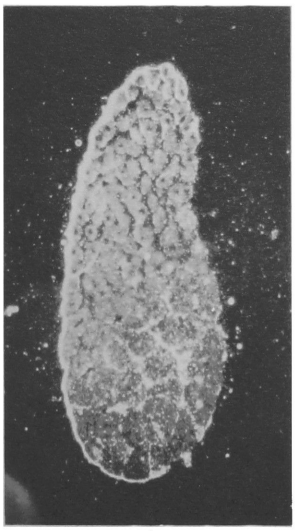
Fig. 7. Dark field photomicrographs of typical dorsal half autoradiographs of various stages. The outer dorsal surface is to the left in all photographs. The progress in formation of the archenteron is apparent in stages 11 and $11\frac{1}{2}$. In stages 12 and 13 the right surface represents the roof of the archenteron. The cranial thickening in the sensorial layer of the ectoderm and the high nuclear activity in all regions can be seen in stage 13. Magnification 48x



Stage 7 (4 hrs.)

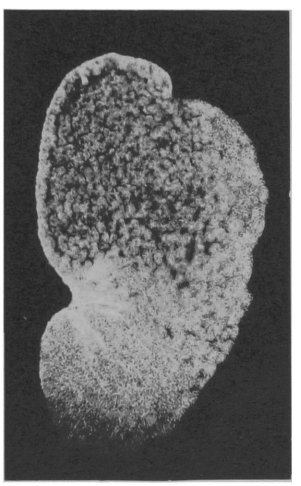


Stage 8 (5 hrs.)



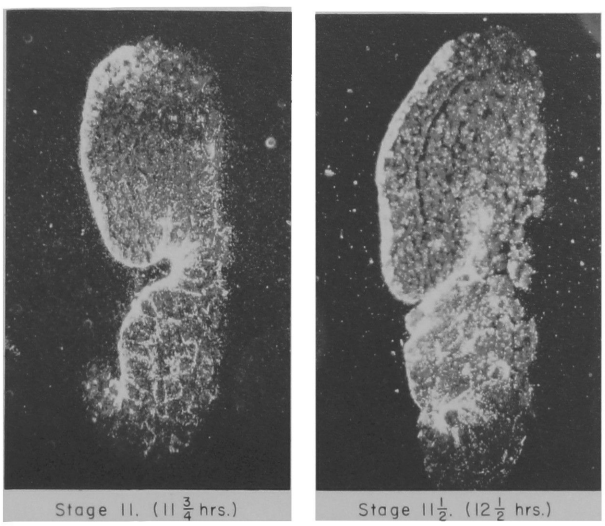
Stage $8\frac{1}{2}$ (6 hrs.)

Stage 9 (7 hrs.)



Stage 10 (9 hrs.)

Figure 7



Stage 11. (11 $\frac{3}{4}$ hrs.)

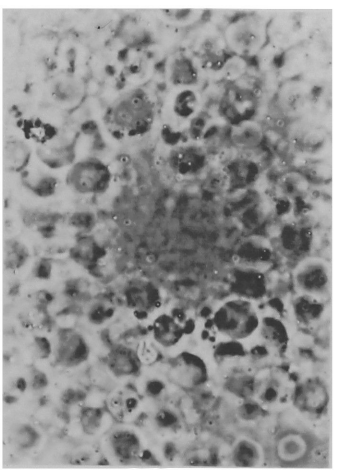
Stage 11$\frac{1}{2}$. (12 $\frac{1}{2}$ hrs.)

Stage 12. (13 $\frac{1}{4}$ hrs.)

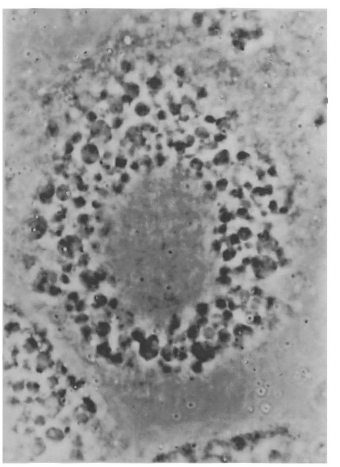
Stage 13. (14 $\frac{3}{4}$ hrs.)

Figure 7 (contd)

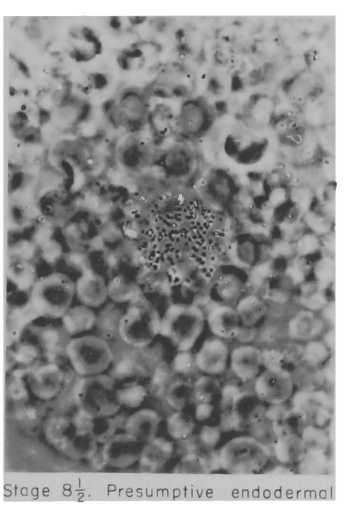
Fig. 8. High magnification photomicrographs of autoradiographs. Intranuclear labeling is demonstrated in various regions at various stages.
Magnification 800x



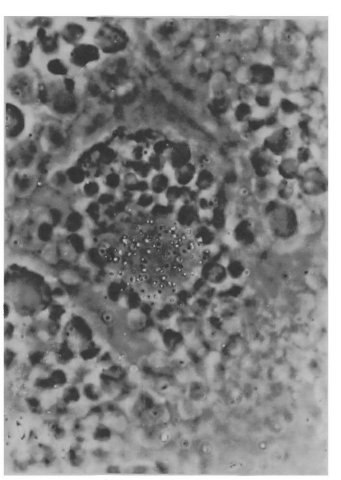
Stage 7. Presumptive endodermal cell



Stage 8. Equatorial cell

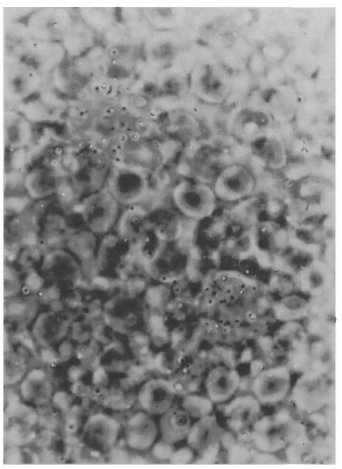


Stage $8\frac{1}{2}$. Presumptive endodermal cell

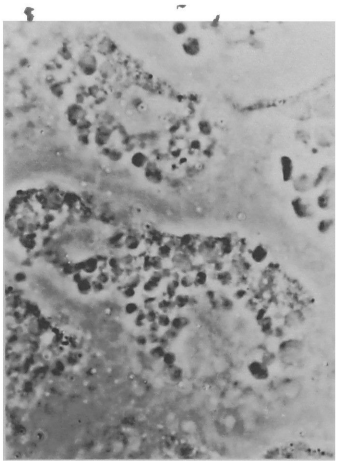


Stage $8\frac{1}{2}$. Equatorial cell

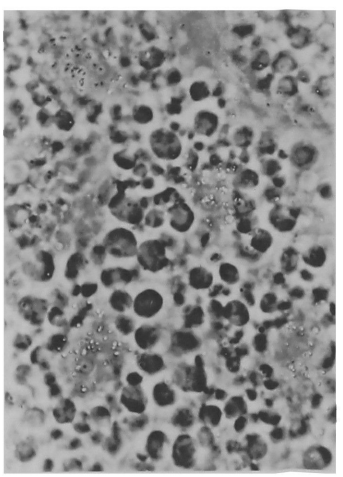
Figure 8



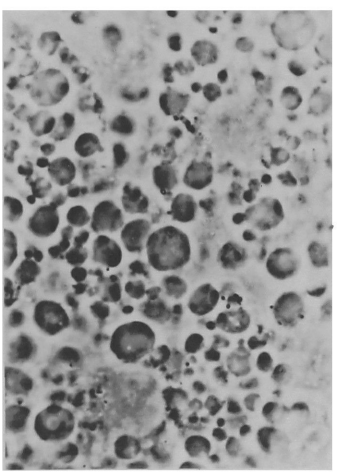
Stage 9 Presumptive endodermal cells



Stage 9. Outer equatorial cells

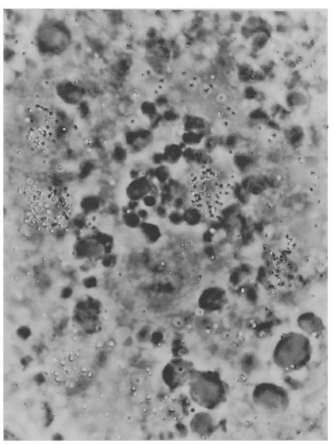


Stage 9. Inner equatorial cells

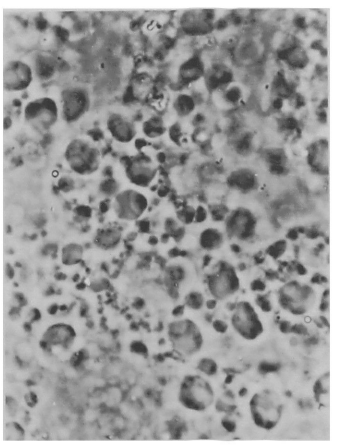


Stage 9. Inner equatorial cells after RNase treatment

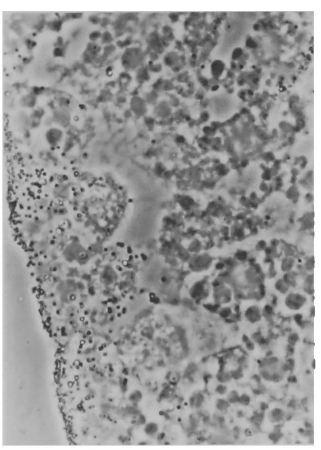
Figure 8 (contd)



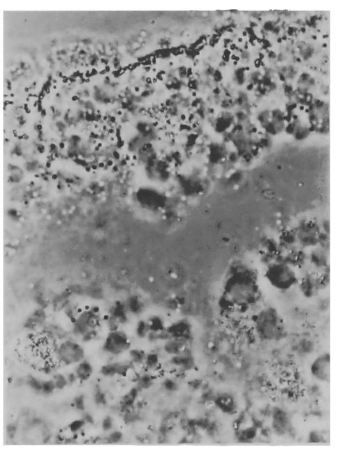
Stage 11 Anterior mesodermal cells



Stage 11. Anterior mesodermal cells after RNase treatment



Stage 11. Ectodermal cells



Stage 13. Epithelial and neural layers

Figure 8 (contd)

Fig. 9. Low magnification photomicrographs of autoradiographs. The lower left stage 10 embryo is the control for the actinomycin D-treated embryos. They were incubated as for the experiment of Table V, but the autoradiographs were exposed for 7 days. The stage 8 and 9 embryos were incubated in uridine-H^3 of specific activity 17.4 mC/μM and concentration of 0.03 μM/ml. Autoradiographs were exposed for 7 days. Magnification 50x

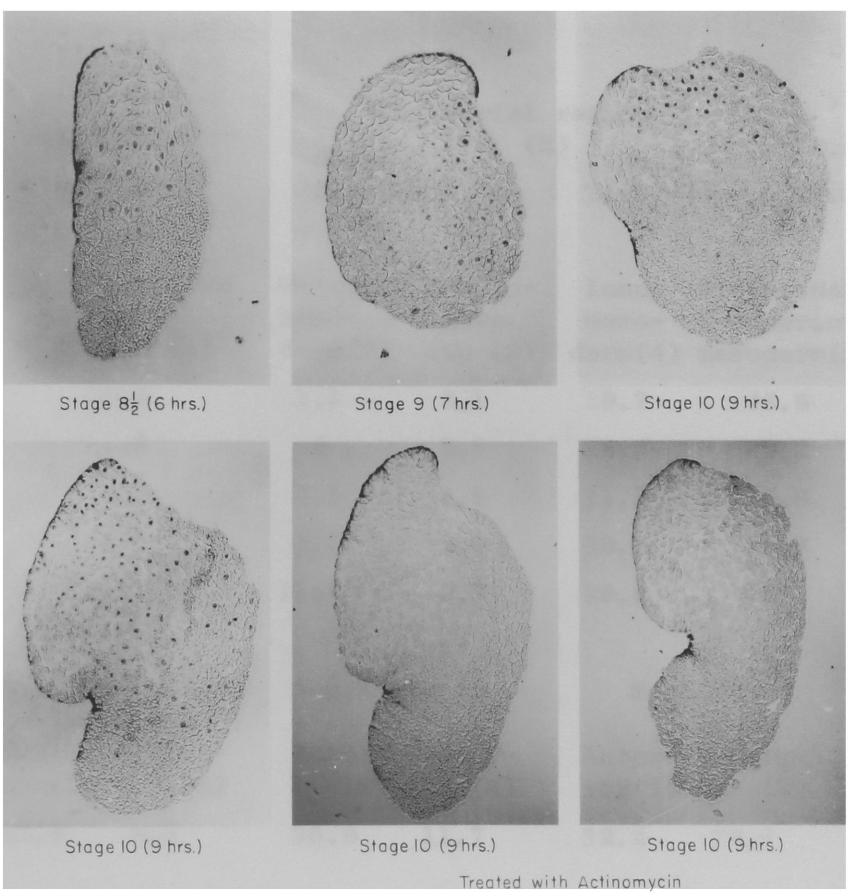

Stage 8½ (6 hrs.)
Stage 9 (7 hrs.)
Stage 10 (9 hrs.)
Stage 10 (9 hrs.)
Stage 10 (9 hrs.)
Stage 10 (9 hrs.)
Treated with Actinomycin

Figure 9

TABLE IIIa

GRAINS PER NUCLEUS REPRESENTING RNA SYNTHESIS IN VARIOUS REGIONS OF DORSAL EMBRYO HALVES FROM MORULA TO LATE GASTRULA STAGES

Stage	Presumptive ectoderm	Equatorial region		Presumptive endoderm
		Outer	Inner	
$6\frac{1}{2}$	.1 (1)			.1 (2)
7	1.5 (1)			0 (2)
8	0 (1)			.7 (2)
$8\frac{1}{2}$	1.8 (1)	17.3 (2)		10.5 (3)
9	6.5 (1)	2.4 (2)	21.9 (3)	23.2 (4)

	Presumptive neural tissue (1)	Outer-meso-derm(2)	Blasto-pore lip (3)	Inner meso-derm(4)	Prechordal & anterior mesoderm(5)	Endo-derm (6)
10	10.6	1.1	2.1	19.7	46.9	25.1
$10\frac{1}{2}$	.6	0	2.5	16.2	41.2	32.3
11	4.5	3.1	5.8	11.5	26.0	28.0
$11\frac{1}{2}$	15.5	1.8	3.9	19.2	33.7	23.5
12	18.1	13.2	10.2	29.8	34.7	13.2

	Epithelial layer		Neural layer		Mesoderm		Endo-derm
	Anter-ior(1)	Poster-ior (5)	Anter-ior(2)	Poster-ior (6)	Anter-ior(3)	Poster-ior (7)	(4)
13	13.4	6.3	38.0	11.7	32.4	19.6	24.1

The data represents a combination of four experiments. Two experiments were averaged for stages $8\frac{1}{2}$, 9 and 11. One experiment was performed for each of the other stages. Numbers in parentheses refer to map areas in Fig. 6. The specific activity of the uridine-H^3 precursor was 4.0 mC/μM and its concentration 0.06 μM/ml in all experiments. The autoradiographs of one experiment were exposed for 5 days, and the grain counts recalculated for an exposure of 7 days. All other autoradiographs were exposed for 7 days. The average standard error of the mean was 8%.

TABLE IIIb

GRAINS PER NUCLEUS REPRESENTING RNA SYNTHESIS
IN VARIOUS REGIONS OF DORSAL EMBRYO HALVES IN BLASTULA STAGES

Stage	Presumptive ectoderm	Equatorial region		Presumptive endoderm
7	2.7 (1)			2.2 (2)
		Equatorial region		
8	1.0 (1)	6.0 (2)		6.7 (3)
		Outer	Inner	
9	1.0 (1)	.4 (2)	12.7 (3)	10.2 (4)

The data represents an experiment using uridine-H^3 precursor of specific activity 17.4 mC/μM and concentration 0.015 μM/ml. Numbers in parentheses refer to map areas in Fig. 6.

released from cytolysing cells. However, the effect was not studied further and grain counts in this region were not included in the results.

The data of Table IV demonstrates a similar activation between stages 8 and 9 of presumptive endoderm and mesoderm in ventral halves. Again the ectoderm is definitively activated between stages 11 and 13.

The effect of actinomycin D on nuclear RNA synthesis in dorsal halves is shown in Table V and Fig. 9. Negligible RNase sensitive grains appear in the nuclei of the presumptive mesoderm and endoderm when actinomycin is present during the incubation.

The permeability of the dorsal halves to the precursor was demonstrated in the preceding section. In addition, the labeling of endodermal and mesodermal nuclei was relatively constant throughout the active region.

Discussion

The experiments of this section have demonstrated a widespread and sudden gene activation of blastula cells of X. laevis. Nuclear RNA synthesis begins within a one hour period between stage 8 and $8\frac{1}{2}$ in the presumptive endoderm and mesoderm. Significant activity in the cells of the preceding morula and early blastula stages was not detected, although a low rate of synthesis is evident when labeled RNA is extracted (see Section V). During stage 8 nuclear grain counts jumped at least twenty- to thirty-fold. Subsequent increases at later stages were relatively small.

The activation in the equatorial region precedes by approximately two hours the beginning of the inrolling of mesodermal cells. This two hour period before gastrulation coincides in time with the expected period of determination of these cells. However, mesoderm cells which are originally on the surface of the embryo do not become activated until they have actually moved into the interior. Although the process of gastrulation is dependent on nuclear activity, it is not known whether the movement of the exterior cells themselves is dependent on accompanying RNA synthesis. Possibly it is caused by the movement of more interior cells or it may be accompanied by a very low level of RNA synthesis not detectable by this method.

The endoderm is determined relatively early and participates in the morphogenic movements of gastrulation (Nicholas, 1945) although its role is not usually emphasized. Since the nuclei of endodermal cells are 15-25% larger in

TABLE IV

GRAINS PER NUCLEUS REPRESENTING RNA SYNTHESIS IN VARIOUS REGIONS OF VENTRAL EMBRYO HALVES FROM BLASTULA TO LATE GASTRULA STAGES

Stage	Presumptive ectoderm			Presumptive endoderm
7	.4 (3)	Inner equatorial region		.5 (4)
$8\frac{1}{2}$	.8 (4)	10.4 (5)		9.3 (6)
9	7.6 (5)	12.3 (6)		21.3 (7)
11	2.0 (7)	11.5 (8)		16.4 (9)
	Outer layer of ectoderm	Sensorial layer of ectoderm	Mesoderm	Endoderm
13	9.0 (8)	21.0 (9)	32.3 (10)	42.4 (11)

Data from two experiments using the same uridine-H^3 precursor as for Table IIIa were combined. The numbers in parentheses refer to the map areas of Fig. 6.

TABLE V

GRAINS PER NUCLEUS REPRESENTING RNA SYNTHESIS IN CONTROL AND ACTINOMYCIN D-TREATED STAGE 10 DORSAL HALVES

	Inner presumptive mesoderm	Presumptive endoderm
Control	17.5	24.7
Actinomycin D-treated	.3	.7

Samples of dorsal halves were taken from those incubated for the experiment of Fig. 18 in Section V. Stage 10 dorsal halves were pre-incubated in 90γ/ml of actinomycin D in Solution X, and then incubated for one hour in the presence of uridine-H^3 of specific activity 5.0 mC/μM and concentration of 0.1 μM/ml and 90γ/ml of actinomycin D. Autoradiographs were exposed for two days.

diameter than those of the presumptive mesoderm, the relative grain count per spherical nucleus is actually higher by this amount in the endoderm than the counts of sections indicate. Tencer (1958) has found that endodermal nuclei are as active in the incorporation of $C^{14}O_2$ as the nuclei of other regions. The coincidence in time of the initiation of nuclear RNA synthesis in presumptive endoderm and mesoderm suggests a generalized activating factor. However, at the level of the genes, the process must be specific and varied from cell to cell.

The fluctuation in activity of the ectoderm until the mid gastrula stage corresponds with the lability of its determination in the late blastula and early gastrula. Unlike mesoderm and endoderm, the patterns of genetic activity initiated in the ectoderm depend on its environment. However, in isolation it is able to differentiate along only two main paths, neural or epidermal. Since this degree of determination is not accompanied by detectable RNA synthesis, it must depend on localization of cytoplasmic factors. The actual determination of neural and epidermal cell type is accompanied by nuclear activity at the mid gastrula stage.

The RNA synthesis of dorsal and ventral nuclei is similar in agreement with the experiments of Friedberg and Eakin (1949) and Flickinger (1954) on the activity of dorsal and ventral explants. This result suggests that at least in X. laevis embryos the progressive determination of cells occurs at an equal rate on all sides of the embryo. Our photographs of median sections of embryos demonstrate that the inward migration of cells occurs at approximately the same time on the dorsal and ventral sides, although it is not as extensive on the ventral side.

In spite of the relatively fast progress of gastrulation, we have not been able to detect RNA label in the cytoplasm after a one hour incubation. Apparently newly synthesized RNA is not immediately exported to the cytoplasm. Moreover, since in the late blastula the average division time is about one hour, some of the labeled nuclei observed must have retained newly synthesized RNA through cellular division. A similar lack of transfer to the cytoplasm of high molecular weight RNA labeled over a two hour period in fish gastrulae was observed by Belitsina et al (1964). This phenomenon deserves further study with longer labeling periods, pulse-chase experiments, and biochemical analysis of fractionated embryos.

In order to correlate the results of the autoradiographic experiments with the results of Section III the number of cells per embryo was determined up to

the late gastrula stage. Cell counts were performed on suspensions of dissociated embryos at stage $8\frac{1}{2}$, 9, $9\frac{1}{2}$, 10 and 12. The cell number of stages 1 through $6\frac{1}{2}$ is known. A logarithmic plot of the number of cells per embryo is presented in Fig. 10. The value at stages 7 to 8 can be confidently determined by interpolation. The exponential increase in the number of cells per embryo continues from cleavage through the period of activation at stage 8 to 9. When the RNA synthesis per cell is calculated using the data of Figs. 4 and 10, it is found that the activity is relatively constant from the late blastula to the late gastrula stage in agreement with the autoradiographic studies. The rapid increase in the rate of total acid insoluble RNA synthesis per embryo half during the blastula and gastrula stages seen in Fig. 4 is therefore due mainly to multiplication of cell nuclei.

Other workers have attempted to describe the synthetic activity of various parts of the embryo by measuring incorporation of labeled precursors into total acid insoluble RNA in explants of different regions. These experiments have already been reviewed and unfortunately the results cannot be accurately converted to a per cell basis for comparison with the present results.

No autoradiographic studies have previously been reported which present quantitative data on the synthesis of RNA around the beginning of gastrulation. The present work clearly defines the time and region of gene activation.

Summary

Nuclear RNA synthesis commences in the presumptive endoderm and mesoderm of the mid to late blastula, preceding the morphogenic movements of gastrulation. The activity per nucleus remains relatively constant throughout gastrulation. By the mid gastrula RNA synthesis commences in the presumptive ectoderm. The pattern of activity is similar in ventral halves. When actinomycin D is present in the medium, no RNA synthesis can be detected in early gastrulae by this method.

V. EXTRACTION AND ANALYSIS OF LABELED RNA FROM *XENOPUS LAEVIS* EMBRYOS

Introduction

In order to describe the gene activation process more fully, experiments were undertaken to characterize the type of RNA synthesized shortly after activation. The autoradiographic studies demonstrated that widespread synthesis of nuclear RNA begins in the late blastula stage. However, the autoradiographic method cannot distinguish between the synthesis of transfer RNA, ribosomal RNA, and messenger RNA. Since genetic activity is required for cell differentiation, and cell deter-

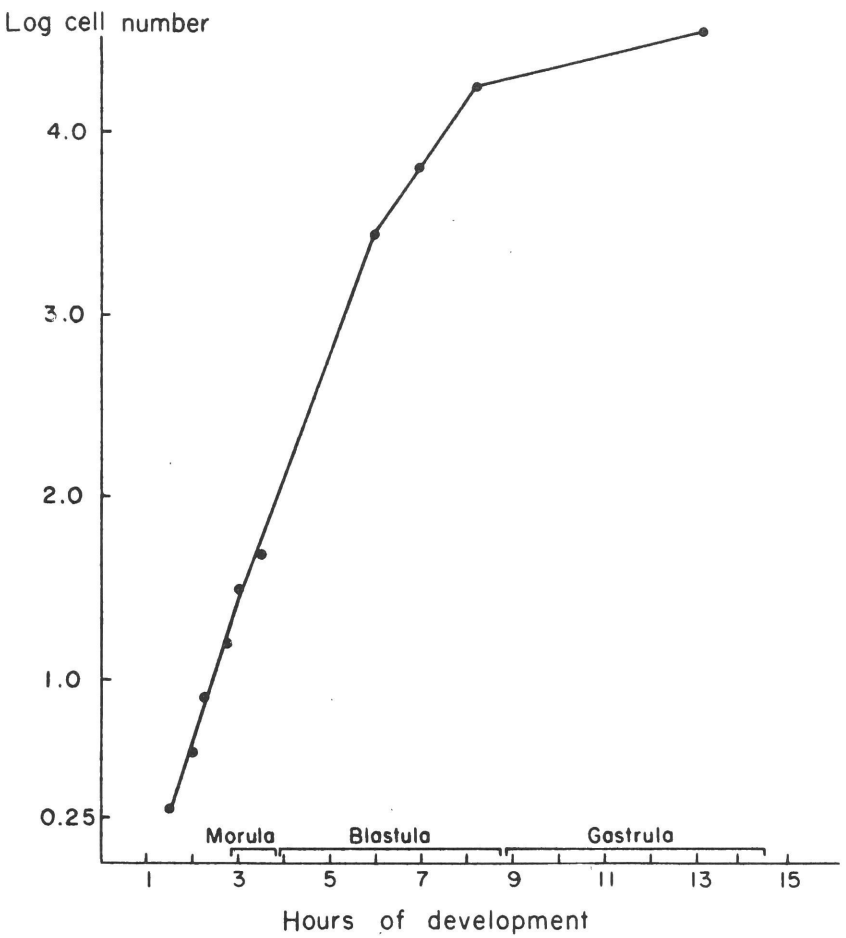


Fig. 10. Log plot of number of cells per embryo at different stages of development.

mination occurs in the late blastula stage, it would be expected that at least some messenger RNA is produced at this stage. Brown and Littna (1964) have demonstrated that a labeled RNA of heterogeneous S value appears during cleavage and blastula stages of X. laevis. However, a large proportion of the radioactivity was found in the region of 4S transfer RNA after centrifugation in a sucrose gradient. Since the distribution of P^{32} among the bases of this peak was similar to the base composition of transfer RNA, these workers concluded that transfer RNA is synthesized de novo during cleavage more rapidly than any other RNA fraction. Ribosomal RNA synthesis was first detected at the gastrula stage, accompanied by continuing synthesis of heterogeneous RNA of base composition distinct from ribosomal RNA and approaching that of DNA.

We have confirmed the synthesis of a messenger RNA-like fraction during the late blastula and early gastrula stages. In order to study the labeling of transfer RNA more accurately we have used Sephadex G-100 columns to separate transfer RNA from higher and lower molecular weight RNA's as described by Schleich and Goldstein (1964). Contrary to the conclusion of Brown and Littna (1964) we found that the synthesis of transfer RNA is insignificant at the early blastula stage, and accounts for only 30% of the incorporation of uridine-H^3 at the early gastrula stage.

In addition in experiments with the specific inhibitor of DNA-dependent RNA synthesis, actinomycin D, performed on the dorsal half preparation, we obtained a complete inhibition of the synthesis of transfer RNA and RNA's of higher molecular weight. Finally activation in the late blastula was again demonstrated by the appearance of increasing amounts of label in heterogeneous RNA between the early blastula and early gastrula stages.

Methods

Dorsal halves labeled with uridine-H^3 were prepared as described in General Methods. At the end of one hour incubation they were transferred to a Tenbroeck all-glass homogenizer. Whole dejelled unlabeled embryos were sometimes added as carrier at this stage. The preparation was frozen overnight at -70°C.

Phenol extraction of embryos was performed essentially according to Brown and Littna (1964). Groups of 20 to 40 labeled embryo halves were homogenized in 1.5 ml of 0.1 M sodium acetate pH 5 containing 4 γ/ml of polyvinyl sulfate (an effective RNase inhibitor). The homogenate was made up to about 3 ml per 100 halves and two 0.1 ml samples were taken for determination of counts in the whole homogenate. 8% sodium lauryl sulfate was added to a final concentration of 0.5%,

followed by an equal volume of water saturated cold phenol. The mixture was vigorously stirred at 0° to 4°C for 30 minutes and centrifuged for 10 minutes at 10,000 RPM in the Spinco SW 39 rotor. The first interface was saved for determination of unextracted counts, and the water supernatant was reextracted as above with phenol. To the second water supernatant were added 0.1 vol of 1 M NaCl and 2.5 vol of cold 95% ethanol, and it was allowed to stand at -20°C for 45 minutes. The precipitated RNA was collected by centrifugation at 40,000 RPM in the Spinco #40 rotor for 30 minutes. The precipitate was dissolved in 0.01 M NaAc, pH 5, containing 1 γ/ml of polyvinyl sulfate, and the RNA precipitated with 0.1 vol of 1 M NaCl and two vol of cold 100% ethanol. The precipitate was collected and redissolved in 0.5 ml of 0.01 M NaAc pH 5 containing 1 γ/ml of polyvinyl sulfate per 40 to 80 embryo halves. 80 to 90% of the RNase sensitive counts were extracted by this method.

Linear sucrose gradients varied from 5 to 20% sucrose in 0.01 M NaAc pH 5. Addition of polyvinyl sulfate to the gradient had no effect.

Sephadex G-100 columns (0.9 x 35 cm) were eluted with 1 M NaCl (Schleich and Goldstein, 1964). Yeast transfer RNA (obtained from Mann Research Labs, N.Y.) was usually added to the embryo RNA solutions as a marker. 0.5 ml fractions were collected, the optical density determined, and aliquots taken for analysis of total counts. In a few cases acid precipitable counts were also measured. Yeast bulk RNA was obtained from Worthington Co., N.J.

Base ratio analyses were performed on RNA labeled by injection of neutralized disodium (P^{32}) phosphate into the female about 15 hours before laying began. The embryos developed in spring water containing 0.1 mg/ml of both penicillin and streptomycin. The phenol-extracted RNA was subjected to alkaline hydrolysis in 0.3 N KOH at 37°C for 18 hours. The hydrolysate was neutralized with perchloric acid and the precipitate removed by centrifugation in the cold. The four 2'3'-ribonucleotides were separated by electrophoresis on cellulose acetate strips (Sepraphore, Gelman Instrument Co., Michigan) in pH 3.42, 0.04 M citrate buffer. The bands were cut out, dissolved in Bray's solution, and the radioactivity determined in a liquid scintillation spectrometer. The distribution of tritium among the four ribonucleotides after labeling of RNA with uridine-H^3, adenosine-H^3, or cytidine-H^3 was determined in the same manner.

Results

Sephadex G-100 effectively separates transfer RNA from lower and higher

molecular weight components. Sephadex G-100 excludes proteins and polysaccharides of molecular weight greater than approximately 100,000. RNA molecules of slightly lower molecular weight are excluded since they are highly negatively charged. Transfer RNA being of molecular weight about 30,000 is only partially excluded (Comb and Brown, 1964; Schleich and Goldstein, 1964; Dirheimer and Ebel, 1964), and under the conditions used in our experiments has a K_D of about 0.67. Low molecular weight nucleotides are hardly excluded with K_D's approaching 1, while nucleosides may actually be absorbed (Hohn and Pollman, 1963). Our columns were eluted with 1 M NaCl; however, the use of 0.2 M NaCl did not change the elution pattern significantly. A trial separation of ribosomal and transfer RNA is shown in Fig. 11. The P^{32}-labeled ribosomal RNA appears in a sharp leading peak, well separated from the broader transfer RNA peak.

The RNA synthesized shortly after activation (3 hours) was thoroughly analyzed in stage 10 dorsal halves. This stage was chosen instead of stage 9 because of the higher level of RNA synthesis and because of the ease with which dorsal and ventral halves can be distinguished at the beginning of gastrulation.

A typical elution pattern from Sephadex G-25 of stage 10 uridine-H^3 labeled RNA is shown in Fig. 12. Sephadex G-25 excludes molecules of greater than approximately 5,000 molecular weight. A small amount of slightly lower molecular weight labeled material is evident. In further experiments, preliminary purification of labeled RNA on Sephadex G-25 was unnecessary since an effective separation of low molecular weight material from transfer RNA was obtained on Sephadex G-100.

The elution pattern of stage 10 labeled RNA from Sephadex G-100 is presented in Fig. 13. Three main radioactive peaks are observed in the curve of total radioactivity; the first sharp peak represents excluded high molecular weight RNA and coincides with the optical density peak of ribosomal RNA, the second low broad peak coincides with the optical density peak of the transfer RNA marker; and the third large peak represents low molecular weight acid soluble polynucleotides. Of the acid insoluble RNA, the transfer RNA peak includes only 29% of the counts, an average value from six experiments.

The sedimentation properties of the labeled RNA in the high molecular weight peak were examined by centrifugation of total labeled RNA in a sucrose gradient (Fig. 14). Here the sharp ribosomal RNA optical density peak seen on the Sephadex G-100 column is spread out and resolved into its two components. No significant breakdown of the bulk RNA has occurred during the extraction. The high molecular weight radioactivity, however, shows no correspondence with the optical density

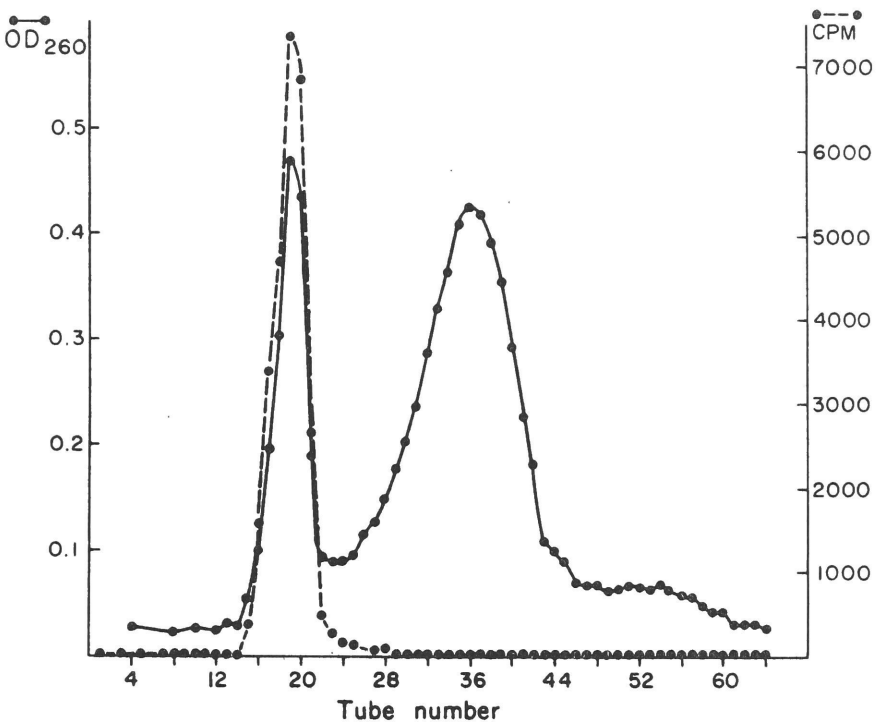


Fig. 11. Separation of P^{32}-labeled Xenopus laevis oocyte ribosomal RNA and yeast transfer RNA on a Sephadex G-100 column. P^{32}-labeled oocyte RNA was prepared by phenol extraction of oocytes after injection of P^{32} into the female, and purified on a Sephadex G-25 column. Unlabeled yeast transfer RNA was obtained commercially.

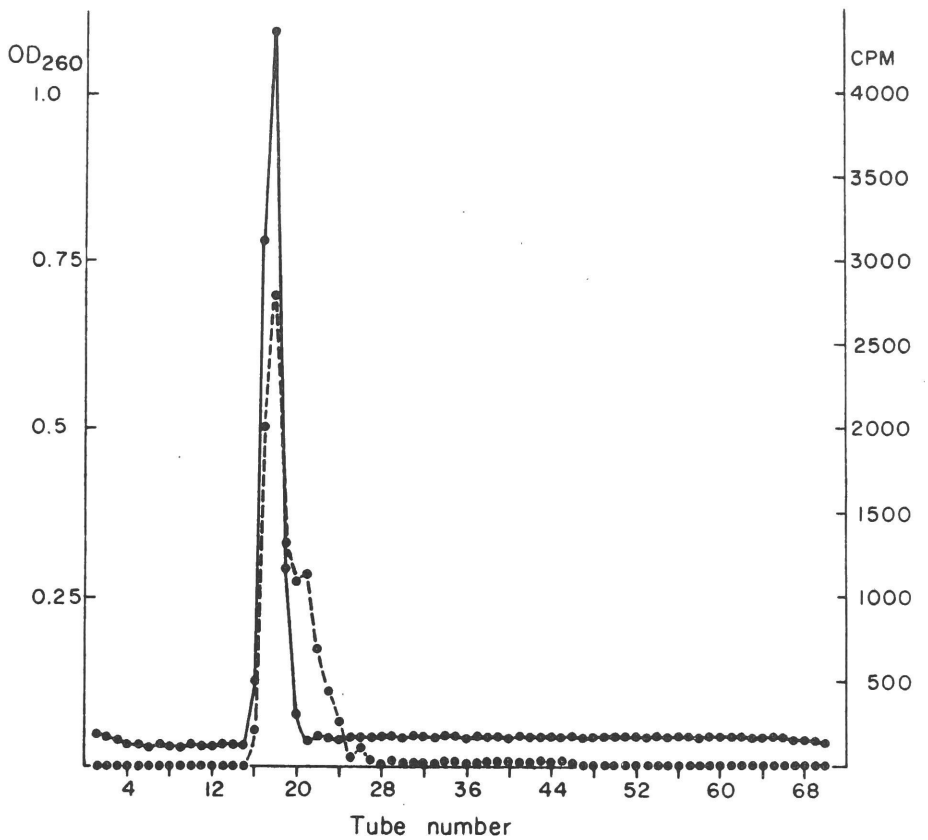


Fig. 12. Elution pattern of uridine-H^3 labeled embryo RNA on a Sephadex G-25 column. Stage 10 embryo RNA was eluted from Sephadex G-25 with water; fractions were collected and analyzed as described in Methods for the G-100 column.

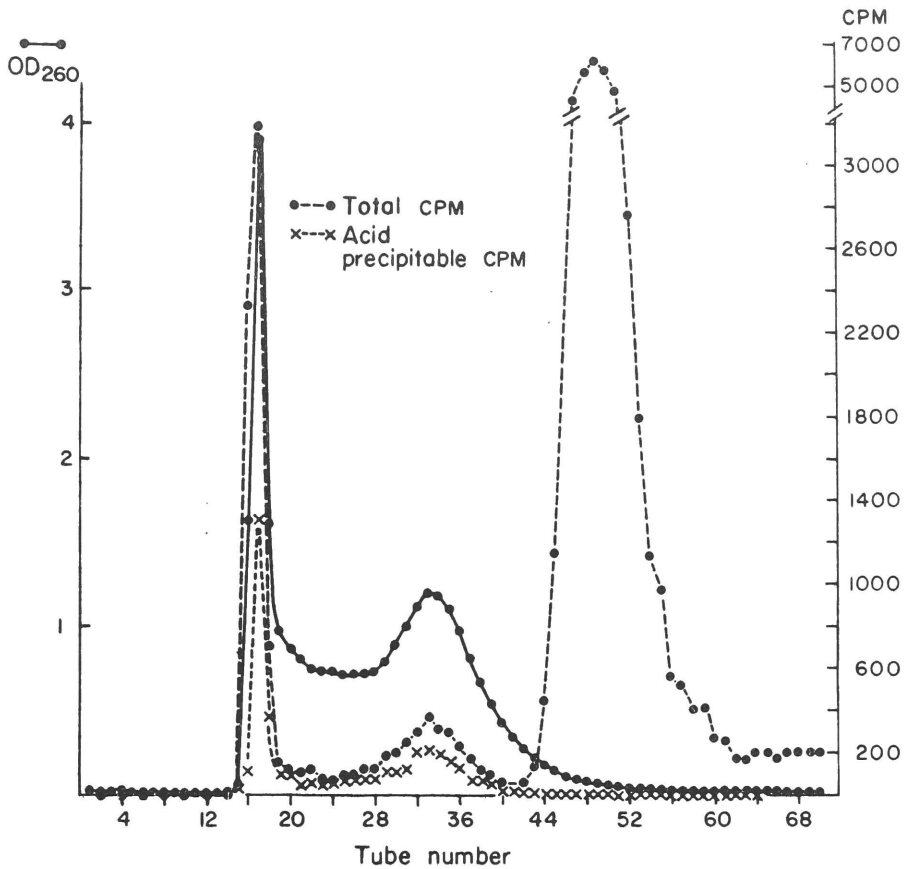


Fig. 13. Elution pattern of uridine-H^3 labeled embryo RNA on a Sephadex G-100 column; total and acid precipitable counts. Labeled RNA was prepared from stage 10 dorsal halves. Bulk yeast RNA was added before the first ethanol precipitation, resulting in a relatively poor separation in the optical density curve between high molecular weight and yeast transfer RNA marker. Acid insoluble counts were analyzed by addition of 0.13 mg of yeast bulk RNA carrier to each tube, precipitation in cold 5% TCA, and filtration on millipore filters. The filters were dissolved in Bray's solution for counting. In this experiment transfer RNA label was considered to lie between tubes 27 and 40.

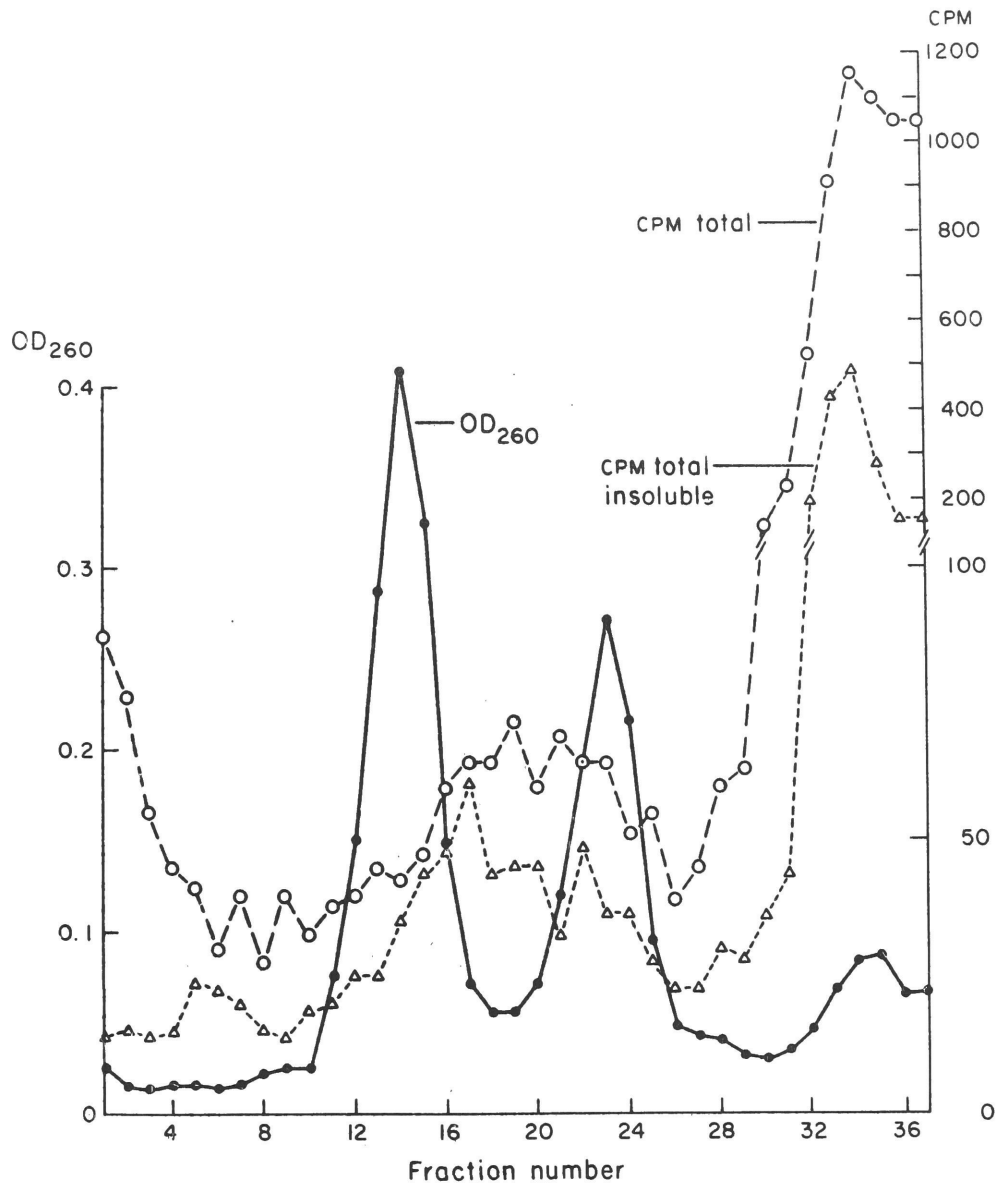


Fig. 14. Sedimentation pattern in a sucrose gradient of uridine-H^3 labeled embryo RNA, total and acid insoluble counts. Labeled RNA was prepared from stage 10 dorsal halves. Acid insoluble counts were analyzed as in the experiment of Fig. 13.

peaks, and is distributed heterogeneously throughout the gradient. No ribosomal RNA synthesis is detectable at this stage. The transfer RNA and acid soluble peaks overlap near the top of the gradient.

RNase effectively breaks down all high molecular weight and transfer RNA as shown by filtration on Sephadex G-100 (Fig. 15) or sucrose gradient centrifugation (Fig. 16) of RNase treated labeled embryo RNA. The absence of DNA labeling under our conditions is apparent.

When stage 10 dorsal halves are incubated in adenosine-H^3 or cytidine-H^3 for one hour, rather than uridine-H^3, and the RNA extracted as usual, a similar elution pattern from Sephadex G-100 is obtained as shown in Fig. 17. In these cases, however, a higher percentage of the acid insoluble counts appears in transfer RNA, being 60% and 53% for adenosine and cytidine, respectively. Presumably, this difference is due to -C-C-A end group turnover of transfer RNA.

To demonstrate that uridine-H^3, adenosine-H^3, and cytidine-H^3 were indeed incorporated into polyribonucleotides, the two excluded peaks of Fig. 17 and a similar uridine-H^3 labeled peak were collected and subjected to alkaline hydrolysis. The distribution of counts among the four 2'-3'-ribonucleotides is shown in Table VI. Essentially all the counts were recovered, predominantly as the corresponding ribonucleotides. Only 3.5% of the uridine-H^3 counts appear as CMP, excluding the possibility of significant end group labeling of transfer RNA with this precursor.

Several properties of the gene products of stage 10 embryos have been established. Approximately 70% of the newly synthesized RNA is material of molecular weight greater than that of transfer RNA and sediments heterogeneously in a sucrose gradient. Ribosomal RNA synthesis is not detectable. The labeled high molecular weight RNA and transfer RNA are entirely digested by RNase. Uridine-H^3, adenosine-H^3, and cytidine-H^3 are incorporated into both types of RNA, and can be recovered from the alkaline hydrolysate of the high molecular weight material as the appropriate 2'-3'-ribonucleotides.

To characterize further the nuclear RNA synthesis of stage 10 embryos experiments were undertaken with the specific inhibitor of DNA-dependent RNA synthesis, actinomycin D. It was not known whether long preincubation periods would be necessary in order to allow penetration of this large molecule. A preliminary experiment was performed to measure the effect of actinomycin D on total acid insoluble RNA synthesis by the methods of Section III. The results are presented in Table VII. Preincubation of 60 minutes did not increase the inhibition obtained

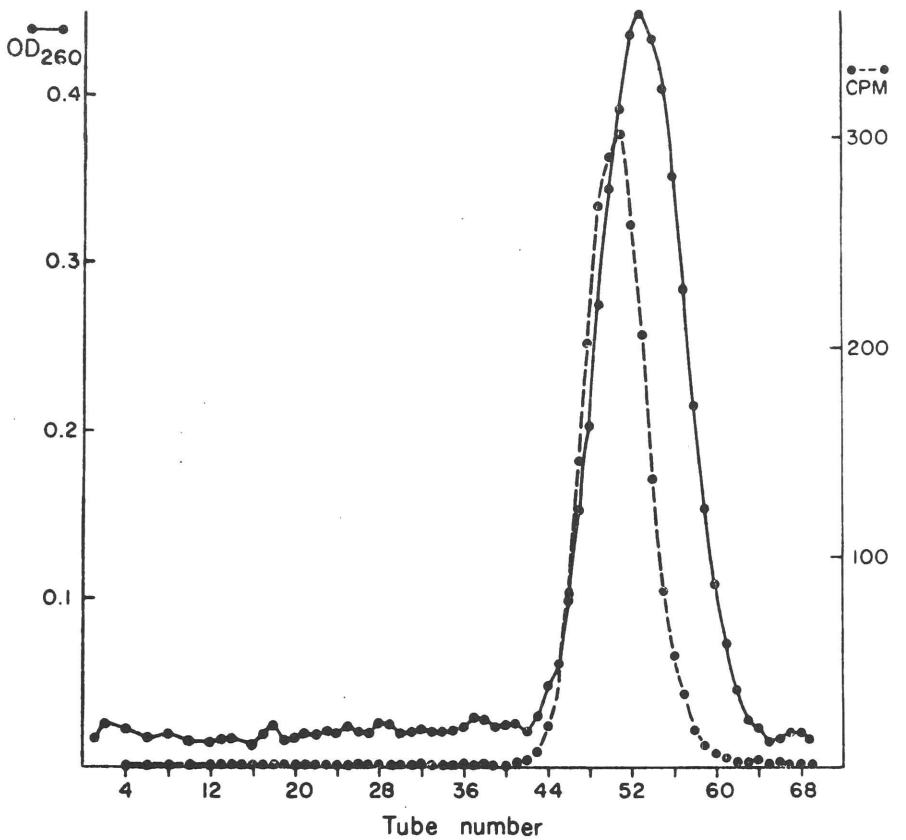


Fig. 15. Elution pattern of RNase treated uridine-H^3 labeled embryo RNA on a Sephadex G-100 column. Labeled stage 10 RNA was treated with pancreatic RNase, 50 γ/ml in 0.1 M Tris pH 7.6 for 30 minutes at 29°C.

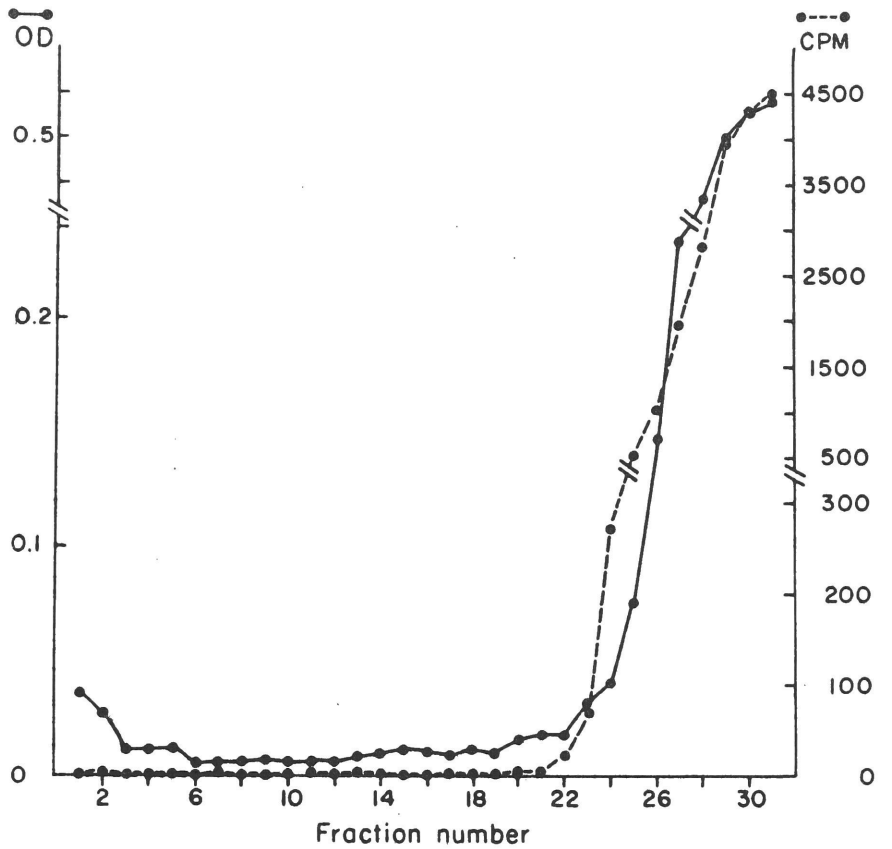


Fig. 16. Sedimentation pattern in a sucrose gradient of uridine-H^3 labeled embryo RNA treated with RNase. Labeled stage 10 RNA was treated with pancreatic RNase, 30 γ/ml in 0.1 M Tris pH 7.6 for 30 minutes at 29°C.

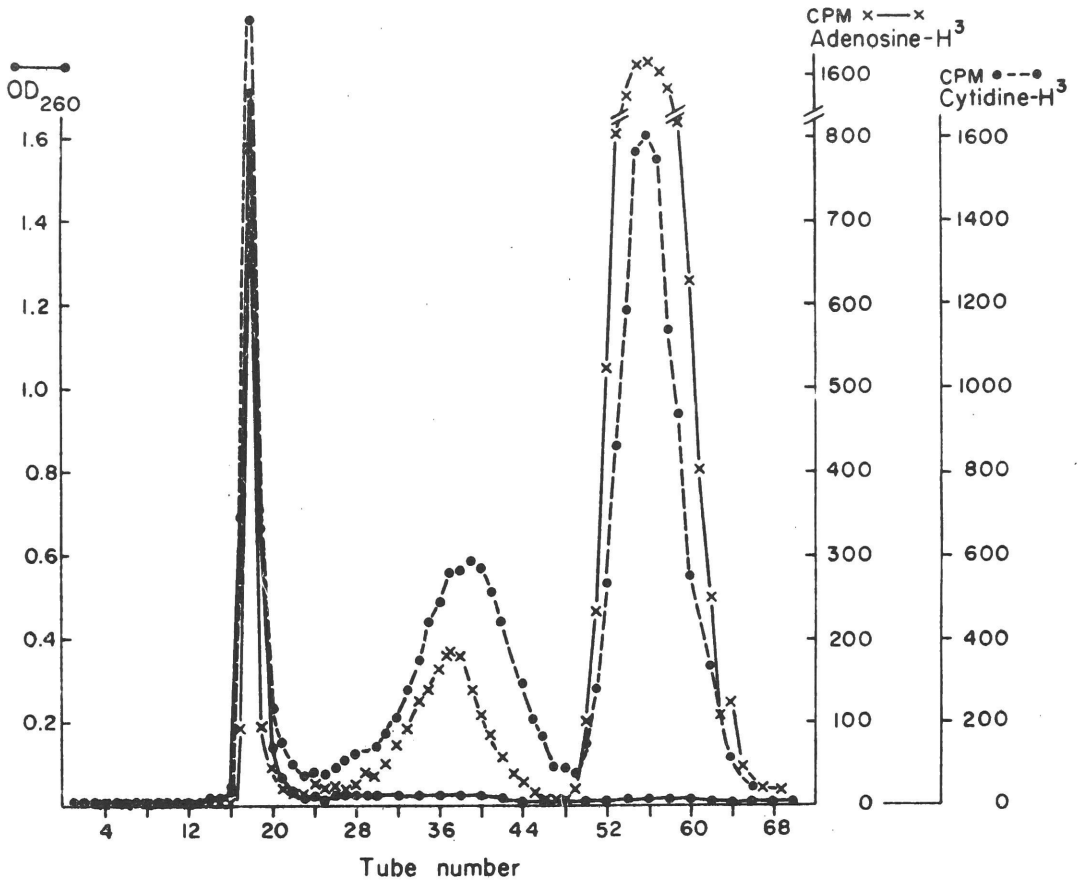


Fig. 17. Elution pattern of adenosine-H^3 and cytidine-H^3 labeled embryo RNA on a Sephadex G-100 column. Stage 10 dorsal halves were incubated as usual except that uridine-H^3 was replaced by adenosine-H^3, 4.0 mC/μM, 0.08 μM/ml (Schwarz Bio Research) or cytidine-H^3, 3.5 mC/μM, 0.08 μM/ml (New England Nuclear Corp., Mass). No yeast transfer RNA marker was added. The optical density pattern was similar in both preparations.

TABLE VI

RECOVERY OF TRITIATED NUCLEOTIDES FROM RNA SYNTHESIZED IN THE PRESENCE OF TRITIATED NUCLEOSIDES

Precursor	% CPM in U band	% CPM in G band	% CPM in C band	% CPM in A band
Adenosine-H^3	0	3	1	96
Cytidine-H^3	2	0	96	2
Uridine-H^3	89	7	4	0

The excluded peaks from the experiment of Fig. 14 and a similar peak obtained with uridine-H^3 as precursor were subjected to alkaline hydrolysis and the four 2'-3'-ribonucleotides determined as described in Methods. The data represents the average counts per minute obtained from four electrophoretic strips.

TABLE VII

INHIBITION OF RNA SYNTHESIS BY ACTINOMYCIN D

Preincubation Minutes	Concentration of actinomycin D, γ/ml	CPM for 10 dorsal halves	% inhibition by actinomycin D
10	0	4056	
10	10	1914	53
10	30	1746	57
60	0	3299	
60	10	1758	47
10	0	1253	
10	90	230	82

Total RNase sensitive counts were analyzed as described in Methods of Section III. The same concentration of actinomycin D was present throughout preincubation and the one hour incubation in the presence of uridine-H^3. Actinomycin D was a generous gift of Dr. E. Reich.

with a 10 minute preincubation. However, a high concentration, 90 γ/ml of actinomycin D, was necessary to block a large percentage of the incorporation of uridine-H^3. In subsequent experiments, a preincubation period of 10 minutes, and a concentration of 90 γ/ml were used.

A comparison between the elution patterns from Sephadex G-100 of RNA from control and actinomycin-treated dorsal halves is shown in Fig. 18. Both high molecular weight RNA and transfer RNA synthesis are over 98% inhibited in the presence of actinomycin D. (Incorporation into acid soluble polynucleotides is actually increased slightly). Sucrose gradient centrifugation of the same preparation is shown in Figs. 14 and 19. The absence of labeling of RNA of molecular weight higher than transfer RNA is confirmed. However, transfer RNA labeling cannot be distinguished from labeling of acid soluble polynucleotides in the gradient.

The base composition of stage 10 embryo RNA was also examined. An attempt was made to prepare P^{32}-labeled embryo RNA by incubation of dorsal halves in Solution X containing neutralized disodium (P^{32}) phosphate. The failure of this experiment is shown in Fig. 20. When both uridine-H^3 and labeled phosphate were present in the incubation medium, the extracted RNA gave the expected tritium pattern on Sephadex G-100; however, no P^{32} appeared in transfer RNA or the high molecular weight peak. P^{32}-labeled embryo RNA was therefore prepared according to the method of Brown and Littna (1964). Neutralized disodium (P^{32}) phosphate was injected into the female before laying. The P^{32}-labeled embryos were collected at stage 10, dissected and incubated as usual in Solution X containing uridine-H^3. Fig. 21 shows the elution pattern from Sephadex G-100 of the phenol extracted RNA. Relatively large amounts of P^{32} appear in the acid soluble and transfer RNA peaks, perhaps partly due to the synthesis of polyphosphate and to -C-C-A end group turnover. Significant labeling of the high molecular weight peak has also occurred. This peak was collected, and the distribution of P^{32} among the four 2'-3'-ribonucleotides determined. The results are shown in Table VIII. The nucleotide composition is distinct from that of ribosomal RNA, approaches that of adult liver DNA, and is similar to that found for the gastrula stage by Brown and Littna (1964) and Decroly et al (1964).

The properties of the high molecular weight RNA synthesized by stage 10 embryos suggest that it is messenger RNA. Its synthesis is inhibited by actinomycin D and the autoradiographic experiments described in Section IV have already shown that all detectable RNA synthesis is nuclear at this stage. Most important,

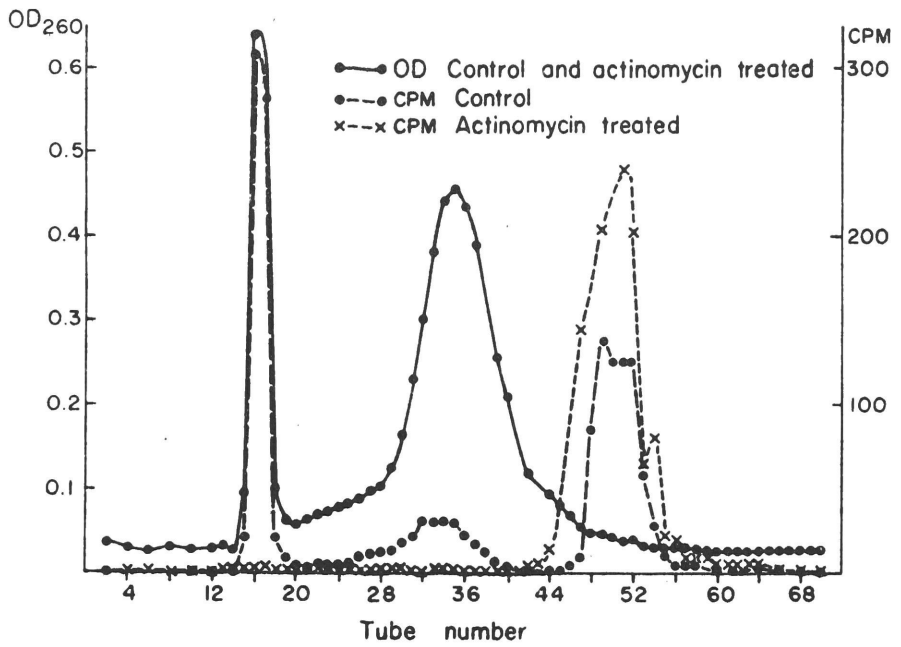


Fig. 18. Elution pattern of uridine-H^3 labeled embryo RNA on a Sephadex G-100 column; control and actinomycin-treated embryos. Stage 10 dorsal halves were preincubated for 10 minutes in 90 γ/ml of actinomycin D in Solution X. Uridine-H^3 was added and the incubation continued for one hour. The control consisted of a duplicate group of embryos incubated without actinomycin D. Whole unlabeled embryos were added as carrier before the extraction. The second optical density peak is due to yeast transfer RNA marker. The optical density pattern for the actinomycin D-treated group was similar to that of the control.

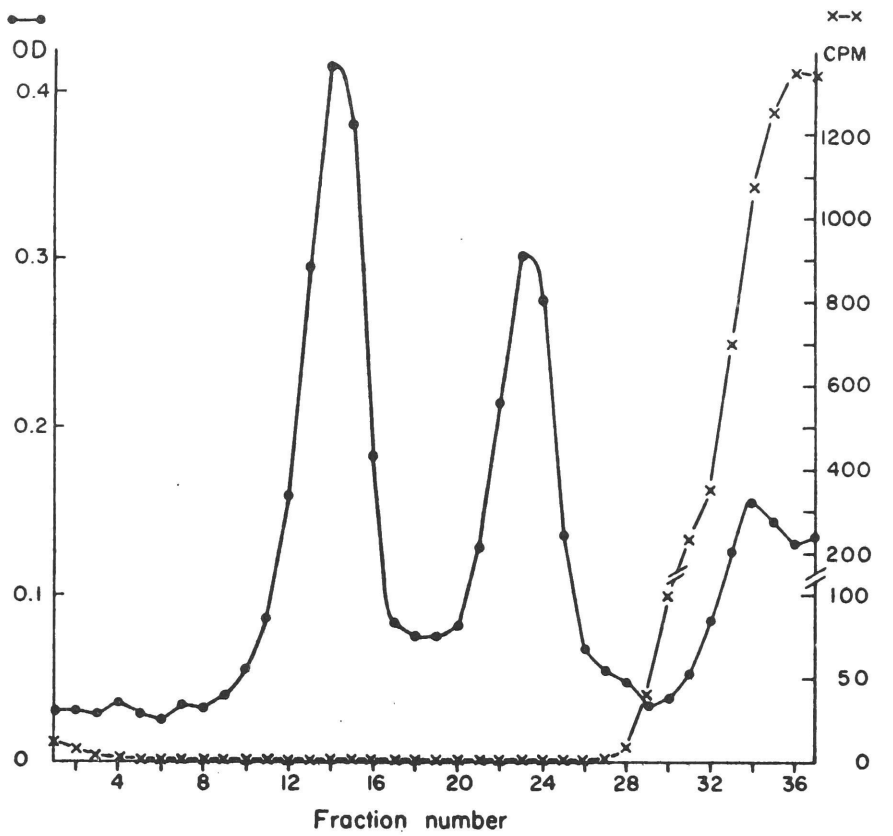


Fig. 19. Sedimentation pattern in a sucrose gradient of uridine-H^3 labeled RNA from embryos treated with actinomycin. Samples of the same RNA preparations described in Fig. 18 were applied to gradients. The control is shown in Fig. 14.

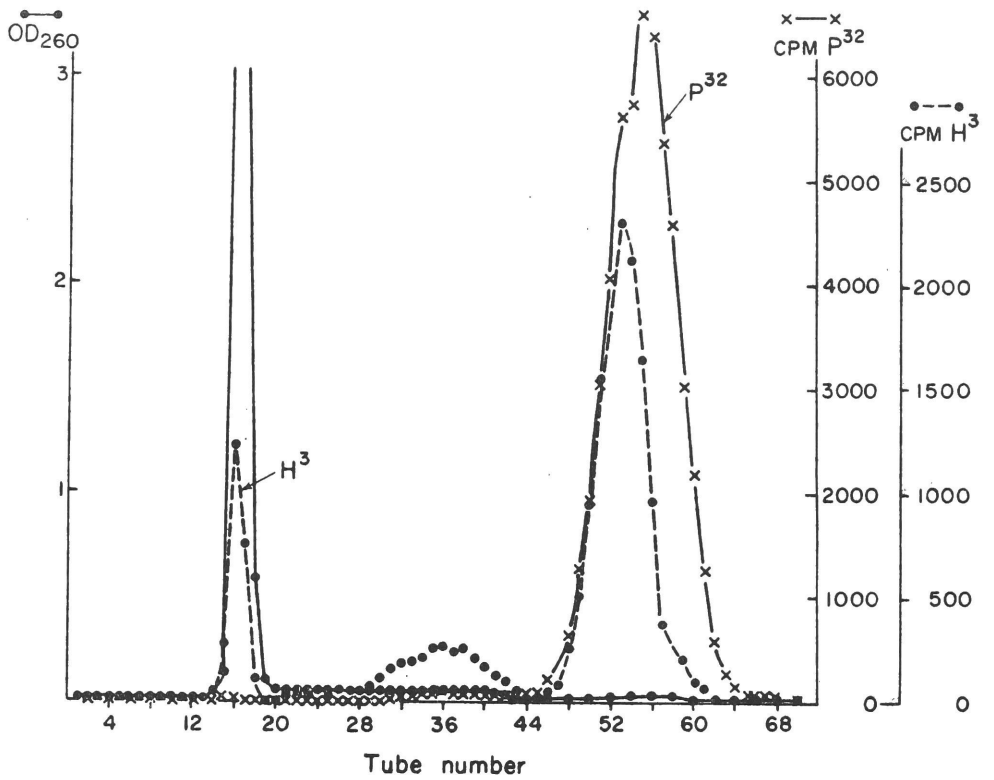


Fig. 20. Elution pattern of uridine-H^3 and P^{32}-labeled embryo RNA on a Sephadex G-100 column. Carrier-free $Na_2HP^{32}O_4$ from New England Nuclear Corp. was neutralized with HCl. Stage 10 dorsal halves were incubated in Solution X containing both 0.018 M labeled phosphate, 0.9 mC/ml, and uridine-H^3, 17.4 mC/μM, 0.012 μM/ml. RNA was extracted as usual. No yeast transfer RNA marker was added.

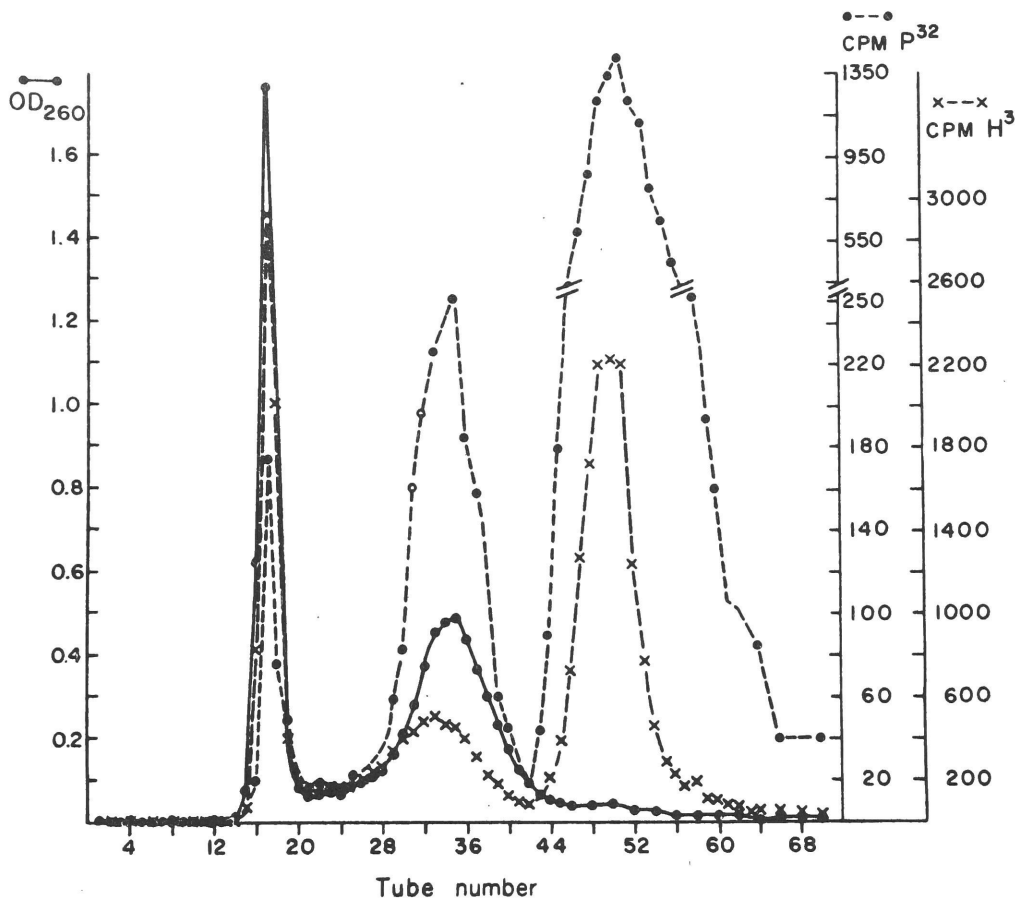


Fig. 21. Elution pattern of uridine-H^3 labeled RNA on a Sephadex G-100 column after injection of P^{32} into the female. P^{32}-labeled RNA was prepared as described in Methods: embryos labeled with P^{32} by injection of label into the female were collected at stage 10, dissected, and incubated as usual in uridine-H^3, and the RNA extracted as in Methods. Yeast transfer RNA was added to the final RNA solution.

TABLE VIII

NUCLEOTIDE COMPOSITION OF P^{32}-LABELED HIGH MOLECULAR WEIGHT RNA

Preparation	% G+C	Mole percent nucleotide			
		U (T)	G	C	A
G-100-excluded stage 10 RNA	50	25	30	20	25
Xenopus total ribosomal RNA (Davidson et al, 1964)	65	19	39	26	16
Xenopus DNA, liver (Dawid, 1965)	41	29	22	19	29

The nucleotide composition was determined as described in Methods

the high molecular weight labeled RNA sediments heterogeneously in a sucrose gradient, and its base composition is close to that of DNA.

The autoradiographic experiments had demonstrated a widespread gene activation on a per nucleus basis occurring between stages 8 and 9, mid to late blastula. In the experiments with extracted RNA a large increase in the synthesis of high molecular weight RNA excluded by Sephadex G-100 can be demonstrated in the same period of development between stages 7 and 10. Dorsal embryo halves from stages 7, 9 and 10 were incubated as usual in the presence of uridine-H^3, and the RNA extracted. The elution pattern from Sephadex G-100 of the three RNA preparations is shown in Fig. 22. Stage 7 embryos synthesize a small amount of high molecular weight RNA: only 13% of the acid insoluble label was in the region of transfer RNA and no peak of radioactivity appears in this region. A five-fold increase in the amount of newly synthesized high molecular weight RNA occurs in stage 9, and an additional two-fold increase at stage 10. 30% and 29% of the acid insoluble label appears under the transfer RNA peak respectively. Thus the abrupt gene activation occurring during stage 8 represents primarily the initiation of messenger-like RNA synthesis.

In all experiments a large proportion of the label was eluted from Sephadex G-100 in the third low molecular weight acid soluble peak. The exact nature of this material is unknown. The present experiments show that it can be labeled by uridine-H^3, adenosine-H^3, cytidine-H^3, and P^{32} (Figs. 13 and 17). It is mostly excluded by Sephadex G-25 (Fig. 12) and therefore its molecular weight must lie between 5,000 and 10,000. Its synthesis continues in the presence of actinomycin D and in the absence of synthesis of higher molecular weight RNA (Fig. 18). Thus it cannot be a breakdown product of high molecular weight RNA.

Discussion

The analysis of phenol-extracted RNA has provided information complementary to that obtained from the autoradiographic studies described in the preceding section. Nuclear activation occurs in large areas of the embryo between stage 8 and 9, mid to late blastula. The RNA synthesized in the initial gastrula stage shortly after activation is a true gene product as demonstrated by actinomycin D inhibition, and is approximately 70% messenger-like RNA and 30% transfer RNA.

The increased permeability of the dorsal half preparation has permitted the use of the highly specific precursor, uridine-5-H^3. Its insignificant incorporation into DNA, demonstrated in all three experimental sections, and its low rate

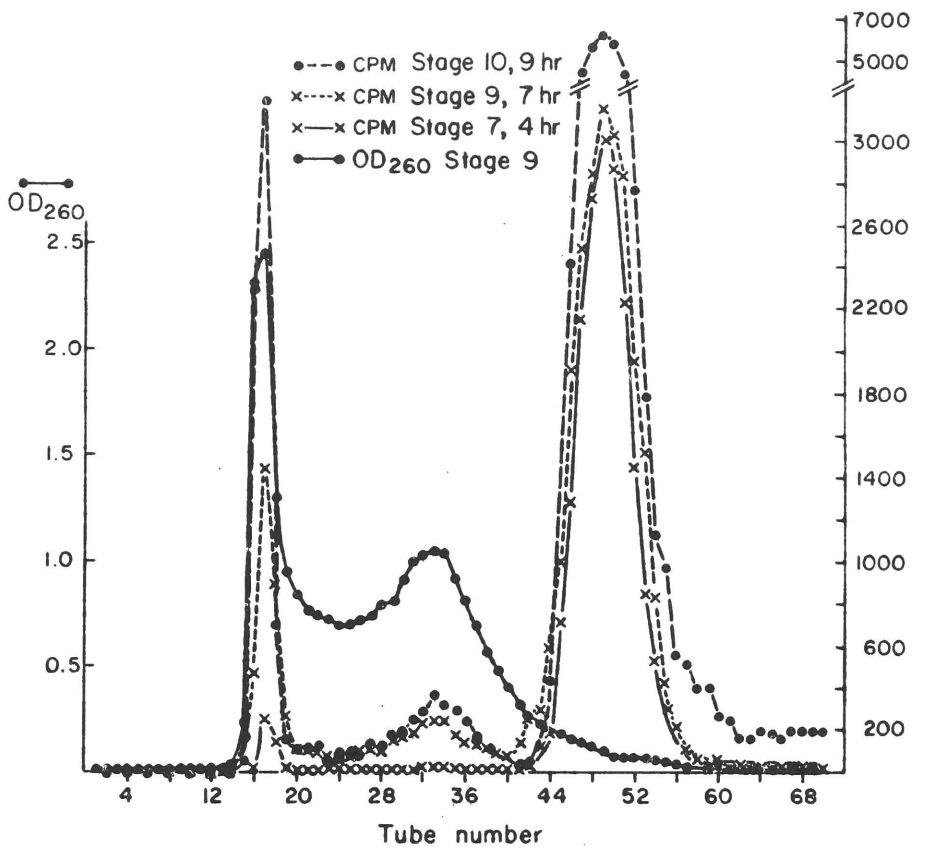


Fig. 22. Elution pattern of uridine-H^3 labeled RNA from stage 7, 9, and 10 embryos on a Sephadex G-100 column. The first optical density peak is primarily due to yeast bulk RNA added as carrier before the first ethanol precipitation. The second is due to yeast transfer RNA marker added to the final RNA solution. The optical density curve was similar for all three stages.

of conversion to cytidine which participates in end group turnover of transfer RNA, allows us to conclude that essentially all incorporation of uridine-H^3 into acid insoluble material represents RNA synthesis. Adenosine-H^3, cytidine-H^3, and P^{32} are all incorporated faster into DNA and transfer RNA than uridine-H^3.

Sephadex G-100 columns clearly separate uridine-H^3 labeled transfer RNA from lower molecular weight labeled material and have allowed us to analyze more precisely the synthesis of transfer RNA than has been possible before.

Within the resolution of the experiments of this section, activation of messenger-like RNA synthesis and transfer RNA synthesis occurs simultaneously. The relatively high labeling of material in the region of transfer RNA in sucrose gradients during cleavage and blastula stages observed by Brown and Littna (1964) must be due to the use of P^{32} injected into the female as precursor, -C-C-A end group turnover, and the presence of lower molecular weight labeled compounds. Under our conditions of short one hour labeling periods in the presence of uridine-5-H^3 no net synthesis of transfer RNA can be detected in stage 7 early blastulae, while a small amount of high molecular weight RNA is synthesized. Labeling of transfer RNA during cleavage and blastula stages of the sea urchin is probably entirely due to end group turnover (Glisin and Glisin, 1964; Comb et al, 1965; Gross et al, 1965). In the activated nuclei of the early gastrula transfer RNA synthesis represents only about 30% of the net RNA synthesis.

Even in stage 7 early blastulae, a low rate of synthesis of high molecular weight RNA was observed. This synthesis was not detected by autoradiography due to the short incubation and exposure times. A similar incorporation of label into a heterogeneous RNA through cleavage and blastulation of sea urchin embryos has also been reported (Gross, 1964; Gross et al, 1964; Wilt, 1964). Actinomycin sensitive nucleolar and non-nucleolar RNA synthesis occurs during cleavage of the mouse (Mintz, 1964).

The identification of the labeled RNA excluded by Sephadex G-100 as messenger-like RNA depends on its sedimentation characteristics and base composition. Although the base composition was determined on RNA accumulated between ovulation and stage 10, the data of Fig. 23 show that most of it was synthesized during stages 9 and 10. A similar fraction has been observed in sucrose gradients by Brown and Littna (1964). However, positive identification of messenger RNA must await demonstration of template activity.

A newly synthesized RNA with base composition approaching that of DNA has

been observed in sea urchin blastulae (Comb and Brown, 1964; Gross et al, 1965). A labeled RNA distinct from ribosomal RNA on methylated albumen columns has been extracted from fish embryos (Belitsina et al, 1963). The increase of label in this peak between the mid blastula and mid gastrula resembles that in the Sephadex G-100 excluded peak of Fig. 20.

Treatment of sea urchin and frog embryos with actinomycin D (Gross and Cousineau, 1964; Wallace and Elsdale, 1963; Brachet et al, 1964) has shown that RNA synthesis during cleavage and blastulation may be required for the initiation and morphogenic movements of gastrulation. Apparently there could be some delay between gene activity and the expression of gene activity. Evidence has recently been obtained that during embryonic development of chicks messenger RNA may be stored for several hours or days before its actual use in protein synthesis (Bell et al, 1965; Reeder and Bell, 1965; Scott and Bell, 1965; Wilt, 1965).

The quiescent nuclei in the presumptive endoderm and mesoderm of the early gastrula are completely activated within a two hour period between stages 8 and 9, mid to late blastula. The synthesis of high molecular weight heterogeneous RNA increases five fold during this period and continues to increase with cell number. The activation process accompanies cell determination in the late blastula. A majority of the RNA synthesized at this stage is messenger-like presumably to be translated into proteins which determine the specificity of cell type. The heterogeneity of the messenger RNA's observed probably represents heterogeneity of patterns of gene activity among different cells as well as the synthesis of a variety of messengers within one cell. The diversity of messages increases as cell multiplication continues.

The origin of differential gene activity among cells cannot lie in the identical chromosome sets. Clear examples of the cytoplasmic control of nuclear activity are well-known during the embryogenesis of nematodes and insects (Boveri, 1910; Hegner, 1911). A precise localization of cytoplasmic substances which control differentiation of tissues has been demonstrated in annelids, molluscs, and ascidians (Novikoff, 1938; Clement, 1962; Conklin, 1905). In the amphibian, a more diffuse but definite localization of cytoplasmic factors defines the three main presumptive regions of the blastula; ectoderm, mesoderm, and endoderm. We may then hypothesize that during the middle to late blastula stage of the amphibian, cytoplasmic substances interact with the nuclei resulting in the activation of specific genes. Progressive differentiation occurs through interaction between nuclear products and cytoplasm. A differentiated structure of the chromosomes

themselves is established which can maintain and control specific patterns of RNA synthesis without loss of genetic potential. The mechanism of this control remains one of the most challenging problems in the molecular biology of multicellular organisms.

Summary

Sephadex G-100 columns effectively separate high molecular weight RNA, transfer RNA, and low molecular weight acid soluble nucleotides. Uridine-H^3 precursor is incorporated almost completely into RNase snsitive material by stage 10 initial gastrulae, and does not participate in end group turnover of transfer RNA. Phenol-extracted acid insoluble RNA synthesized by stage 10 embryos is mostly excluded by Sephadex G-25. Its synthesis is completely inhibited by actinomycin D. It is composed of 70% high molecular weight RNA of heterogeneous sedimentation rate with base composition approaching that of DNA, and 30% transfer RNA. Uridine-H^3, adenosine-H^3, cytidine-H^3 and P^{32} are incorporated into both types of RNA, and can be recovered from the alkaline hydrolysate of the high molecular weight RNA as the appropriate 2'-3'-ribonucleotides. A marked activation of high molecular weight and transfer RNA synthesis occurs in the late blastula stage.

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