ACTION OF RNA ON THE DEVELOPMENT OF THE NEURAL TUBE OF NOTOCHORD-LESS CHICK BLASTODERMS CULTURED IN VITRO

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A THESIS

Submitted to the

AMERICAN UNIVERSITY OF BEIRUT

In partial fulfilment of

the requirements for the

degree of

MASTER OF SCIENCE
IN
BIOLOGY

May 1967

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ACTION OF RNA ON CHICK AXIS

AKRUK

ACKNOWLEDGMENTS

The author wishes to extend his sincere thanks to Dr. Joseph Butros, thesis advisor, for his continuous help, advice and suggestions during the course of this study. Thanks are extended to Drs. Levon Babikian, Joseph Powell and Samir Deeb, members of the supervisory committee, for their constructive criticisms and comments throughout this work.

The author is especially indebted to Drs. Joseph Butros and Samir Deeb for their donation of pure RNA samples.

AN ABSTRACT OF THE THESIS OF

Samir R. Akruk for the M.S. degree in Biology

Title: Action of RNA on the development of the neural tube of notochord-less chick blastoderms cultured IN VITRO

Notochord-less chick blastoderms were treated with various exogenous RNA solutions and were cultured in vitro. The RNA was used at a concentration of 0.5 mg/ml. The presumptive ectoderm was induced to form neural tubes as well as certain non-specific tubules in both the control and experimental groups. The RNA-treated explants revealed an enhanced ectodermal thickening accompanied by healthier neural tubes with duplication of structures in some cases. Non-specific action for RNA was noted since brain-RNA, liver-RNA and boiled brain-RNA all "induced" neural tubes. No other structures were found to be induced.

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I. INTRODUCTION

Induction in Amphibia:

There have been several advances made in the study of embryonic induction since the "organizer" experiment of Spemann and Mangold (1924). They transplanted a piece of tissue from the dorsal lip of the blastopore of an unpigmented amphibian gastrula, Triton cristatus, into the ventral ectoderm of a pigmented, Triton taeniatus. The implant and the overlying ectoderm developed into a secondary embryo. The dorsal lip tissue was found to induce the ectoderm to form a neural tube. It also self-differentiated mostly into somites and notochord. This showed that the implant tissue served not only as an inducing stimulus but contributed materially to the substance of the extra embryo. The dorsal lip of the blastopore was designated as the "organizer".

The vital activity of the organizer was not found to be essential (Bautzmann, Holtfreter, Spemann and Mangold, 1932). The organizer was capable of induction after boiling, freezing and alcohol or petroleum ether treatment. Holtfreter (1934) observed that embryonic, adult or even dead tissues of various kinds were capable of performing the function of Spemann's "organizer". All these tissues were generally referred to as inductors. Toivonen (1940, 1953) implanted guinea pig tissue into the blastocoel of Triton gastrula. It was found that kidney regularly induced a tail-like structure (spinocaudal). The liver induced a head-

like structure (archencephalic) and bone marrow induced mesenchyme and pronephros (mesodermal). These experiments demonstrated the presence of qualitatively different inducing agents.

Induction in Birds:

Graper (1929) argued that endoderm formation should have an organizing function. Wetzel (1929), Hunt (1929)and Willier and Rawles (1931) held that the role of the organizer in birds was played by Hensen's node. The introduction by Waddington (1930) of *in vitro* blastoderm cultivation, facilitated the technical difficulties usually encountered in the transplantation experiments. These experiments were needed to demonstrate the effect exerted by one part of the embryo on other parts in its neighbourhood.

Waddington (1930, 1932) demonstrated the occurence of embryonic induction in the primitive streak stage. Two blastoderms were used; their hypoblasts were removed while their epiblasts were retained. The blastoderms were cultured *in vitro* on a plasma clot with the mesodermal faces of the double epiblasts in contact but not throughout the whole length of the primitive streak. Four embryonic axes occured in such cultures each comprising a neural plate with associated mesoderm. The neural plates assumed full "anatomical continuity" with one or the other of the epiblasts. Necrosis was reported in the lower blastoderm (nearer to medium) which did not play an important role in these experiments.

Induction by grafts of the primitive streak and other regions of the blastoderm was demonstrated by Abercrombe, Schmidt, Taylor, Waddington

and Waterman (1933). In some grafting experiments, differentiation of the graft without that of the host was observed; in others a clear-cut inductive effect was exerted by the graft (Waddington and Schmidt, 1933; Woodside, 1937). The following results were reported from the second group in which the inductive effect was manifested: First, induction could be performed by pieces of the anterior part of the streak. Second, Hensen's node was not necessary. Third, no induction was obtained from the posterior third of the fully grown streak. Fourth, the boundary between the inducing and the non-inducing region appeared to be approximately at the mid-point of the streak. The presumptive axial mesoderm of the inducible regions of the streak rather than the presumptive neural tissue accounts for this phenomenon of induction. "This brings the chick strictly in line with the amphibia" (Waddington, 1952). Induction in the chick was further demonstrated by the immediate product of the head process and the sinus rhomboidalis, (Waddington, 1933). Induction can be performed by pure neural tissue, free of mesoderm, derived from early somite embryos, (Waddington, 1952).

There seems to be little inducing capacity in the chick notochord after its differentiation. The inductive action of the various "organizer grafts" is not limited to the formation of neural tissue. Axial mesoderm may be stimulated as well. Waddington and Taylor (1937) demonstrated that the presumptive ectoderm could be induced to form mesoderm. Their technique consisted mainly of introducing fragments of presumptive ectoderm grafts into holes made in the primitive streak of the host blastoderm.

This is analogus to the grafting experiments of Spemann and Geintiz (1927) with Triton.

In the chick, the presumptive axial tissues undergo a great deal of elongation connected with the retreat of Hensen's node. Abercrombie (1937) revealed that morphogenetic movements could be achieved by induction. He explained it on two levels elongation and centering. However, the morphogenetic movement was not a necessary part of the induction process.

Waddington (1932) in trying to analyse the phenomenon of induction resulting from his grafting experiments in birds, explained it on the level of competence. Self differentiation can be another interpretation. This stems from the original chorio-allantoic grafting experiments where a systematic exploration of the blastoderm had been undertaken with the establishment of the presumptive maps (Willier and Rawles, 1935; Rawles, 1936; Rudnick, 1944). Waddington (1941) and Needham (1942) suggested that the induction phenomenon could be attributed to evocation and individuation. The plasmagene or cytogene hypothesis that had been invoked by Medawar (1947), might account for the process of self-reproducing tissue, a by-product of the phenomenon of induction, i.e., neural tissue by neural tissue (Mangold and Spemann, 1927). However Waddington (1952) suggested that it would be simpler to explain this embryonic case on the level of competant cells containing inactive complex from which an effective inducing agent could be easily released. This suggestion is highly probable in connection with the evocation of the neural tissue in Amphibia (Needham, 1942; Waddington, 1940; Holtfreter, 1948).

Chemical Nature of the "Inductor":

As soon as it was shown that the vitality of the organizer was not essential, it was realized that induction must depend upon a chemical agent. It was suggested that "diffusible substances" could be liberated from the inducing tissue. Attempts were made to isolate and identify the "active" inducing agent. Waddington, Needham and collaborators (1934) succeeded in preparing active ether extracts from inducing tissues. Its solubility in ether suggested that it was of a fatty or lipoid nature. Further experiments by Needham (1934) suggested that the "primary evocator" was a substance of steroid nature. Wehmeier (1934) prepared an active aqueous extract, by prolonged boiling of muscle in water, which induced large neural tubes. This proved that the active inducers released by the tissues included substances other than steroids and ether soluble compounds. Fischer, Wehmeier and collaborators (1935) found that weak organic acids were also good inductors. Isolated ectoderm could be stimulated to form neural tissues by substances of non-biological origin, such as weak solutions of methylene blue (Waddington, Needham and Brachet, 1936). This lead the latter to the conclusion that the ectoderm already contained the inducing substance, but in a masked and inactive form. Any treatment with the above mentioned agents would unmask the inducing agent to provoke neural differentiation. Similar results were obtained by Barth and Graff (1938) and Flickinger (1958). It was also found that presumptive ectoderm might be caused to develop into nervous tissue by short exposure to a saline solution whose pH is lower than 5.0 or higher than 9.2, (Holtfreter 1947). Treatment of the presumptive ectoderm with

ammonia (Yamada, 1950) not only induced, but also increased the developmental potentiality of ventral mesoderm, which would differentiate into chorda mesoderm. Neural induction by cytolyzing agents such as acids or bases was demonstrated by Karasaki (1957). "These substances may have nothing to do with normal induction but cause neural differentiation through their toxic action, which causes sublethal cytolysis of cells" (Balinsky, 1965).

Parallel to the attempts to identify the natural inducing substances, Niu and Twitty (1953) developed a new biochemical approach for the study of induction. They ascertained that the inducing tissues released inducing substances into the surrounding medium. They succeeded in cultivating a piece of inducing tissue in a modified saline solution, later called "Niu and Twitty solution", by the hanging drop method. It was discovered that the saline solution was "conditioned" to induce neural differentiation. It was referred to as "conditioned medium". Niu and Twitty (1953) demonstrated that the "conditioned medium" contained a macro-molecular substance of the nature of the nucleoproteins. The nucleic acid component was mainly ribonucleic acid. In later experiments, Niu (1956) found that addition of ribonuclease to the "conditioned medium" reduced its inductive capacity. Addition of trypsin or chymotrypsin prevented induction completely. These results suggested that the inducing substances were nucleoproteins.

Role of RNA in Embryonic Induction:

Brachet (1941, 1942, 1943) was the first to demonstrate the abundance of RNA-rich cytoplasmic granules (microsomes) in the dorsal lip

of the blastophore. He observed that during gastrulation the concentration of microsomes decreased in the chorda mesoderm, but increased in the nerve plate. This lead him to conclude that the RNA - containing particles migrated from the inductor to the reacting ectoderm. Brachet (1950) succeeded in isolating the RNA - rich cytoplasmic granules by differential centrifugation of the homogenized inducing tissue. Brachet (1944) also demonstrated that induction of neural tubes was exhibited by tobacco mosaic virus-RNA. He also found that treatment of the "organizer", microsomes or T.M.V.-RNA with ribonuclease resulted in a conspicuous decrease in the inductive capacity (Brachet, 1947). Moreover Brachet (1949, 1950) showed that there was a direct correlation between the RNA content of the implant and the percentage of inductions obtained. In later experiments, Brachet et al. (1952) could not confirm the inhibitory effect of the RNA-ase. Kuusi (1953), Yamada and Takata (1955a), Englander et al. (1953), Hayashi (1955), Englander and Johnen (1957) and Vahs (1957) all confirmed Brachet's (1944) finding on the inductive role of the T.M.V.-RNA, but could not confirm his findings of the inhibitory effect of RNA-ase. Brachet (1957) confessed that most of his RNA-ase used in the 1944 experiments was a crude preparation which was contaminated with proteolytic enzymes that effected the protein. Toivonen (1949, 1954), Yamada and Takata (1955a), Tiedmann and Tiedmann observed the loss of the inductive activity upon the limited proteolytic digestion of their proteins (1955). Furthermore, Yamada and Takata (1955b) could not obtain any neural induction when kidney RNA was placed in a "sandwich" of ectoderm cells. Brachet (1957) attributed these negative results to the difficulties encountered in the isolation of

intact RNA. The fact that the abnormal inductors lost their activity when treated with formalin, ketene or nitrous acid, substances which block the $-NH_2$ groups of the proteins (Lallier, 1950, Smith and Schechtman, 1954), strengthened the conclusion made later by Brachet (1957) that the evocating activity in the case of abnormal inductors was greatest in ribonucleoproteins and that the integrity of the protein rather than the RNA was necessary.

Despite that fact that Tiedmann and Tiedmann (1955) held that the proteinaceous part of the inducing substance was essential, they also reported that ribonuclease had an inhibitory effect on tail induction by chick embryo extract (Tiedmann and Tiedmann, 1955). Vahs (1957) made the interesting observation that Gram positive bacteria could induce neuralization of presumptive ectoderm while Gram negative bacteria were inactive in that respect. He suggested that this could be explained by the fact that Gram positive bacteria contain more RNA in their membranes than Gram negative bacteria. According to Vahs, treatments with ribonuclease or U.V. irradiation (which inactivate or destroy RNA) suppressed the inducing activity of the Gram positive bacteria (Vahs, 1957).

Further chemical analysis of the inducing substance and preparations of purified extracts of the inducing tissues were performed by Yamada and Takata (1961). Their extracts contained fairly pure macromolecular substances of molecular weights ranging from 43,000 - 100,000. Some extracts showed all the chemical and spectrophotometric reactions of proteins, others showed those of ribonucleoproteins. Very minute quantities were sufficient to cause induction when introduced into the

blastocoele of a Triturus embryo (Tiedmann, 1962). The general conclusion that these investigators drew was that the inducing principle was of protein nature, and that the protein might or might not be coupled with ribonucleic acid.

Specific vs. Non-Specific Action of Exogenous RNA:

Niu (1958, 1959, 1960, 1961, 1963) favors the inductive role of RNA. He reported that saline extract (SE) and ribonucleoprotein fraction (RF) prepared from calf thymus induced the neural differentiation of the presumptive ectoderm of A. tigrinum (Niu, 1958). The extracts contained 30-50 ug RNA/ml., while the control series gave rise to 0-15% of cultures showing neural differentiations, SE and RF induced the formation of a much greater percentage (73%) of the neural and non-neural structures. Moreover he showed that the reduction of RNA content after RNA-ase treatment correlated with the loss of inductive ability. The finding that RNA isolated from different sources was capable of inducing the presumptive ectoderm to differentiate into specific structures, attributes to RNA certain specific inductions (Niu, 1959). It was experimentally shown that when presumptive ectoderm cells were cultured in salt solution containing appropriate concentrations of RNA prepared freshly from various sources, differences in the pattern of differentiation were obtained. Cultures grown in kidney RNA developed into either tubule structures alone or tubule and neural structures, and those in liver RNA developed into gut and neural structures (Niu, 1959). These results showed that specific RNA's from certain tissues would induce the differentiation of their respective organs. Along with this finding it was also recently reported by Hillman

and Niu (1963) that brain and notochord RNA could initiate, respectively, the development of brain and notochord. Double brain vesicles were produced when 0.5 mg B-RNA were added to a stage 5 (head-process) chick embryo whose head process was removed and cultured *in vitro*. Two notochords were also found upon treatment of similar embryos with 0.5 mg N-RNA. Moreover, it was also found that experimental stage 10 (ten somites) intact chick embryos showed increased size of notochord upon treatment with 0.5 mg N-RNA. These results lead Hillman and Niu (1963) to postulate that brain and notochord RNA's carry information which initiated the differentiation of their respective tissues.

Butros (1963) cultured posterior fragments of head process blastoderm *in vitro* for two days on a protein - deficient medium supplemented with various RNA's. The cultures were then grafted over the chorio-allantoic membrane for 8-9 days. It was found that embryonic brain RNA produced a papillomatous epidermis with giant epidermal cysts that were keratinized and fibrillar as in feather formation. Besides, liver (embryonic) RNA had no visible tissue modifications. No keratinization, nor papillary formation of the epidermis was reported in the controls, the epidermal cysts were very small, unkeratinized and devoid of fibrillar organization. Eisenberg and Van Alten (1964) showed that lens RNA promoted a "better subsistence" of cephalic ectoderm cells of 4-7 somite chick embryo and a compact arrangement of cells within the culture tissue. No specific cytological transformations related to lens or liver RNA were observed. Eisenberg (1964) employed the agar diffusion technique of

Ouchterlony (1949) to determine if lens specific antigens were being synthesized in the cells of the cultured explants. This immunochemical method was based on the fact that chick embryo extract cross reacted with the lens antiserum. No precipitin line could be detected in any of the experimental groups. Butros (1965) cultured post-nodal fragments of definite streak or head process chick blastoderms for eight days on a basic medium containing TC 199, whole egg suspension, embryo extract, agar and penicillin. The experimental group received a drop of RNA twice daily, the control receiving a drop of the solvent. He noted that heart RNA prepared from chick embryos by a modified procedure of Kirby (1956), promoted twitching of certain localized areas. Butros (1965), also reported that liver RNA stimulated the folding of the entoderm layer and the modification of the cells to columnar, goblet form. In that work the RNA action was not as specific as the previous works of Hillman and Niu (1963). However, Butros (1965) mentioned that his procedures deviated basically from those of Hillman and Niu (1963) in two aspects. First, while Butros (1965) used complex tissue fragments (postnodals), Hillman and Niu (1963) used dissociated individual cells. Second, exposure to the nucleic acid in the transformation experiments of Hillman and Niu (1963) was much shorter than that of Butros (1965), i.e., one day vs. eight days. The argument for use of eight days being that histogenesis could not be observed within the one day period. Finnegan and Biggin (1966) studied the influence of calf spleen RNA on amphibian histogenesis. They held that neither embryos nor competent gastrula ectoderm of Xenopus lacuis demonstrated any tissue - specific inductive response, but an enhanced

epithelial development was observed in the experimental system of the RNA-conditioned medium. They reported that their results best confirmed those of Butros (1963, 1965) of non-specific effects.

Very recently, Hillman and Hillman (1967) reevaluated the specific response of competent chick ectoderm to RNA. Hillman (1963) who previously reported with Niu the specific action of RNA, now maintained that RNA had no specific effects. Hillman and Hillman (1967) reported that neural tubes differentiated upon the treatment of chick competent ectoderm with brain RNA (B-RNA), heart RNA (H-RNA) or boiled brain RNA. The ectoderm did not, however, respond to liver RNA (L-RNA). They suggested that the ectoderm might not have responded to the RNA itself but rather to a possible contaminant present in the H-RNA, B-RNA and boiled B-RNA, but not found in the L-RNA. Their results further suggested that a Folin-positive material in the RNA preparation might be responsible for the nonspecific induction. They concluded that "it appears that the specificity of the induction is open to question and that the role of RNA in the inductive process should be further tested experimentally."

Purpose of this work: With the specific inductive capacities of various RNA's as reported by Niu and the subsequent differentiation to specific organs on one hand, and with the nonspecific effects claimed by Butros, Eisenberg, Finnegan and very recently by Hillman and Hillman, on the other hand, the main purpose of this work was to reinvestigate the problem of induction by RNA with more effective methods of getting under-graded RNA, denudation and culturing techniques and ask the following questions:

1. Does exogenous RNA from different sources induce the presumptive ectoderm of a Hamilton stage 5 chick blastoderm (denuded of its endmesoderm at the region of the brain presumptive areas) to form a neural tube? In other words, can the RNA substitute the inductive effect of the notochord?
2. Do specific RNA solutions from certain embryonic tissues induce the differentiation of their respective organs?

II. MATERIALS AND METHODS

Eggs: Fertile white Leghorn eggs were generously supplied by the A.U.B. farm. They were stored at 18°C for no more than a week before use.

Solutions and Media: The following Ca^{++}-free solutions were tried for the splitting of the endmesoderm from the ectoderm:

1. Merchant's Ca^{++}-free solution (Merchant et al. 1961).

NaCl	8.00 gm.
KH_2PO_4	0.20 gm.
KCl	0.20 gm.
Na_2HPO_4	1.15 gm.
Distilled H_2O	1.00 liter.

2. 3% EDTA (Versene) in Merchant's solution.
3. 0.05% Trypsin in Merchant's solution.
4. Moscona's (1952) Ca^{++}-free solution constituted as follows:

NaCl	8.000 gm.
KCl	0.200 gm.
$NaH_2PO_4.H_2O$	0.005 gm.
$NaHCO_3$	1.000 gm.
Glucose	2.000 gm.
Distilled H_2O	1.000 liter.

Moscona's Ca^{++}-free solution proved to be superior in disaggregation to the other three, and consequently was used throughout the work. The pH of the solution was checked with a pH meter, but phenol red indicator was used in the media. New's (1955) original albumin-Ringer medium was used. It was made up of two volumes of albumin to one volume of Ringer. All the solutions were autoclaved before use at fifteen pounds per sq. inch of pressure and at 120°C for fifteen minutes. Sterile technique was used throughout all the work.

RNA Preparation and Characterization: The RNA extraction procedure was Whiteley and McCarthy's (1966) modification of Kirby's (1956) original method. Brains and livers from over a hundred chick embryos, of 8-13-day incubation were separately frozen on solid CO_2 and all subsequent operations were performed in the cold. The tissues were homogenized for one or two minutes in three volumes of 0.05 M Na Acetate, pH 5.2, to which DNA-ase (10 gamma/ml. final concentration) and bentonite (30 gamma/ml.) were added. The homogenate was then incubated with pure 1.5% SDS (sodium dodecyl sulfate) for one minute at 37°C. An equal volume of distilled phenol saturated with 0.05 M sodium acetate pH 5.2 was added. Re-extraction with buffer saturated phenol was performed two more times; the RNA was finally precipitated by adding three volumes of absolute alcohol at -20°C. The precipitate was dissolved in 0.01 M acetate buffer pH 5.2. It was later incubated with DNA-ase (20 gamma/ml.) for one hour at 25°C. Extraction with phenol was performed once more. It was precipitated with ethanol as before; then it was dissolved and reprecipitated for a second time (this being the third precipitation from the start). Finally the precipitate

was dissolved in a minimal volume of 1/10 SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) and the residual phenol was extracted with ether. The ether was evaporated by bubbling with nitrogen gas. The RNA product was stored in sterile vessels at -20°C. The orcinol test for RNA was positive yielding a green color. The diphenylamine test for DNA and the Biuret and Folin-Ciocaltew tests for protein were all negative. The ultraviolet absorption spectra of the RNA samples were measured with the Beckmann spectrophotometer. The optical density (OD) was measured at wavelengths varying from 200-300 mu; the readings furnished a curve typical of the nucleic acid absorption spectrum (Fig. 1). The maximum absorption was at wavelength 260 mu and the minimum at 230 and 280 mu. The quotients 260/230 and 260/280 were 2.56 and 2.10 for the liver and brain-RNA samples respectively.

The concentration of the RNA sample solution was determined by first diluting the sample 100X in SSC and then measuring the optical density at 260 mu using a one centimeter quartz cell. The measurement obtained was multiplied by 100. RNA has an approximate concentration of 1 mg/ml. for 20 OD units. The concentration of the sample RNA was determined on that basis. For all culturing experiments calling for the use of exogenous RNA, a concentration of 0.5 mg/ml. was maintained. The original samples were diluted with Ca^{++}-free solution. Ringer's solution was found to precipitate the RNA by forming the calcium salt of the latter.

Operational Procedure:

Denudation: The eggs, upon being taken from the 18°C. cold room,

were incubated for 21 hours in a constant air draft incubator at 37.5°C. This brought them to Hamilton stage 5: definite streak-head process stage. The blastoderms, which were handled in Ringer's in a finger bowl, were transferred by a wide mouth pipette to a Syracuse dish containing Ca^{++}-free solution. A second transfer to Ca^{++}-free solution was made to ascertain that no traces of Ringer's solution remained. The embryos were then incubated for 15 minutes at 37.5°C. Then the endmesoderm layers were peeled with very fine steel needles and a specially designed, broom-like, camel-hair loop. The latter made it possible to sweep away the loose mesoderm without considerably damaging the underlying ectoderm. The denuded area extended lengthwise from 0.4 mm. below Hensen's node up to the end of the streak or process depending on the stage, thus assuring complete stripping of the notochord. The endmesoderm in the immediate vicinity of the streak (0.3 to 0.4 mm. on each side) was also denuded (Fig. 2). The prepared blastoderms were then transferred to another dish containing Ringer's solution.

Application of the RNA: Treatment of the blastoderms with the exogenous RNA consisted of transferring them to a watch glass containing the desired RNA and placing them in a culturing dish on a wetted piece of cotton. They were incubated for almost an hour; at the end of this time, they were washed in Ringer's solution for five minutes and set ready for culturing. Boiled RNA samples were used for controls instead of RNA-ase treatment (Hillman and Hillman, 1967).

Culturing Technique (Rings): Butros' recent modification of New's (1955) culturing procedure was followed. This double ring technique (DRT) employed two glass rings 1 mm. thick, one being 2.2 cm. in diameter, the other 1.8 cm. (Fig. 3). A piece of dialysis cellophane membrane was pulled like a drum across the opening and between the two concentric rings. The rings rested on a watch glass that was placed on a soaked piece of cotton in a Petri dish. The albumin-Ringer 2:1 liquid medium was introduced below the membrane; the blastoderm was cultured on top of the membrane, thus getting its nutrients by diffusion. The exogenous RNA for the experimental group was also added on top of the membrane, which was maintained wet throughout the culturing period. Controls received the solvent of RNA instead. The blastoderm was cultured at 37.5°C for 24 hours.

Macroscopic Observation and Morphology: At the end of the culturing period, macroscopic observations were recorded and followed immediately by fixation with Bouin's solution. Macrophotographs were also taken to show the morphological changes.

Histology: The fixed cultures were transferred to vials which contained Bouin's. After twenty four hours the fixative was washed with 50% alcohol and the cultures were dehydrated, infiltrated with 50 to 52°C. paraffin, and then embedded. They were then serially sectioned anterioposteriorly at 10 micra, mounted on embryological slides, stained with Hematoxylin-Eosin, and finally mounted with Kleermount solution. The slides were studied serially and microphotographs taken of particular regions.

III. EXPERIMENTAL RESULTS

A total number of 115 blastoderms furnished the data for this work. They were distributed almost uniformly among the following five control and experimental groups receiving various treatments:

1. Intact blastoderms cultured on albumin-Ringer basic medium.
2. Denuded blastoderms cultured on albumin-Ringer basic medium.
3. Denuded blastoderms treated with brain-RNA, cultured on albumin-Ringer basic medium.
4. Denuded blastoderms treated with liver-RNA, cultured on albumin-Ringer basic medium.
5. Denuded blastoderms treated with boiled brain-RNA, cultured on albumin-Ringer basic medium.

Ten experiments were performed. A minimum of ten blastoderms were cultured in each experiment. The morphological and histological results of each group will be discussed separately.

Morphological Results:

1. Intact Blastoderms cultured on Basic Medium:

Table 1 shows that out of the 23 explants from this group, 19 embryos developed while the others disaggregated. The brain developed normally with conspicuous optic vesicles in eleven out of the 19 differentiating blastoderms. The mid-brain (mesencephalon) and hind brain (mylencephalon) developed in all explants.

The blastoderms showed profound morphogenetic movements and elongations with an increase in the blastoderm size. Numerous blood islands differentiated in the majority of the explants. The number of somites at the end of the culturing period varied from two to seven. The appearance of single normal beating hearts seems to be correlated with the appearance of numerous blood islands. Three blastoderms revealed a unique result by developing two beating hearts anterior to the brain.

2. Denuded Blastoderms Cultured on Basic Medium:

Despite the cracks that resulted from the various mechanical operational procedures used for the denudation of the endomesoderm, the blastoderms healed after 24 hours of culturing. The mid and hind brains were observed in $\frac{12\text{-}13}{23}$ of the cases. The appearance of the forebrain in this group is suppressed as compared to the intact series (Table 1). The frequency of the appearance of the two beating heart tubes anterior to the brain (plate 1-A) has increased.

3. Denuded Blastoderms Treated With Brain-RNA:

The midbrains and hindbrains have developed in 14/16 of the explants. The forebrain development was enhanced upon treatment with B-RNA. There were 8/16 cases with forebrains observed. The latter appeared in 6/23 of the cases in the control, while it appeared in 3/17 and 4/15 of the liver and boiled brain-RNA treated explants respectively. The appearance of the two heart tubes anterior to the brain was more pronounced in this group than in any of the preceding groups.

4. Denuded Blastoderms Treated With Liver-RNA:

As shown in table 1, this group demonstrated the least amount of disaggregation of cultures. The blastoderms have in most of the cases elongated (15/17) and developed numerous blood islands. The double heart condition appearing anterior to the brain was also observed in 12 out of 17 embryos. The mid. and hind brains developed in 16/17 of the cases. There was a considerable decrease (3/17) in the appearance of the forebrain.

5. Denuded Blastoderms Treated With Boiled Brain-RNA:

Despite the fact that 15 out of 20 of the explants developed in this group, the mid. and hindbrain developed in all (table 1). The appearance of the two beating heart tubes anterior to the brain was most frequent in this group. The somites developed beyond the five-somite stage in 14 out of 15 of the cases. The blastoderms have enlarged in all cases.

Histological Results:

1. Intact Blastoderms Cultured on Basic Medium:

Histological observations of this group reveal normal development. Consistent with the morphological results, plate 1-B shows the appearance of the forebrain with conspicuous optic vesicles, the mid and hindbrains and several healthy somites. The normal appearance of the heart, foregut and neural tube with the notochord is revealed in plate 1-D. At the cephalic region neural tubes were observed in 17/19 explants with an equal percentage for the notochords which

appeared in their normal position, mid-ventral to the brain (table 2). One remarkable observation made in some embryos in this group, was the appearance of a flat neural plate and notochord at the level of the heart (table 2-D). This flat neural plate continued to appear as far down as the trunk region. It showed signs of tubulations at the level of hind brain and heart, but developed into lateral cup-like foldings or semi tubules, instead.

2. Denuded Blastoderms Treated With Basic Medium:

The forebrains and optic vesicles didn't develop as normally and frequently as those of the intact cultures. Only four out of twenty three optic vesicles were observed. These were associated with abnormalities, such as optic defects, twisting of brain and neural tubes. As shown in table 2, 18/23 of the explants developed neural tubes, while the rest developed non-specific tubules of various kinds. The notochord reformed in 16/23 of the cases. In one case (Specimen DRT 17) the notochord at the level of hind brain and somites was half as big as the neural tube itself. The number of cells constituting the notochord tripled and quadripled, (plate 2-H). An estimated 37 to 45 cells were counted as compared with 10 in the normal notochord. In specimen DRT 30, two thickened ectodermal convolutions and semi tubular structures were observed eleven sections (110 micra) after the posterior limit of the heart, (plate 2-F). These could have been unsuccessful attempts towards the formation of three neural tubes. However, in specimen DRT 43 two neural tubes were

observed, (plate 2-A). In several explants the same condition of the appearance of flattened neural plate at the region of somites was observed. This thick neural plate semi-tubulates in three sections, (30 micra) but reflattens after six sections (60 micra) then becomes thinner and convolutes again at the trunk region. The frequent occurence of the two heart tubes anterior to the brain that was previously mentioned in the morphological results is demonstrated in the serial sections shown on (plates 1-C and 2-C).

3. Denuded Blastoderms Treated With Brain-RNA:

Serial sections showed that the excised portion of the notochord had reformed in 14/16 of the cases with an identical frequency for the appearance of neural tubes. Normal optic vesicles were observed in half of the differentiating explants. One of the commonly encountered results was the enhanced thickening of the neural ectoderm. The appearance of a flattened neural plate was also manifested in this experimental group. The neural plate attempted to tubulate and convolute but reflattened after 6-7 sections. The notochord reformed in 14/16 of the cases. The position of the notochord varied with respect to the neural tube or plate. It not only appeared mid-ventral to the neural tube or plate, but also ventrolateral. Two notochords were observed in one of the cultures. Enlargement of the notochord size was also encountered in the trunk region of this group. Certain non-specific tubules, as observed in specimen numbers DRT 29 and DRT 65, developed as many as three or four ring-like tubules which appeared

beside each other in the somite region. In several sections brain cells were seen proliferating into the neurocoele. This situation was marked by the appearance of thickened ectoderm in the healthier neural tubes. The walls of the neural tube developed lateral constrictions which projected into the brain cavity and later resulted in a bridge which divided the neural tube into two halves resulting in an eight-shaped structure (plate 2-E).

4. Denuded Blastoderms Treated With Liver-RNA:

Explants of this series revealed the development of neural tubes in 14/17 of the cases. Though several forebrains were observed, optic vesicles appeared in only 3/17 of the cases. The notochord was seen in 12/17 of the cultured explants and was in a few cases enlarged. Two notochords were observed one being ventro lateral to the neural tube, the other lateral to it (plate 2-G). In most of the other cases the notochord asumed a slender, conical shape particularly in the explants that revealed a flattened neural plate which looked like a bow. Ectodermal thickenings with attempts towards the formation of non-specific tubules were frequently encountered.

5. Denuded Blastoderms Treated With Boiled Brain-RNA:

As shown in table 2, the appearance of neural tubes was least frequent in this group (10/15). However, the quality of the neural tubes observed was better and healthier than those treated with liver-RNA or denuded with no treatment. In several proliferation of brain cells into the neurocoel resulted in a constriction that divided the

neural tube into two tubules. Flattened neural plates were continuously encountered in many of the embryos. These formed in certain limited sections (30-70 micra) incomplete tubes and cup-like structures. In 7/15 of the cases there is a notochord. Optic vesicles were observed in 3/15 of the blastoderms. Specimen DRT 118 was one of the three specimens in which appearance of four non-specific tubules were observed. Specimen DRT 102 had two neural tubes and two notochords (plate 2-B).

Taken together these results point out the following highlights:

1. Brain-RNA treatment enhanced the development of the forebrain (8/16 blastoderms). The latter appeared in 6/23 of the cases in the control, while it only appeared in 3/17 and 4/15 of the liver and boiled brain-RNA treated explants respectively.

2. A small number (4/23) of optic vesicles developed in the denuded blastoderms; they were associated with abnormalities, such as optic defects, twisting of brain and neural tubes. On the other hand in brain-RNA treated ones a larger number (8/16) of optic vesicles developed that were definitely normal.

3. The midbrains and hindbrains appeared in greater proportions in the RNA-treated groups than in the control. While they appeared in 14/16, 16/17 and 15/15 of the brain, liver and boiled brain-RNA-treated explants respectively, they appeared in $\frac{12\text{-}13}{23}$ of the cases in the control.

4. The duplication and/or enlargement of neural tubes was higher in the brain-RNA-treated explants than in any other control or RNA-treated groups. These neural tubes appeared in 14/16 of the cases in the Brain RNA-treated blastoderms, while it appeared in 18/23, 14/17 and 10/15 of the blastoderms in the control, liver and boiled brain RNA-treated explants respectively.

5. The notochord reformed in 14/16 cases in the brain RNA-treated blastoderms, while it reformed in 12/17, 7/15 and 16/23 of the cases in liver RNA-treated, boiled brain RNA-treated and control blastoderms respectively.

6. Enlarged notochords were observed in 1/10 cases in the denuded controls, while two notochords were observed in 1/16, 1/17 and 1/23 of the cases in brain-RNA, liver RNA-treated and control respectively, and therefore there does not seem to be a significant difference in this respect.

7. RNA from brain and liver-RNA stimulated the formation of the two beating heart tubes anterior to the brain by a threefold increase over the control.

IV. DISCUSSION AND CONCLUSIONS

Effect of RNA on Forebrain and Optic Vesicles Formation

It is evident from the results at hand that noticeable differences were encountered between the RNA-treated explants and some of their controls. The enhanced development of the forebrain in the brain RNA-treated blastoderms (8/16) as compared to 6/23, 3/17 and 4/15 of the control, liver, and boiled brain RNA-treated explants respectively, suggests that there is a correlation between the brain RNA and the development of the forebrain. There is also a correlation between the appearance of optic vesicles and treatment with exogenous brain RNA. With brain-RNA treatment, 8/16 developed normal optic vesicles. A smaller number of optic vesicles (4/23) developed in the denuded blastoderms. These four showed optic defects and other abnormalities.

In a similar work Hillman and Niu (1963), using New's culture technique (New, 1955), reported the development of abnormalities in the forebrain and midbrain (microcephaly, acleiencephaly and eye defects) in notochordless chick blastoderms that did not receive RNA. They reported that upon treatment of 45 embryos, whose head processes were extirpated, with brain RNA, 23 embryos had normal brain development and three had developed two complete brains. These results agree with those reported in this work.

The appearance of the forebrain in the absence of the notochord was explained by Rawles (1936). She transected the chick blastoderm at the head-process stage into 16 pieces and tested the potency of each piece by transplantation to the chorioallantoic membrane. The transplants were found to possess specific developmental capacities. This helped in mapping the organ

forming areas in the chick blastoderm. It was found that the notochord appeared in two grafts out of 18 median grafts which contained a part of the head-process at the level of the forebrain. A like situation might account for the occurrence of the forebrain in 6/23 of the cases in the control group.

Effect of RNA on Midbrain and Hindbrain Formation

The midbrains and hindbrains appeared in greater proportions and looked healthier in all the RNA-treated groups than in the control. This result partly deviates from Hillman and Niu's (1963) finding, where normal development of the brain in the operated blastoderms occurred only upon treatment with brain RNA. In none of the 35 chick liver RNA-treated blastoderms did the forebrains and midbrains develop normally in their work. A possible explanation of the stimulation of midbrains and hindbrains by liver RNA in this work could be that the applied RNA was depolymerized and resynthesized in the neural tissue in accordance with the cell type involved (Butros, 1965).

Neural Tube Development

The cephalic ectoderm posteior to the hindbrain presumptive region was induced to form neural tubes or spinal cords to almost the same extent in both the experimental and control groups. Microscopic investigations of the spinal cords at these regions showed that they have not well-differentiated.

The neural tubes induced in the RNA-treated explants were healthier and more numerous than in the control. Of all the RNA-treated blastoderms, the neural tubes appeared most frequently in the brain RNA-treated group (14/16). The control group developed neural tubes in 18/23 of the cases.

The appearance of neural tubes in 18/23 of the cases in the denuded controls might receive an explanation similar to that of the appearance of the

forebrain in the absence of notochord (Rawles, 1936). However, since it was proved that induction was mainly a chemical process, this last phenomenon might also be explained as being due to the active diffusion of chemical substances which could have been liberated by the excised notochord cells during the various operational procedures at the time of denudation, and resulted in the unmasking of the active inducing substances already found in the presumptive ectoderm as reported previously by Waddington, Needham and Brachet (1936). Another possible explanation might be the incomplete denudation of the endmesoderm, which left some unscraped notochord tissue; this tissue might have regenerated and induced the formation of neural tubes.

The appearance of healthier (enhanced ectodermal thickenings) and more numerous neural tubes that duplicated in some regions in the RNA-treated ones, might be attributed partly to either one of the previously mentioned processes and partly to the inductive influence of the extraneous RNA.

One of the remarkable observations made in this region was the appearance of flattened neural plates instead of the usual neural tubes. The lack of tubulation in these flattened neural plates might be attributed to the stage of culturing the blastoderms, which varied from the short streak to the definite streak. These early blastoderms required certain morphogenetic movements before they can give rise to axial structures, and consequently they stick on the cellophane membrane of albumin-agar and cannot tubulate. This phenomenon, however, was not observed in the explants that were cultured in a more advanced stage (head process to headfold) using the same culturing technique.

Notochord Formation

Hillman and Niu (1963) reported the appearance of two notochords upon treating a process-less blastoderm with 0.5 mg/ml. of notochord RNA. They also reported a three- and fourfold increase in the size of a normal notochord of a ten-somite intact blastoderm, when treated similarly. While the normal notochord has an average of ten cells, the experimental has three and four times that number.

The author attempted to isolate notochord RNA from 11- to 13-day chick embryos in order to repeat Hillman and Niu's (1963) results. All attempts to remove the notochord at that stage were unsuccessful, since it has an average diameter of 0.15 mm and is surrounded by cartilage. Whether the N-RNA used by the investigators mentioned above was purely notochordal is questionable.

Enlarged notochord appeared in this work with no RNA treatment. Two notochords were also observed in 1/16, 1/17 and 1/23 of the cases in the brain and liver RNA-treated and control blastoderms respectively. The appearance of two notochords in a few of the control and the experimental blastoderms in limited sections that didn't exceed 30 micra, might be attributed to chance errors in the denudation procedure whereby some notochord cells are not completely excised and so regenerate and develop two notochords instead of one.

The notochord had reformed in 14/16 of the cases in the brain RNA-treated blastoderms in this work. This result is in complete disagreement with Hillman and Niu's (1963) finding that the notochord has not reformed in the regions from which it had been extirpated while receiving similar treatment. However, notochord reformation of operated embryos was reported by the same investigators after treatment with 0.5 mg/ml. of notochord RNA.

Double Heart Formation

Another commonly encountered feature that demands further explanation is the appearance of the two heart tubes anterior to the brain in both experimental and control groups. It was observed that denuded blastoderms cultured on Spratt's (1947) albumin-Ringer medium could still develop two beating hearts anterior to the brain. This eliminated the possibility of explaining the occurrence of this phenomenon as being due to the present culturing technique.

The maps of Willier and Rawles (1935), Rawles (1936) and Rudnick (1948) revealed that the heart presumptive areas have not been denuded in this work. Dehaan (1964) observed that the precardiac mesoderm migrates in an anteromedial direction from stages five to seven by surgical intervention. The author believes that due to the denudation of the endmesoderm in the brain presumptive areas and the consequent extirpation of the notochord, the route of migration of the precardiac mesoderm cells is interferred with and instead of the cells' fusing to form a singular heart tube, they follow a migratory pattern around the denuded area; consequently each is separately induced by the available entoderm to form a tiny heart tube anterior to the brain.

RNA from brain and liver stimulated the formation of the two beating heart tubes by a threefold increase over the control. This might be explained as follows:

Dehaan (1965) reported that several pulsating vesicles or masses of heart tissues resulted from treatment of chick embryos in stages similar to those of this work with 40 mM sodium citrate for 40 hours of incubation. One explanation that might account for the stimulation of the double hearts is the presence of citrate in the solvent. The threefold increase in the appearance of the double hearts in the brain and liver RNA-treated blastoderms might be

attributable to an enhancing effect produced by the solvent and the RNA when present together.

RNA Specificity

Whether specific RNA solutions used in this work induced the differentiation of their respective organs, is another point that requires further elaboration. The works of Niu (1958, 1959, 1963) and of Hillman and Niu (1963) suggested specific actions for various RNA preparations. As previously mentioned, brain RNA enhanced the formation of the brain, while notochord RNA enhanced that of the notochord (Hillman and Niu, 1963). In this work no specific inductive responses were observed since liver RNA-, brain RNA- and boiled brain RNA-treated blastoderms all developed neural tubes. No other tissue specific structures were observed. Moreover the fact that brain development and notochord reformation was not restricted to one kind of RNA in this work points to a non-specific response to the RNA. This result doesn't agree with that of Hillman and Niu (1963) but is in agreement with the results of Butros (1963, 1965), Eisenberg (1964) and Finnegan and Biggin (1966) and the recent work by Hillman and Hillman (1967). The absence of any specific inductive effects with isolated RNA in experimental procedures was attributed to the fact that the added RNA is depolymerized and the nucleotides then used in the synthesis of homologous RNA (Butros, 1965; Finnegan and Biggins, 1966).

Moreover, Amos and Moore (1963) interpret the non-specific action of RNA as being due to the failure in obtaining a biologically "active" preparation.

Mode of Action of Exogenous RNA in Embryonic Differentiation

A prerequisite to the action of exogenous RNA is the entrance of the macromolecule into the reacting cells. Niu (1963) showed that the exogenous RNA molecules enter the ectodermal cell by the use of autoradiographic methods

described by Schwarz and Rieke (1962). Carbon 14-treated liver-RNA were found to be distributed in different cellular components of Ehrlich ascites cells. Niu (1963) further suggested that the exogenous RNA could have entered the ectodermal cells in a way similar to the phenomenon of pinocytosis. The exogenous RNA seems to have some sort of affinity for chromosomal material. The association between C^{14} activity and the chromosomal material was analyzed with the aid of ribonuclease, deoxyribonuclease, and a combination of both enzymes (Niu, 1962, 1963). An average of 90 to 97 percent of the radioactivity was eliminated by digestion with ribonuclease, 10 to 18 percent by deoxyribonuclease, and 99 to 100 percent by both enzymes. Further, Niu (1963) suggested that once RNA molecules enter the ectodermal cell, they may break down into nucleotides and then be used as substrates for resynthesis of RNA. If so, how can the action of RNA be specific? Niu (1963) goes on contradicting himself by saying: "The breakdown products of RNA isolated from various sources would contribute to the common pool of nucleotides inside the ectoderm cells. The resynthesized RNA should induce the ectoderm to differentiate into only one kind of structure". There seems to be no question that the exogenous RNA acts as template or catalyst for protein synthesis and therefore leads to the morphological identity of the induced cells (Brachet, 1957), but whether the RNA is depolymerized or not needs to be settled in the future. The author believes that it is more likely for the exogenous RNA to be depolymerized and then the nucleotides used in the resynthesis of homologus RNA in the neural tissue. This mode of action supports the non-specificity in the action of RNA.

Comments and Conclusions

From the preceding discussion it could be concluded that the effect of exogenous RNA in embryonic differentiation and test systems is not specific. RNA-treatment of presumptive brain ectoderm resulted in certain quantitative

and qualitative improvements in some cases as in forebrain, optic vesicles and neural tube development. However, no definite inductive effect could be attributed to the various RNA's used in this work.

More accurate results might be achieved with the following improvements in methodology:

1. More efficient methods of denudation need to be developed together with techniques for estimating the degree of denudation.
2. New techniques need to be developed for preparing notochord RNA. Micromanipulators can be used.
3. More extensive characterization of the RNA, especially as far as molecular weight is concerned, needs to be done. This will throw some light on whether the RNA is degraded or not.
4. During culturing by the double ring technique (DRT), albumin-Ringer was used as a medium. A control experiment needs to be done to study the effect of dialyzable materials found in this medium on the development of the explants and on the permeability of the cellophane membrane. This could be done by dialyzing the medium against water and checking the dialyzate for proteins and nucleic acids.
5. The effects of boiled and enzyme-treated RNA's on the results need to be compared in the future in order to check possible inductive differences.

In conclusion, the effect of RNA on embryonic induction needs to befurther elucidated and subject to experimentation with the least number of variables in order to remove as much controversy as possible.

V. SUMMARY

1. Axis-less blastoderms from stages varying between definite streak to head process were cultured on the albumin-Ringer medium by the double ring technique.
2. The denuded blastoderms were treated with various RNA samples from chick embryonic brain and liver to study their "inductive" action on the presumptive ectoderm.
3. Intact, denuded and RNA-treated blastoderms developed neural tubes and certain non-specific tubules.
4. The denuded blastoderms developed neural tubes in 78.2% of the cases, but were in most of the time associated with certain abnormalities. Brain-RNA-treated blastoderms developed healthier neural tubes and axial structures with enhanced ectodermal thickening in 87.5% of the cases.
5. The appearance of an enlarged notochord as well as two notochords in few cases of the control and experimental groups was mainly attributed to denudation errors. It does not, however, eliminate possible inductive roles played by RNA.
6. The appearance of the two beating heart tubes anterior to the brain in the denuded blastoderms is attributed to the interference in the route of migration of the precardiac mesoderm. They follow another migratory pattern around the denuded area and consequently are induced separately by the available ectoderm to form tiny tubes anterior to the brain. There seems to be a high correlation between the appearance of the two hearts and treatment with RNA.
7. No specific inductions by the RNA were observed. Liver-RNA, brain-RNA and boiled brain-RNA-treated blastoderms all developed neural tubes. No other tissue specific structures were observed.

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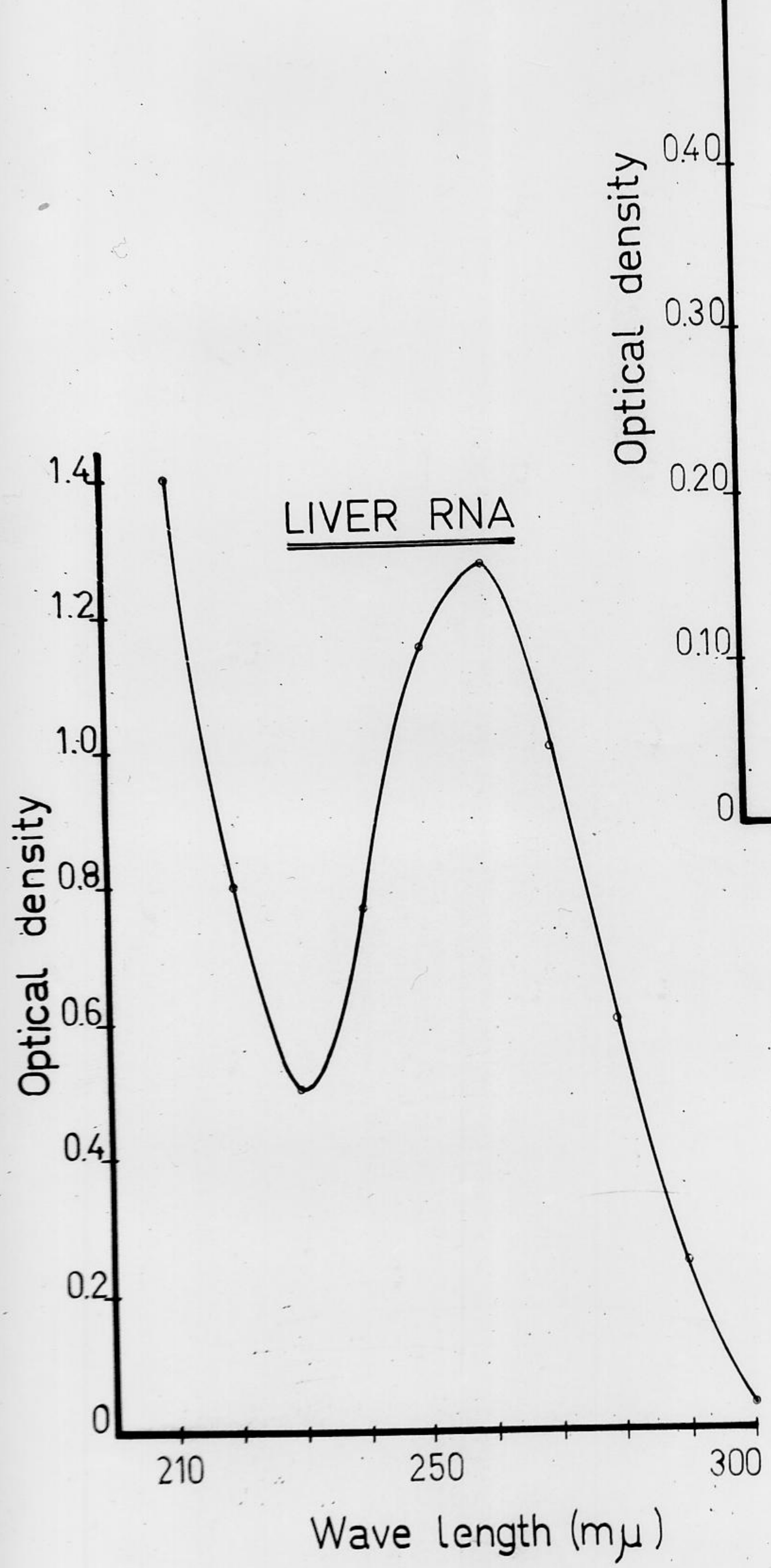
Table 1. Frequency of the various morphological structures in control and experimental groups.

TREATMENT	NUMBER OF EXPLANTS	DEVELOPED EXPLANTS	BRAIN			HEART			SOMITES	BLOOD ISLANDS		ENLARGED BLASTO-DERMS
			Fore.	Mid.	Hind.	One beating	Two beating	Not beating	Above 5	Few	Numerous	
Intact with Basic Medium	23	19	11	19	19	12	3	4	11	5	13	16
Denuded with Basic Medium	29	23	6	12	13	10	9	4	5	11	12	13
Denuded with Brain-RNA	23	16	8	14	14	4	12	0	5	2	14	10
Denuded with Liver-RNA	20	17	3	16	16	4	13	0	5	4	12	15
Denuded with Boiled Brain-RNA	20	15	4	15	15	2	13	0	1	2	13	15
Total No. of Explants	115											

Table 2. Frequency of the appearance of axial structures in Control and Experimental Groups.

TREATMENT	NO. OF EXPLANTS	DEVELOPED EXPLANTS	EXPLANTS WITH NEURAL TUBES	EXPLANTS WITH NON-SPECIFIC TUBULES	NOTOCHORD	OPTIC VESICLES
Intact with Basic Medium	23	19 (82.6%)	17 (89.4%)	2 (10.6%)	17 (89.4%)	10 (52.6%)
Denuded with Basic Medium	29	23 (79.3%)	18 (78.2%)	5 (21.7%)	16 (69.5%)	4 (17.4%)
Denuded with Brain-RNA	23	16 (69.5%)	14 (87.5%)	2 (12.5%)	14 (87.5%)	8 (50%)
Denuded with Liver-RNA	20	17 (85%)	14 (82.3%)	4 (23.5%)	12 (70.5%)	3 (17.6%)
Denuded with Boiled Brain-RNA	20	15 (75%)	10 (66.6%)	3 (20%)	7 (46.6%)	3 (20%)

Total No. of Explants 115



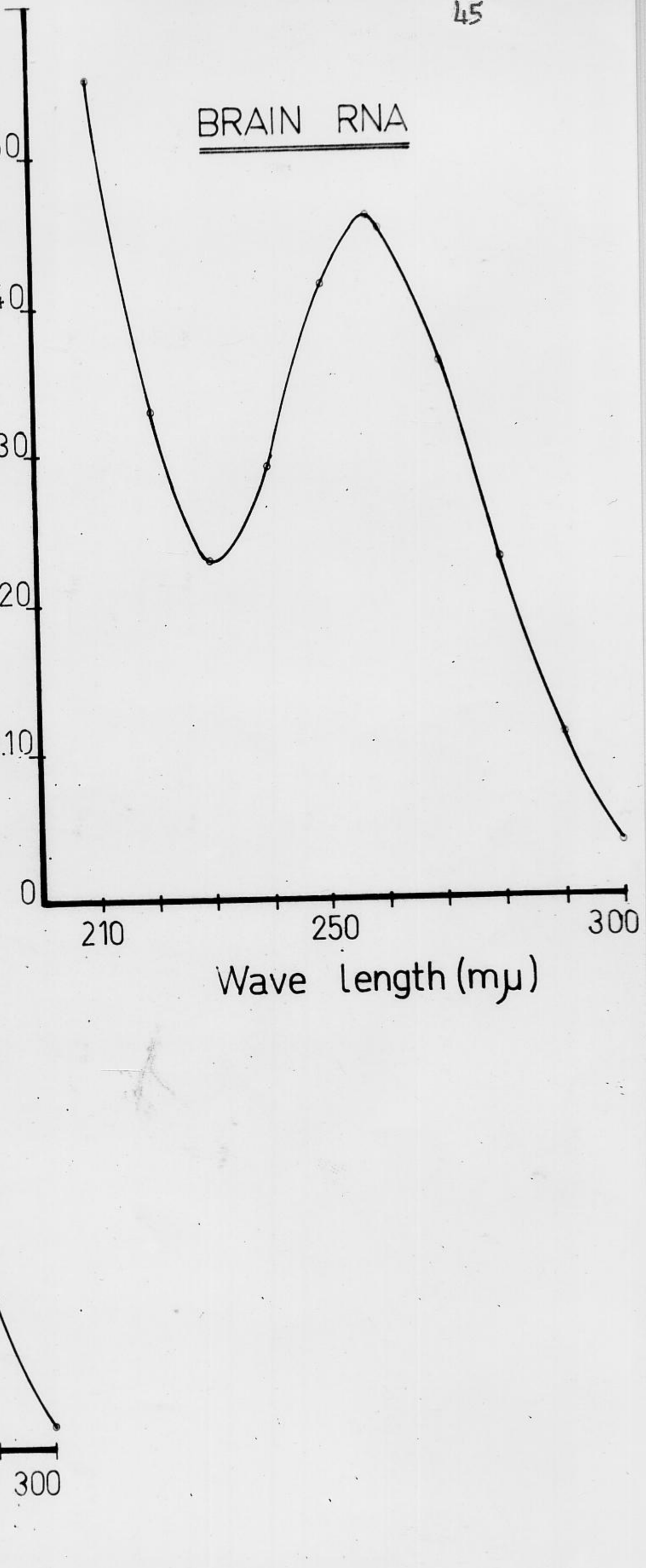


FIG. 1. – Ultraviolet absorption spectra of the ribonucleic acid preparations. (A) RNA extracted from chick brain. – (B) RNA extracted from chick liver.

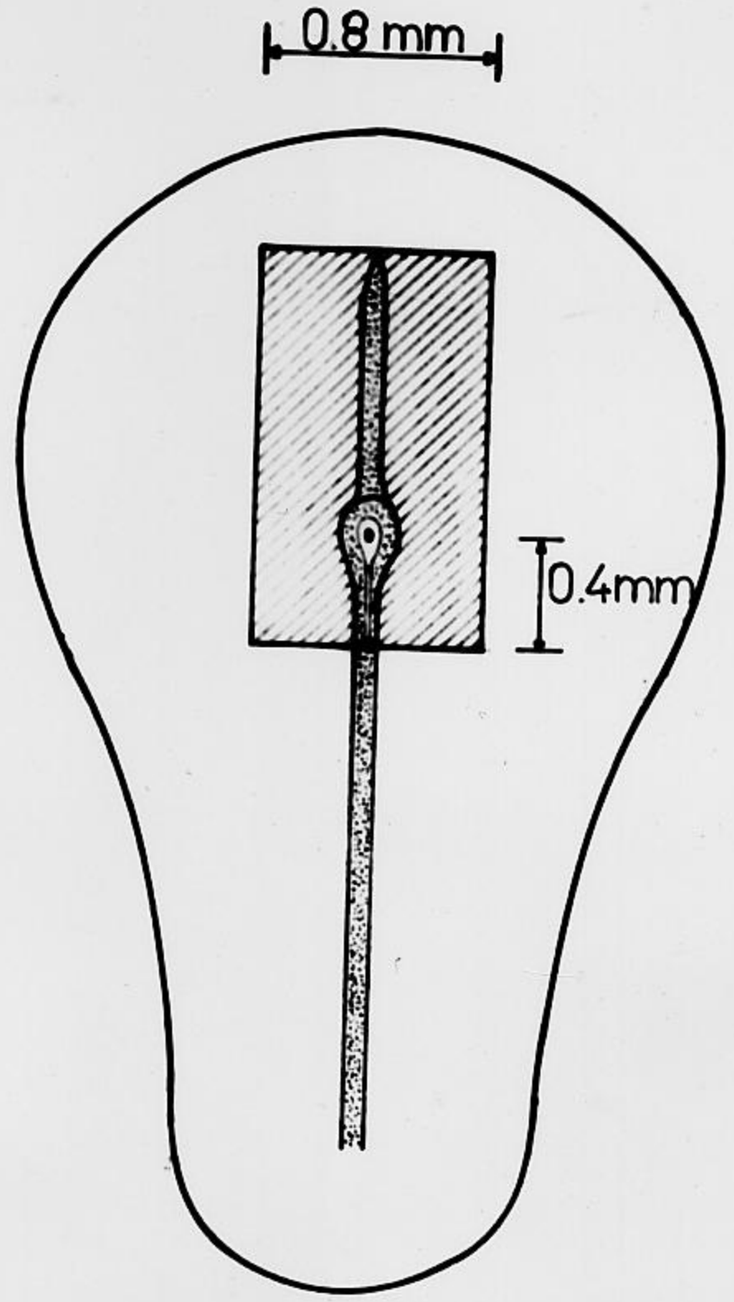
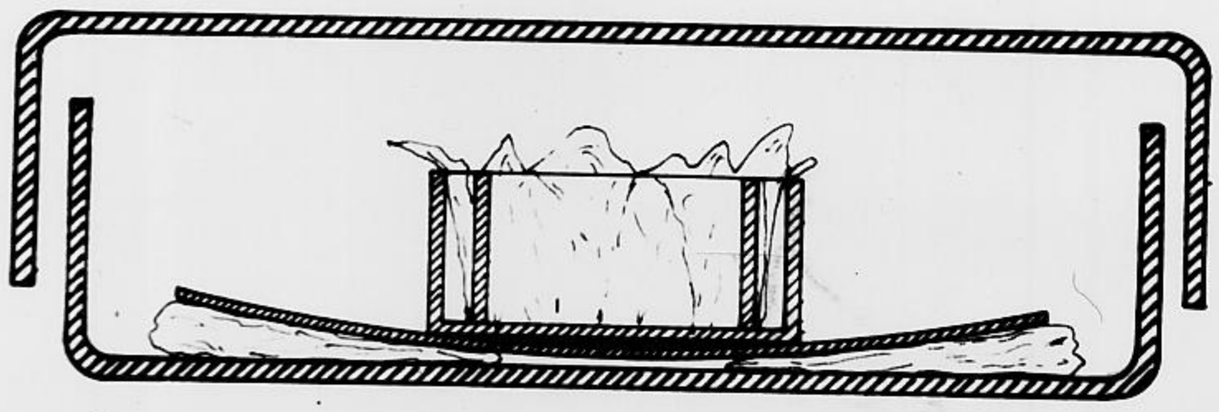
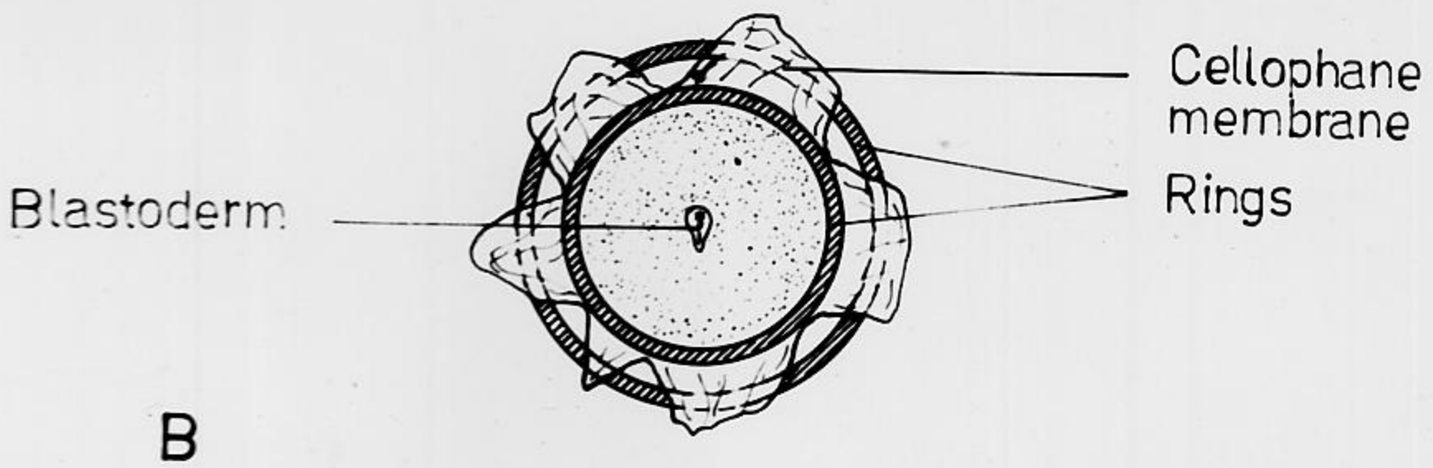


FIG. 2. Blastoderm showing the denuded area of the endmesoderm.



A



B

FIG. 3. Diagrammatic representations of the culturing technique :(A)– Side view of the rings resting on a watch glass inside a Petri dish.(B) – Top view of the two concentric rings showing the cellophane membrane & the Blastoderm.

Plate 1.

Explanation of Figures

A. Photomacrograph of a denuded explant showing the development of two heart tubes anterior to the brain.

B. Longitudinal section of an intact blastoderm. Note appearance of forebrain (f. br.), optic vesicles (opt. ves.) and somites (so.). 75X.

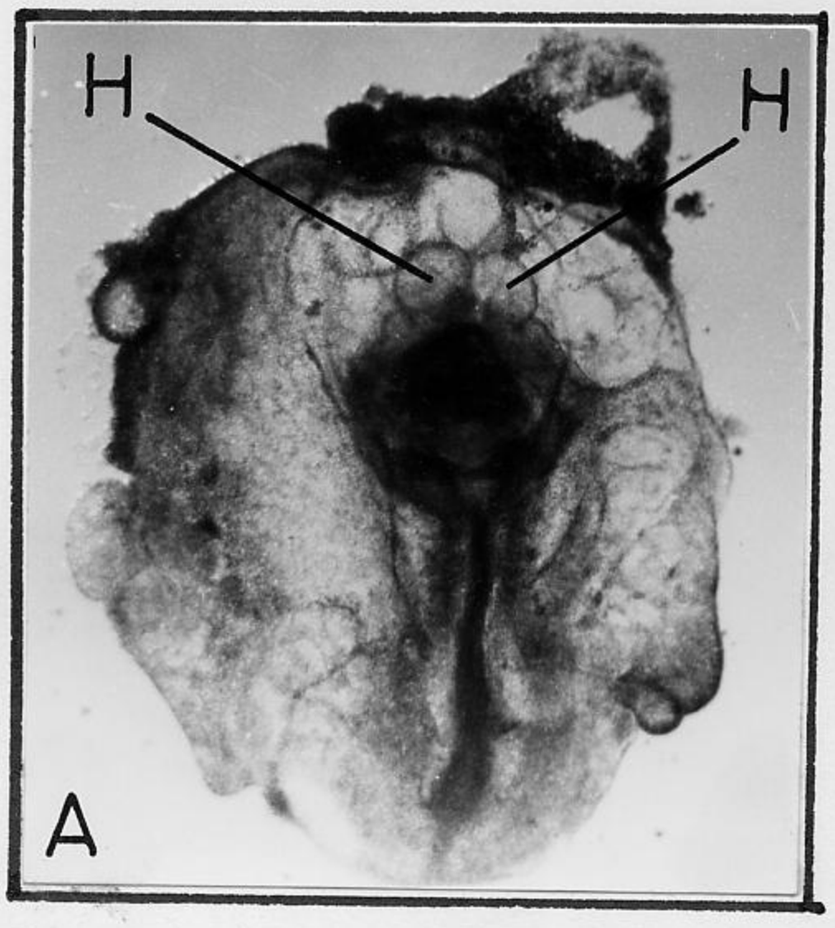
C. Cross section through the two heart tubes of a denuded culture. Note forebrain in the middle. 75X.

D. Cross section through the heart region of an intact blastoderm. 75X.

E. Cross section of a denuded blastoderm at the level of the forebrain showing abnormal appearance of optic vesicles. 75X.

PLATE 1.

1.0 mm



0.2 mm

Scale for figs. B, C, D & E

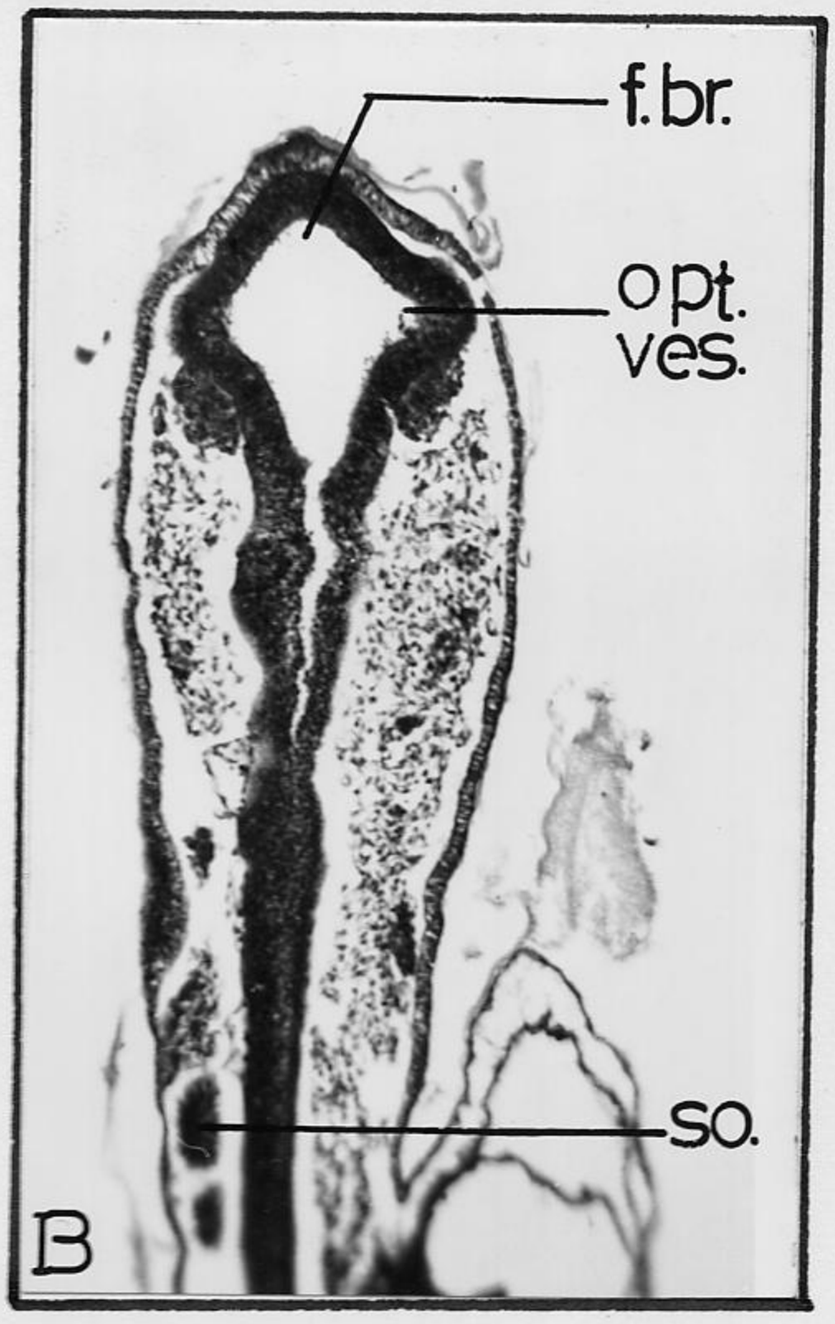


Plate 2.

Explanation of Figures

A. Appearance of two neural tubes in specimen No. DRT 43. Denuded cultured on basic medium. 75X.

B. Specimen No. DRT 102, denuded, treated with boiled brain-RNA. Note appearance of two neural tubes and two notochords. 75X.

C. Section showing two heart tubes in a denuded blastoderm. 75X.

D. Appearance of flattened neural plate in an intact explant. 75X.

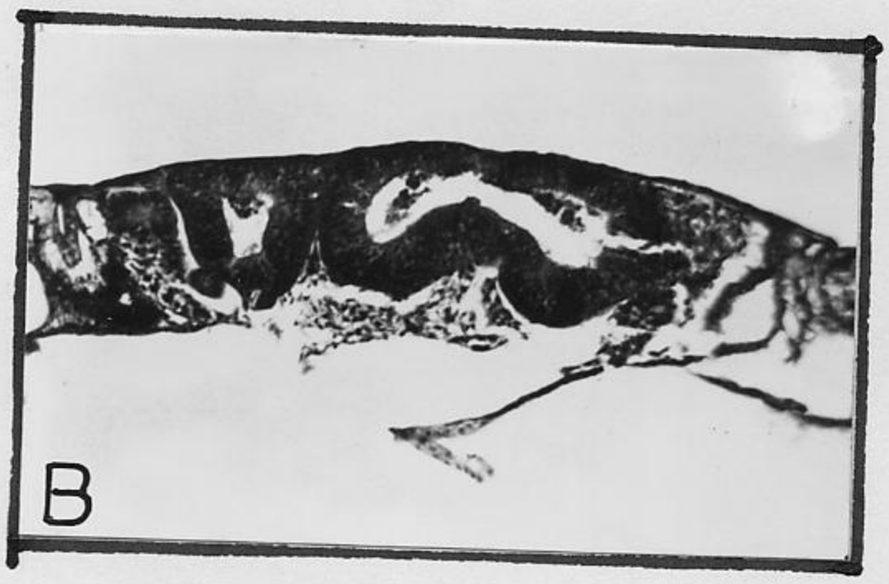
E. Brain-RNA-treated culture. Note the duplication of neural tube. 75X.

F. Specimen No. DRT 30 (Denuded with basic medium). Note ectodermal convolutions and attempts towards tubulation. 75X.

G. Liver-RNA-treated blastoderm. Note appearance of two notochords. 150X.

H. Enlarged notochord appearing in a denuded control. 150X.

PLATE 2.



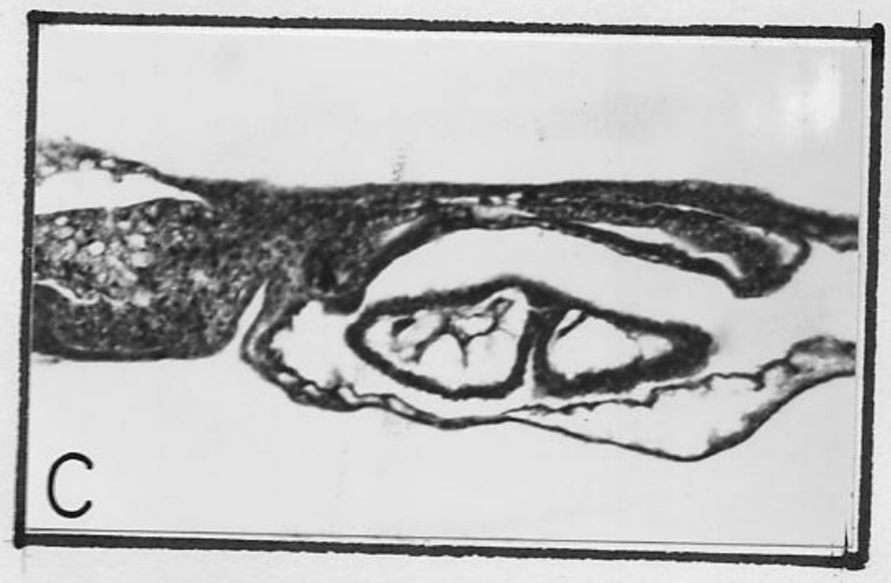


PLATE 2.