THE EFFECT OF ACTINOMYCIN D
on
EARLY DEVELOPMENT OF THE CHICK EMBRYO

by

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ABSTRACT

The morphological, histological and biochemical effects of actinomycin D on early development of the chick embryo were described. The results varied with the stage of development and the concentration of the drug. The antibiotic, at a concentration of 0.15 ug/ml, inhibited all organ primordia at the medium streak stage; from that up to the four-five somite stage partial inhibition was observed. Blood islands were found to be the most resistant to the antibiotic, then came, in decreasing order, heart, notochord, hindbrain, forebrain and somites. Partial recovery from the effects of drug was observed when embryos were transferred from the antibiotic-containing medium to a control medium. Complete recovery resulted on addition of calf thymus DNA to the antibiotic. The mechanism of action of the drug, correlation between resistance of different organ primordia to it and their embryonic determination were studied.

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INTRODUCTION

Actinomycin D (AD) is an antibiotic produced from a soil Streptomyces called Streptomyces parvullus (Manaker et al., 1955). It was isolated 26 years ago by Waksman and Woodruff (1940), and was shown to consist of red rhomboid prisms. It has a melting point of 243°C and a molecular formula of $C_{60}H_{70}O_{15}H_{12} \cdot 3H_2O$ (Manaker et al., 1955). Paper chromatography shows the presence of basic components common to all actinomycins plus five other components particular to AD (Vining and Waksman, 1954).

Action of AD on Microorganisms

During the past twelve years the effects of AD had been analysed in a variety of organisms. It is a growth inhibitor in microorganisms, such as Neurospora crassa (Kersten et al., 1960, Escherichia coli (Woodruff and Waksman, 1961), Staphylococcus aureus (Kirk, 1960), Lactobacillus arabinosus and Lactobacillus leichmanni (Wheeler and Bennet, 1962). The same phenomenon was observed in gram positive bacteria, gram negative bacteria, and fungi (Pugh, Katz, and Waksman, 1956). In presence of AD, nucleate fragments of Acetabularia sp. failed to form caps (Denis, 1964).

Action of AD on Eggs and Embryos

Eggs and embryos of some animals experience growth inhibition by the antibiotic. Ascidia nigra eggs placed in 200 ug/ml of AD failed to undergo blastulation, while in 20 ug/ml they developed

into swimming larvae with abnormalities like humped- or crooked-tails (Market and Cowden, 1965). Sea urchins of Lytechinus variegatus in 200 ug/ml AD were arrested at the blastula stage (Market and Cowden, 1965); those of Paracentrotus lividus in 40 ug/ml were slowed at the morula and stopped at the blastula stage (Lallier, 1963).

Amphibian, avian, and mammalian embryos showed the following effects: a concentration of 100 ug/ml caused cesation of development in Rana pipiens embryos by mid gastrula and subsequent death. However, when a concentration of 7.5 to 10 ug/ml was used, gastrulae developed to the tail bud stages. Neurulae and tail bud stages were more resistant (Flickinger, 1963). Xenopus sp. eggs treated with AD showed exogastrulation, and removal of vitelline membrane resulted in absence of nervous systems. Concentrations of 2.5 to 5 ug/ml produced microcephally (Brachet and Denis, 1963). Suppression of the inducing power of the blastopore and dedifferentiation of ectoderm, after neurulation, resulted when Pleurodeles sp. were exposed to AD for two hours (Denis, 1964).

Research of the effects of AD on avian embryos began five years ago with emphasis on the chick. Pierro (1961) observed that rumplessness, trunklessness, absence of notochord and nerve chord resulted when AD was injected into the yolk sac of chick embryos of 48 hours of incubation age. Cells in the trunk-tail region and in the unsegmented somite mesoderm were degenerated. Concen-

trations of 0.125 to 0.5 ug/ml inhibited development and growth of the axial skeleton posterior to the 12th somite on culturing embryos for 48 hours. Head and trunk portions derived from segmented somite mesoderm were not affected (Klein and Pierro, 1963). Chick embryos at the primitive streak stage, exposed to 0.1 ug/ml AD showed dedifferentiation of the nervous system and lysis of the posterior region (Pohl, 1964). Inhibition of haemoglobin synthesis, to about 40 per cent, was noticed in chick embryos between the definite streak and eight somite stage when exposed to 0.25 ug/ml of AD (Hell, 1964).

Rat and rabbit embryos were the most commonly used. Intraperitoneal injection of rats on the 7th, 8th, and 9th days resulted in embryos with abnormal nervous systems and facial skeletons. Malformations of the skeleton, viscera, and cardiovascular systems appeared in rat embryos on injection with the antibiotic (Duplessis and Parot, 1958). Besides its effects on embryos, AD has been used with mammalian cells, tissues, organs, and organisms as a whole. Mammalian cells treated with AD lost their nucleoli and their histochemical RNA. The cytoplasm of mouse fibroblast cells shrank, nuclei enlarged, and nucleoli disappeared on exposure to the drug (Reich et al., 1962, Shatkin et al., 1962). Yafee and Feldman (1964) studied the effects of AD on cardiac and muscle cells of rats. They showed that nondifferentiated cells were destroyed after 24 hours of AD application; however, differentiated cells were

somewhat resistant. Weights of submandibular and parotid glands and body weights of rats showed a decrease during the first week of injection of AD. Cytoplasmic vacuolation and decrease in number of nuclei, after 24 hours of injection, were observed (Jhee, 1965; Angeletti, Salvi, and Tachinni, 1964). Mice treated with AD became thin and showed a remarkable decrease in spleen weights (Manaker et al., 1955; Pugh, et al., 1956). Inhibition of tubulogenesis in mouse foetus was reported when AD was added to both spinal cord and mesenchyme 30 minutes prior to their recombination (Jainchill, Saxen, and Vainio, 1964).

Action of AD on Mitosis

Mitosis in Allium cepa root meristem ceased in 10 ug/ml AD (Ball and Gross, 1963). A concentration of 150 to 250 ug/ml inhibited second mitosis in Physarum polycephalum (Mittermayer, Braun, and Rush, 1965). Inhibition of cell division in Tetraphymena pyriformis resulted on exposure to 100 ug/ml of AD (Whitson and Padilia, 1964). Mitosis in mouse fibroblasts was completely blocked by low concentrations of AD (Reich et al., 1962).

Action of AD on Tumors

The drug inhibited the appearance of melanotic atypical growth in irradiated males of Drosophila melanogaster (Budeti, 1961). Six out of thirteen patients with methotrexate tropho-plastic disease were relieved when treated with AD (Rose et al., 1962). The antibiotic produced regression in African children

with malignant lymphoma involving the jaw (Oettgen et al., 1963). A recovery rate of 62 per cent in children with Wilm's tumor was observed (Howard, 1965; Sitarz et al., 1965).

Action of AD on Viruses

A concentration of 0.05 ug/ml of AD inhibited influenza virus formation in chicken fibroblasts (Burrinskay and Vorkonova, 1964). Thormer (1965) found that Visna virus in cultures of sheep fibroblast was destroyed 24 hours after infection. Formation of the DNA-containing vaccinia virus was blocked by AD (Shatkin, 1962), while Mengo virus, made of RNA, was unaffected (Reich et al., 1962).

Effect of AD on Regeneration

Complete arrest of oral regeneration in tail pieces of Stentor coerulus resulted on treatment with 250 ug/ml of AD. When regeneration was initiated, no effect of the drug was noticed (Whitson, 1965). Yamada and Roesel (1964) found that injection of AD, at a concentration of 1(one) ug/ml, into the body cavity of Triturus vividescens suppressed regeneration and caused disintegration in the lens epithelium.

Metabolic Effects of AD

The metabolic effects of AD have also been studied, especially by radioautography. Recent, but significant evidence shows that the drug interferes with synthesis of RNA, DNA, and proteins.

The suppression of RNA synthesis by AD has been demonstrated by the inhibition of the incorporation of :H^3- uridine into chicken fibroblasts to about 70 per cent (Scott and Bell, 1965); P^{32} - orthophosphate in all "species" of RNA in rat liver (Meritz, 1963); C^{14} - orotic acid in rat diaphragm (Sovik, 1965; Tamaoki, Gerald, and Muller, 1962); and C^{14} - guanine into RNA purines of Ehrlich ascites tumor cells (Harber and Muller, 1962). Aggregates of ribosomes in rat liver, held together by mRNA, are broken down into monomers and dimers when exposed to AD (Theophill, 1963). Cytoplasms of Cloud melanoma cells were devoid of RNA on treatment with the antibiotic (Shatkin et al., 1962).

Inhibition of DNA synthesis has been noticed in a number of vertebrates. This was demonstrated in: Pleurodeles sp. (Denis, 1964); chick (Klein and Pierro, 1963; Pohl, 1964), and mouse embryos (Wessels, 1964). Also, AD caused a decrease in chromosome puffing and formation of Balbiani rings in Chironomus thummi within 24 hours (Laufer, Nakase, and Vanderberg, 1964).

Haywood and Sinsheimer (1963) observed inhibition of protein synthesis in E. coli protoplasts by AD. The same phenomenon was noticed in Pleurodeles sp. blastulae, gastrulae, and neurulae (Denis, 1964), in the posterior parts of chick embryos (Klein and Pierro, 1963), and in rat liver (Korner and Monro, 1963).

Inhibition of incorporation of C^{14}-histidine into calf lenses was observed after 30 hours exposure to the drug (Spector and Kinoshita, 1965).

Purpose of the Work

From the above considerations, it is clear that, inspite of the great deal of work with AD, yet there is very little information available on its effects on the very early development of vertebrate embryos. Also, it seems that there has been no comprehensive study of the morphological, metabolic, and histological effects of this antibiotic on any vertebrate embryos so far studied. The question of whether a concentration of AD applied at an early stage of development would inhibit all organ formation, or would have a differential effect at a slightly later stage has not been answered. Furthermore, determination of the critical stage which is not affected by AD has not been investigated. Thus, it seemed necessary to compare the effects of different concentrations of AD on different stages of development to elucidate the above questions. In this study, the chick embryo was used for this purpose.

MATERIALS AND METHODS

Source of Eggs

Fertile Leghorn eggs supplied by Agrico, A.U.B. farm, and Vitasni, were stored at 17 to 18°C and were used within five days.

Preparation of Media

Spratt's culturing technique (Spratt, 1947) was used with slight modifications. A whole egg was homogenised in a 100 ml

flask and 10 ml of the homogenate were put in a 50 ml flask and kept at 40°C. 80 mg of Bactoagar were placed in a 50 ml flask containing 10 ml of chick Ringer's solution. This was boiled and cooled down to 42°C, then added to the 10 ml portion of the egg homogenate. The mixture was placed in a water bath at 40 to 42°C, with occasional shaking, for 10 to 15 minutes. Then the medium was poured into sterile watch glasses within petri dishes surrounded by rings of cotton moistened with chick Ringer's solution.

The antibiotic-containing medium was prepared in a similar way. Three different concentrations of the antibiotic were used. These were: 0.15, 0.05, and 0.01 ug/ml of the final agar-egg mixture. These were prepared by adding 0.6, 0.2, and 0.04 ml from a stock solution of the drug, containing 5 ug/ml, to the 20 ml of the egg-agar mixture.

Calf thymus deoxyribonucleic acid (DNA) was added to the drug-containing medium to give a final concentration of 10 ug/ml of the final mixture. The DNA was added to the antibiotic 24 hours prior to their mixing with the egg homogenate.

Operation

Fertile eggs incubated for 22 to 28 hours were opened in finger bowls containing about 200 ml of chick Ringer's solution at 37 to 38°C. A cut was made along the circumference of the blastoderm. The blastoderm was removed with a pair of forceps and was allowed to settle in the finger bowl. The vitelline membrane

was removed with fine forceps, and the blastoderm was transferred with a wide-mouthed pipette to a syracause dish containing about 10 ml of warm Ringer's solution. Most of the area opaca was trimmed off with a glass needle. Embryos were examined under a dissecting microscope to determine their stage of development. They were classified as follows: definite streak (DS), head process (HP), head fold (HF), two-somites (2S), and four-somites (4S). Then, embryos of the five classes were transferred, with a wide-mouthed pipette, onto the media labelled: control (C), actinomycin D(AD), and actinimycin D-deoxyribonucleic acid (AD+DNA). The embryos were placed ventrally onto the media, and excess fluid was removed. Petri dishes were covered and incubated at 38°C.

Sterile technique was followed in preparation of media, glass ware, and explantation of embryos.

Recovery from AD

Possibilities of recovery of embryos from the effects of AD were tried in two ways: either by addition of DNA to the AD-containing medium, as explained before, or by transferring embryos (DS) to control media after culturing them on AD-media for about seven hours. Viability of embryos was tested by staining them with neutral red.

After the incubation period, embryos were flooded with chick Ringer's solution, removed from petri dishes, transferred to syracause dishes and examined under a binocular. Detailed

observations on each embryo were recorded. Following this, embryos of the five stages and under different treatments, were examined for the following purposes:

1. Morphological Study

Representative embryos were fixed in Bouin's solution for about 30 minutes, stained with alum cochineal for five minutes, and mounted with kleermount.

2. Histological Study

Embryos were fixed with Bouin's solution for about 30 minutes, serially sectioned at 10 micra, stained with Delafield's hematoxylin and eosin, and mounted. In the above two studies, photomicrographs of representative whole mounts and sections were taken by a Carl Zeiss microscope.

3. Quantitative Biochemical Study

This is divided into three categories: determination of RNA, DNA, and protein nitrogen (PN). Before determination, the above metabolic constituents were extracted according to the following procedures:

A group of embryos (six to eight) were placed in two ml of 5 per cent trichloroacetic acid (TCA), and were thoroughly homogenized. The sample was washed with two-ml portions of 5 per cent TCA, three times, to remove amino acids and small polypeptides. The precipitate was washed with two-ml portions of 5 per cent TCA in a water bath (90 to 95^{o}C) to extract the nucleic acids. The supernatent

containing the nucleic acids was saved for later separation of RNA and DNA. The residue was extracted with two ml of three: one ethanol chloroform at 70°C to remove the lipids. The final precipitate contains the proteins (Hell, 1964).

The RNA was extracted with 10 per cent perchloric acid (PCA) from the nucleic acid fraction at 4°C for about 18 hours. DNA was extracted with 5 per cent PCA at 70°C for 20 minutes (Hell, 1964).

Quantitative Determination of RNA

Miller's method (1956) was used to one volume of the RNA-containing fraction, an equal volume of the orcinol reagent was added. The mixture was boiled in a water bath for about 30 minutes. The beaker containing the tubes was then placed under running water for green color development. Readings were taken with DU Beckman Spectrophotometer at 675 mu against a blank. RNA from Nutritional Biochemical Corporation was used as a standard.

Quantitative Determination of DNA

One volume of the DNA-containing sample was mixed with a double volume of the diphenylamine reagent. The reaction mixture was heated in a boiling water bath for about 10 minutes for blue color development, and the optical density was read with a DU Beckman Spectrophotometer at 595 mu against a blank (Siebert, 1940). Calf thymus DNA from Man Research Laboratories was used as standard.

Quantitative Determination of PN

Chaney's and Marbach's method (1962) modified by Shahinian (1966) was used. One ml of the protein fraction was pipetted into a 30 ml Kjeldahl digestion tube graduated at 25 ml mark. One ml of 10 N H_2SO_4 and two glass beads were added. The tubes were heated on microdigestion flames until water evaporated and dense fumes of SO_3 appeared. The tubes were covered with glass stoppers and digestion was continued for 20 more minutes. After cooling, five ml of distilled water were added to each tube, thoroughly mixed, and four ml of 2.5 N NaOH were added to neutralize the H_2SO_4. Contents of the tubes were then diluted to 25 ml with distilled water, and mixed by inversion. From each tube, one ml was taken and to it one ml of five per cent phenol nitroprusside and sodium hypochlorite, respectively, were added. The ingredients were mixed and incubated at 37°C for 15 minutes for blue color development. Contents were diluted by adding 10 ml of distilled water. Absorbance was read with DU Beckman Spectrophotometer at 630 mu. Ammonium sulphate (Lang, , 1958) was used as a standard.

EXPERIMENTAL RESULTS

AD added to the culture medium of chick embryos affects the morphology, histology, and biochemical activity of these embryos. The effects are diverse and characteristic of the stage of development and the dose used.

I. MORPHOLOGICAL EFFECTS

A. Effects on Elongation of the Embryos:

1. Definite Streak Stage: A supression of elongation of the embryos was noticed. The most serious effect was experienced by medium and definite streak stages, where embryos of the first and second group had an average length of 1.8 and 2.0 mm, respectively. This was noticed when 0.15 ugm/ml of the drug was used. This effect was reduced with decrease in concentrations of AD. When concentrations of 0.05 and 0.01 ug/ml were used embryos had an average length of 2.8 and 3.6 mm, respectively. Embryos treated with 0.15 ugm/ml of AD plus 100 ugm/ml of DNA and control embryos had approximately the same length (4.1 mm in the first case, and 4.2 mm in the latter).

2. Head Process Stage: Here, lengths of the embryos were: 3.1, 3.5, 3.8, 4.1, and 3.9 mm, corresponding to the treatments 0.15, 0.05, 0.01 ugm/ml, AD + DNA and controls. Notice that embryos of this stage treated equally (0.15 ugm/ml of AD) as embryos of DS-stage, showed an average increase in length of 1.1 mm. (Table I).

3. Head Fold Stage: The results showed a difference of 0.9 mm between 0.15 ug/ml AD treated and control embryos. The lengths were: 3.4, 3.5, 3.8, 4.5, and 4.3 mm, corresponding to of 0.15, 0.05, 0.01 ugm/ml AD, AD + DNA, and control treatments.

4. Two-Somite Stage: Only the 0.15 ugm/ml AD treated embryos showed a slight decrease being 0.4 mm shorter than controls; embryos of the first group had an average length of 3.8 mm and those of the second 4.2 mm. Lengths of embryos treated with 0.05, and 0.01 ugm/ml AD, and AD + DNA were 3.9, 4.1 and 4.5 mm, respectively.

5. Four-Somite Stage: Embryos exposed to 0.15, 0.05, and 0.01 ugm/ml had lengths of 4.1, 4.1, and 4.3 mm, respectively. Control and AD + DNA treated embryos had lengths of 4.7 and 5.0 mm. (Table I).

B. Effects on Blood Islands:

Some arbitrary symbols are used to designate approximate number of blood islands. These are: + indicating around 25, and ++ indicating around 50 blood islands, and so on. On this basis, the different effects of the drug are compared. In this study, the consecutive symbols correspond to the following treatments: 0.15, 0.05, 0.01 ug/ml AD, AD DNA, and control.

Definite Streak Stage: Embryos of this stage had a very small number of blood islands (+) when cultured on 0.15 ug/ml AD. However, the number increased as the dosage decreased as shown; ++, in the second third treatments, and +++ in the last two treatments. Notice that embryos treated with 0.15 ug/ml AD showed about 65 per cent less blood islands compared

to controls (Table I).

Head Process Stage: A noticeable increase in the number of blood islands was observed. Notice that embryos treated with 0.15 ug/ml AD had ++. Thus the inhibition of blood islands formation amounts to about 50 per cent, compared to control embryos. The amount reached +++ in the second and third treatments, and ++++ in the fourth and fifth treatments.

Head Fold Stage: Embryos treated with 0.15 ug/ml AD developed +++ under the first three, and ++++ under the last two treatments. Notice that the percentage of inhibition goes down to about 25 on treatment with 0.15 ug/ml AD.

Two Somite-Stage: As table I shows, only embryos treated with 0.15 ug/ml AD developed +++ while under other treatments they developed ++++.

Four Somite-Stage: The relative amounts of blood islands ranged from ++++ in the first treatment to +++++ in other treatments. Thus, there is about 20 per cent inhibition.

C. Effects on the Nervous System:

1. Definite Streak Stage: Embryos treated with 0.15 ugm/ml AD had an undifferentiated mass of nerve cells, more or less bulk-like in shape (Fig. 2). In no case was the brain divided into vesicles. In all cases (10), tips of brains were opaque and

cytolyzed. On treatments with 0.05 ugm/ml AD, embryos showed slightly differentiated brains, with tiny lateral protrusions, as eye vesicles primordia. Mid brains, with convex walls, and linear hindbrains were present. Conspicuous eye vesicles, mid brains, and better outlines of hind brains appeared in embryos exposed to 0.01 ugm/ml. Small, but clear optic vesicles, mid brains, and hind brains with three neuromeres were observed in control and AD-DNA treated embryos. Constrictions of the three brain divisions were clearly seen. Embryos of the medium streak stage developed no brains on treatments with AD (Fig. 1).

The posterior part of the nervous system, namely the neural folds and the unsegmented somite mesoderm around them, were seriously distorted on treatment with 0.15 AD. These were cytolyzed, chalky, and largely vacuolated (Fig. 2). The effect was less severe as the dosage decreased. In embryos treated with 0.05 ugm/ml AD, only the very posterior parts of the embryonic axes showed the above phenomena and in one case, vacuolation was highly accentuated (Fig. 5). In those treated with 0.01 ugm/ml, the axes and the nodal areas were healthy with no indications of cytolosis or opaqueness. In control and AD+DNA treated embryos, the post-embryonic axes and the nodal areas plus the surroundings were in good shape (Fig. 4).

2. Head Process Stage: A common feature common to all treatments was the presence of brains having three linear

vesicles, with clear constrictions between the fore-, the mid-, and the hind-brains. Embryos exposed to 0.01 ugm/ml AD, AD + DNA and control embryos had, in addition to the above, relatively big optic vesicles (0.6 mm distance between the two vesicles). In the latter two cases, the hind brains were divided into five and three neuromeres, respectively. Posterior embryonic axes were present, although somewhat opaque and cytolysed in embryos treated with 0.15 ug/ml. In five out of six cases, embryos treated with this concentration showed five small vesicles in the nodal area. Similar responses appeared in embryos treated with 0.05 ug/ml. Normal embryonic axes were present in embryos cultured on 0.01 ug/ml AD, AD + DNA, and control media.

3. Head Fold Stage: Embryos exposed to different treatments had forebrains with optic vesicles (0.6-0.7 mm apart), convex-walled mid brains, and hind brains with three neuromeres. AD + DNA treated embryos had hind brains with five neuromeres. Post embryonic axes and nodal areas of embryos treated with 0.15 ug/ml and 0.01 ugm/ml of AD showed signs of breakdown and opaqueness. A characteristic rhomboid-like axis was observed in one embryo treated with 0.15 ug/ml. Embryos treated with 0.05 ug/ml had two-three big vacuoles in their posterior axes; in one case two big membraneous vacuoles were noticed (Fig. 6) . AD+DNA treated and control embryos had normal, healthy posterior portions.

4. Two-and Four-Somite Stages: Embryos belonging to these

stages shared the following common effects on different treatments: relatively big forebrains with optic vesicles 0.7 mm apart, a more or less circular mid brains, and hind brains with three to five neuromeres (Fig. 11). Their axes and nodal areas were quite normal in shape. An exceptional case was one when an embryo at the two-somite stage treated with 0.15 ug/ml AD had a cytolyzed posterior axis with two small vacuoles around it. Also, the brain was opaque and poorly differentiated (Fig. 7).

D. Effect on Somite Formation:

1. Definite Streak Stage: Somite genesis was completely blocked in embryos treated with 0.15 ug/ml AD. However, concentrations of 0.05 and 0.01 ug/ml left five and thirteen somites, respectively. Control and AD + DNA treated embryos had an average of 12 and 14 somites. Thus, it was noticed that 0.01 ug/ml of the drug had no effect on somite formation (Table I).

2. Head Process Stage: The effect was less pronounced at this stage. Treatment with 0.05, 0.01 ug/ml, and AD + DNA resulted in embryos with 10, 16, and 14 somites. In case of the 0.15 ug/ml AD treatment, no remains of the somites were seen. Control embryos had 14 somites (Table I).

3. Head Fold Stage: Embryos exposed to 0.15 ug/ml AD had remains of 3 to 4 somites, with 10 and 14 on treatment with 0.05 and 0.01 ug/ml (Fig. 6). Control and AD-DNA treated

embryos had 15 and 16 somites, respectively.

4. Two- and Four-Somite Stages: Only embryos of the two-somite stage exposed to 0.15 ug/ml AD had eight unhealthy somites (Fig. 7). Other treatments resulted in clear-cut somites ranging from 16 to 20 in number (Figs. 8, 9, 10, and 11).

E. Effect on Heart Formation:

The heart seemed to be one of the most resistant organs to the antibiotic. Inhibition of heart formation was noticed in medium and definite-streak stages. In all later stages and under different treatments of the drug, the heart was persistant. Treatments of the later stages resulted in S-shaped hearts which varied from 0.2 mm in embryos (HP) treated with 0.15 ug/ml AD to 0.8 mm in control embryos at the four somite stage (Table I, and figs. 1-12, plate 1).

II. HISTOLOGICAL RESULTS

A thorough study of the histological effects of the antibiotic at a concentration of 0.15 ug/ml on the six early stages of development showed the following results:

Medium Streak Stage

In all embryos sectioned (6) big vacuoles were observed among masses of cells. Cells were round with distinct nuclei, but with very few mitotic figures. In middle and posterior sections vacuolation was highly pronounced. At these levels, cells lacked

distinct nuclei and cytoplasmic membranes. No notochords, guts, dorsal aortae, hearts and somites were noticed.

Definite Streak Stage

Two out of seven embryos had neural plates. The other five had ectodermal elevations made up of cytolysed cells. At level of the heart, embryos of the first group had tiny round notochords (with 6 cells each), and very small hearts. However no dorsal aortae were found. In the second group, the ectodermal elevations persisted. Here, a series of vesicles occupied the area between ectodermal and endodermal layers. Fig. 13, plate 2, shows an example of such a case.

Lateral vacuoles replaced somites in all embryos. At the level of Hensen's node the three germ layers joined, each was made of one single layer of cytolysed cells.

Embryos of the same stage but treated with AD + DNA developed round brains with optic vesicles. Fig. 18, plate 2, is a section of an embryo at level of the heart. Notice the closed neural tube, the two dorsal aortae, the gut and the heart. Somites differentiated into dermatome, myotome, and scleretome were present (fig. 22, plate 2). At the level of Hensen's node, neural grooves and folds with distinct round cells were seen. Mitotic figures were much more than in 0.15 ug/ml AD treated embryos. The above characteristics applied to control embryos.

Head Process Stage

Two out of five embryos developed closed neural tubes at level of forebrain. The other three had neural plates. In all embryos, and at the level of the heart neural folds failed to meet, thus showing unclosed neural tubes. Small round notochords, with 10 cells each, were noticed. Ventral to that guts and hearts were present (fig. 15, plate 2). Cells in the above structures had distinct nuclei and clear cytoplasms. However, mitolic figures were few.

Posterior to the hearts, small aggregations of somatic cells appeared on either side of the notochords. These looked more mesenchymal in nature. Ectodermal depressions were observed at level of Hensen's node. Cells looked more triangular than round. Also, they were not as healthy as the anterior ones. Big lateral vesicles occupied the mesodermal layer.

Head Fold Stage

Anteriorly, neural tubes were closed only in four out of nine sectioned embryos. The cavities of the tubes were very small, and in three cases were filled with cytolysed cells. Failure of closure of neural tubes was observed in all embryos before, at, and after the level of the heart (fig. 16, plate 2). Oval to round notochords, with fine cells each, wide guts, and hearts were present in all embryos. These organ primordia had cells with distinct nuclei.

At the level of somites, two masses of cytolysed degenerated cells were present on either side of notochord. About 70 per cent of the cells lacked clear cytoplasmic membrane and distinct nuclei. As fig. 20, plate 2, shows, cells in the intermediate mesoderm looked healthier. Notice the triangular-shaped closed neural tube and the cytolysed notochord.

Posteriorly, at level of Hensen's node, round vesicles appeared on the two sides, occupying place of mesodernal layer. Cells lacked distinct nuclei and cytoplasmic membranes.

Two-Somite Stage

In six sectioned embryos closed neural tubes were present. In three of these, small optic vesicles were noticed as lateral extensions of the forebrains. Also, they had otic vesicles at level of hindbrains.

Fig. 17, plate 2, shows a section at the level of the heart. Notice the triangular-shaped neural tube, the round notochord with 12 cells, anterior cardinal veins, dorsal aortae, gut, and a heart. The above characteristics applied to all embryos sectioned, except two where the neural tubes were round at level of the heart.

Although somites were represented by groups of compact cells, they were not differentiated into dermatome, myotome and scleretome as in control embryos. Cytoplasmic membranes and nuclei of cells were not clear-cut. (Figs. 21, 22, plate 2)

On either side of Hensen's node disintegrated masses of cells, separated by big space, were noticed. Cells looked round with distinct nuclei; few had mitotic figures. (Fig. 23, plate 2)

Four-Somite Stage

The eight sectioned embryos showed characteristics quite close to control embryos. Embryos sectioned at level of forebrain showed distinct optic vesicles, and at level of hindbrain otic vesicles were noticed. At level of the heart, normal organ primordia, exactly as in fig. 18, plate 2, were observed. Somites were in a normal shape: a dermatome on the outerside followed by a myotome then by a scleretome, were present. At the level of Hensen's node, cells looked very healthy with distinct cytoplasmic membranes and nuclei. The above characteristics were noticed in embryos with five to seven somites before culturing on 0.15 ugm/ml AD media.

III. BIOCHEMICAL RESULTS

Under this section, amounts of RNA, DNA, and PN in ug/embryo were determined.

A. Effect on Amount of RNA

A great decrease in amount of RNA was noticed in embryos, up to the two-somite stage, when cultured on media

containing 0.15 ug/ml AD compared to control embryos of the same stages. Inhibition reached about 83 per cent in embryos of DS-stage, about 56 per cent of HP-stage, about 40 per cnet at HF-stage, and about 34 per cent of 2S-stage. In embryos of four somite-stage, the percentage of inhibition was about 8 per cent (Table II).

A decrease in the dosage caused a reduction in the percentage of inhibition. When a concentration of 0.05 ug/ml AD was used, embryos at DS-, HP-, HF-, and 2S-stage, showed an inhibition of 50, 34, 26, and 15 per cent, respectively. Embryos exposed to 0.01 ug/ml AD showed a 20 per cent reduction at DS-stage, and five per cent at 2S stage (Table II).

In all stages of development studied and under AD + DNA treatments, amount of RNA not only approached that in control embryos, but exceeded it in two cases: HP which had an increase of about seven per cent, and 4S-stage which showed an increase of four per cent (Table II).

B. Effect on Amount of DNA

Embryos at DS-stages experienced a marked decrease in the amount of DNA. The inhibition of DNA formation reached about 78 per cent on treatment with 0.15 ug/ml AD, as compared to control embryos of the same age. Under the same treatment the blocking of DNA was 42 per cent in embryos at HP- and HF-stages,

and about 32 per cent at 2S-stages. 4S-embryos showed a decrease of about 15 per cent compared to controls of the same stage.

When a concentration of 0.05 ug/ml AD was used, the percentage of inhibition decreased to about 65, 34, 28, and 15 per cent at the DS-, HP-, HF-, and 2S-stages, respectively. Embryos at the 4S-stage showed a decrease of about seven per cent compared to control embryos. A concentration of 0.01 ug/ml reduced the inhibition to about six and 16 per cent at the DS- and 2S-stages (Table II).

It is worth pointing out that embryos took up DNA when cultured on media containing AD+DNA. The increase in the amount of DNA was 12 per cent at DS-stage, 36 per cent at HP-stage, and 15 per cent at the 4S-stage, compared to controls of the two stages.

C. Effect on Amount of PN

The correlation between the effects of AD on PN and RNA is quite clear. Thus embryos at DS- and HP-stages cultured on media containing 0.15 ug/ml of the drug showed a decrease of about 75 and 55 per cent, respectively, in amount of PN, compared to control embryos. To recall, the percentage of RNA inhibition in embryos of same stages was about 83 and 56 per cent. Under the same treatments, the reduction in PN dropped down to about 29, and 38 per cent in embryos at HF- and 2S-stages. Embryos at the 4S-stage showed an increase of about three per cent over controls (Table II).

Further decrease in suppression in amount of PN was correlated with the decrease in concentration of the antibiotic. The inhibition went down to about 38, 34, 10, and 33 per cent in embryos at DS-, HP-, HF- and 2S-stages. The concentration used was 0.05 ug/ml. Here, too, 4S-stage embryos showed an increase of about eleven per cent over controls. The inhibition reached a minimum when 0.01 ug/ml of the drug was used. Thus, at DS- and 2S-stage, embryos experienced a reduction of about 14 and seven per cent (Table II).

In four out of the five stages studied, amount of PN in AD + DNA treated embryos exceeded that in controls of the same stages. The percentage increase reached about 14, 4, 7, and 16 in DS-, HF-, 2S-, and 4S-stages, respectively. (Table II).

IV. VIABILITY DETERMINATIONS

Morphological Results

Nine embryos at definite streak stage were grown for seven hours on media containing 0.15 ug/ml AD, transferred to control media, and left there for about 15 hours. At the end of the culturing period, embryos were transferred to syracuse dishes. Neutral red was added to test viability. Viability determination was also run on embryos cultured for the 24 hours on 0.15 ug/ml AD. In both

cases embryos took the stain, thus showing the nontoxicity of the drug at this concentration. In all cases, transferred embryos developed forebrains with small optic vesicles, mid- and hindbrains. Fig. 3 shows one of the embryos thus treated. Two to three somites, which were difficult to see in whole mounts, were present in every embryo. Small fingerlike hearts were noticed. The axes, although not very healthy, looked better than those in embryos left on AD for the 24 hours. Two lateral kidney-shaped vesicles were observed in one of the embryos studied (Fig. 3). Approximate amounts of blood islands were ++.

Histological Results

Five out of seven sectioned embryos had brains with tiny eye vesicles. The other two showed flat neural plates below which tiny round notochords (with nine cells) and wide triangular-like guts were noticed. At the level of the heart concave neural plates were present in all embryos. Walls of hearts consisted of three layers (Fig. 14). Cells in the above organ primordia were either round or oval; they looked much more healthier than cells of embryos of the same stage left on the 0.15 ug/ml AD.

At the level of somites two semi-compact somatic undifferentiated cells were observed (Fig. 19). Posterior to that, vacuolation was seen but highly reduced.

DISCUSSION AND CONCLUSIONS

The above results show that the antibiotic AD exerts either partial or complete effects, depending on the dosage of the drug and the stage of development of the embryos. Total inhibition of all organ primordia appeared in embryos at the medium streak stage (about 18 hours old). From the definite streak onward, partial inhibition of all organ primordia was observed. Thus, at the definite streak, two out of seven sectioned embryos had small hearts (0.1 mm in length); however, all had notochords. In the two cases, the anterior parts of brains were plate-like, with tiny inward foldings at the two ends. Posteriorly, small unclosed neural tubes were noticed. Small amounts of blood islands were present in the seven embryos. In all embryos, somites, dorsal aortae, guts (except in embryos which had hearts), and well differentiated brains were lacking. In the head process stage, embryos had notochords, guts, poorly differentiated somites, and hearts. Although neural tubes were observed in two embryos, in three embryos neural folds failed to meet and form a tube. No optic vesicles were noticed. More or less similar results were found in embryos at the head fold stage. At the two-somite stage, round brains with no distinct optic vesicles were present. Notochords, guts, hearts, and dorsal aortae were observed. Although five to six somites were present, these were in a poor shape. Posteriorly, embryos were cytolysed and vacuolated. The above were noticed on treatment with 0.15 ug/ml AD.

The critical stage was in embryos with four to five somites, where all looked like controls. Somites and posterior axes, which were severly affected in younger embryos, seemed healthy. Embryos at this stage showed an average increase of 14 to 16 somites in about 22 hours of culturing period. Well differentiated brains with eye vesicles, clear-cut round notochords, triangular-shaped guts, dorsal aortae, and relatively big hearts (0.6 to 0.8 mm in length) were very similar to those in controls. Blood islands were approximately equal in amount to those in control embryos.

Pierro and Kleine (1963) found that chick embryos with about 12 somites and cultured for 48 hours, showed cytolysis in the unsegmented somites mesoderm posterior to the 12th somite. In other words, no somite formation took place. Also, reduction in length and thickness of the tail and its absence in some cases, and disruption of the embryonic axes posterior to the 12th somite were reported. Comparing these results with the above ones, one notices that what Pierro and Kleine found applied to embryos up to (but not including) the five somite stage, but not to embryos with more than five somites.

As for the mechanism of action of AD, one group proposes that it inhibits DNA-dependent RNA (messenger or mRNA) and shows evidence that AD inhibits incorporation of radioactive RNA precursors (Harber and Muller, 1962; Pohl, 1964; Scott and Bell, 1965). Another group suggests that AD acts on DNA itself. Their

evidence is that C^{14}-AD was found in the nucleus of cells bound to DNA (Kersten et al., 1960; Kawamata and Imanish, 1960; Goldberg, Rabinowitz, and Reich, 1963).

The results of this work suggest that the second alternative is quite possible. When a small concentration of calf thymus DNA was added to the antibiotic-containing medium (0.15 ug/ml of AD), 24 hours before culturing the embryos, partial recovery of embryos at the definite streak stage was observed. However, when the concentration of DNA was raised to 100 ug/ml, a more or less complete recovery of embryos took place. Embryos developed brains with optic vesicles, notochords, dorsal aortae, guts, somites, and hearts. Their posterior axes looked very healthy. One of the characteristics of metabolic inhibition is its removal by addition of large amounts of the inhibited substance. The validity of this suggestion is further observed by reviewing the metabolic effects of AD. AD-treated embryos showed a reduction in amounts of RNA, DNA, and protein up to 75 per cent (in embryos at the definite streak stage treated with 0.15 ugm/ml AD). When DNA was added to the drug-containing medium, it was noticed that the amounts of the above components not only approached that in control embryos, but exceeded it in some cases.

A possible theoretical explanation of the mode of action of AD might be related to what Jacob and Monod (1961) propose about the gene action in microorganisms. Before dealing with this, a review of

the role of genes in protein synthesis is worth mentioning at this point. Protein formation is mediated by a structural genes through mRNA. The structural genes in turn are under the control of operator genes. Furthermore, there are regulator genes which control the information about protein, or other metabolites, formation through their action on operator genes. Transfer RNA, ribosomes, amino acids and their activators play an important role in the synthesis. From this one can conclude that an interference in any of the different steps involved in this intricate system would lead to suppression of the metabolite formation. In this system, Jacob and Monod suggest that a certain metabolite may act as either a suppressor or an inducer of gene action. The suppression is brought about by an interaction between the inactive substance called repressor, produced by the regulator gene, and the metabolite in question. Now being active, the repressor blocks the operator gene, and as a consequence formation of mRNA and proteins will be abolished. There might be some interaction between AD and this system.

What can be concluded from the fact that 0.15 ugm/ml AD-treated embryos (DS) were about twice smaller than control embryos of the same stage. Also, what does the failure of neural folds to meet and form closed neural tubes mean. In the first place, decrease in length of embryos was due to low rate of mitosis on exposure to AD. In cross sections of treated embryos (up to the two-somite stage), mitotic figures were few compared to those in control

embryos. So, slowing down of mitotic activity of cells seems to be one of the direct effects of AD. In the second place, a failure of the two neural folds to meet and form a neural tube results from inhibition of morphogenetic movements of the two folds. Morphogenetic movements, as known, are of utmost importance in organ differentiation. In addition, failure of heart formation at the medium and definite streak stages (on treatment with 0.15 umgs/ml AD) could be explained on this basis. 0.15 ugm/ml AD-treated embryos at the DS stage had two mesenchymal masses on either side of unclosed gut. These were supposed to meet in the midline and form a heart primordium, but due to the action of AD they did not.

Does the differential permeability of different parts of embryos to the antibiotic play a role in its effects? This seems reasonable: as embryos grow up, their differential permeability becomes more and more accentuated. This idea is suggested by Haywood and Sinsheimer (1963) who found that, although Escherichia coli is resistant to AD, mRNA could be inhibited in protoplasts prepared from E. coli. In the present work, it was noticed that in the same embryos (DS) treated with AD (0.15 umg/ml) ventral cells, mainly endodermal, and mesodermal, were not clear and lacked distinct nuclei. Also, much vacuolation was observed in ventral parts of posterior sections.

In mentioning differential permeability, it is worth citing this striking result of AD-treatments. Four embryos at the head

fold stage cultured on media containing 0.15 umg/ml AD for about eight hours developed four clear-cut somites. About 14 hours later, embryos showed very slight traces of two somites. From this, one can conclude that it takes about 10 hours for AD to penetrate cells and exerts its effects.

Is the differential effect of the antibiotic on different organ primordia related to their determination? What does the resistance of blood islands to the drug at a stage where most other structures disappeared mean? Attempts to answer these questions were made by Tyler, (1965), and Hell (1964). Tyler believes that "specification is brought about by intraction with other tissues and the environment, and occurs sometime before there are manifest signs of differentiation. There are no distinctly visible changes that occur at the time a tissue becomes determined, the tests for this state being the type of development that results when the tissue is transplanted to another location." He suggests that determination is characterized by formation of what is called masked mRNA which, when later activated, produces specific substances that characterize a specific differentiation. If a tissue is resistant to AD at a certain stage this means that the tissue is determined at that stage, as could be known from transplantation or explantation experiments.

Further light may be thrown on the idea of determination by dealing with blood islands and the appearance of hemoglobin. An

immunological study was made by Wilt (1962) to determine the time of hemoglobin formation. By the use of precipitating antibodies specific to globin, he noticed that globin-like molecules were present in unincubated embryos. He also found hemoglobin synthesis increased greatly at the eight-somite stage. Hell (1964) found that three agents (AD, azaquanine, and bromodeoxyuridine) inhibited hemoglobin specifically. Also, he found that it could be synthesized in embryos even at one somite stage in the absence of net synthesis of DNA, RNA and protein.

Klein and Pierro (1963) attribute the effects of AD to regional specificity. To them, it is possible that the synthesis of mRNA in cells of somite mesoderm is completed before segmentation. In other words, a relatively stable mRNA has to be synthesised in the resistant cells.

From the above ideas one can extract a common point of view. It is the time of appearance of mRNA which is directly related to determination, as Tyler says, that is important in differentiation of tissues or organ primordia. This would mean that blood islands, notochord, and heart would be determined earlier than nervous tissues, somites, postembryonic axes. One can go further and say that the degree of resistance of the different tissues to the drug is in direct proportion to the degree of determination: the more determined an organ primordium is, the more mRNA it has synthesised, consequently the more protein, and the more resistant

to AD it is. Thus, although blood islands are resistant to AD at DS stage, their number is about three times less than that in controls. However, the amounts of blood islands in both AD + DNA treated and control embryos, say at the two-somite stage, is more or less the same.

It is hoped that this survey and study will encourage further work which will lead to a better understanding of the action of this drug on metabolic activities and the subsequent effect on development.

SUMMARY

1. The effects of actinomycin D, at different concentrations, on different stages of early development of the chick embryo have been studied.

2. Morphological and histological observations showed that blood islands were the most resistant to the drug followed, in decreasing order, by heart, notochord, hindbrain, forebrain, and somites.

3. Quantitative biochemical studies showed that levels of RNA, DNA, and PN are inhibited to about 75 per cent in embryos at definite streak stages on treatment with 0.15 ugm/ml AD. The percentage of inhibition decreased with decrease in dosage of the drug and increase of age of embryos.

4. Embryos at and above the four-somite stage were not affected by AD at a concentration of 0.15 ugm/ml. Morphological, histological, and biochemical results were more or less the same as in control embryos of the same stage.

5. Addition of calf thymus DNA to the AD medium relieved embryos (from definite streak to the two-somite stages) from the effects of the antibiotic. This was shown by comparing the morphological, histological and biochemical results of both AD+DNA treated and control embryos.

6. Partial recovery of embryos treated with 0.15 ugm/ml AD was noticed when embryos at the definite streak stage were transferred to control media after eight hours of culturing on AD-media.

7. The results were discussed in relation to the time of synthesis of mRNA and determination of different organ primordia.

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TABLE I

Average number of somites, relative amounts of blood islands, average length of hearts, and average length of embryos cultured on Ad-, AD+DNA-, and control media for about 20 hours.

Stage	Dose***	Embryos used	No. of somites	Blood islands*	Length of heart**	Length of embryo**
DS	0.00	14	12	+++	0.40	4.2
	0.15	12	00	+	0.10	2.0
	0.05	06	05	++	0.20	2.8
	0.01	07	13	++	0.30	3.6
	0.15+ DNA	12	14	+++	0.30	4.1
HP	0.00	08	14	++++	0.55	3.9
	0.15	08	00	++	0.38	3.1
	0.05	07	10	+++	0.38	3.5
	0.01	06	15	+++	0.40	3.8
	0.15+ DNA	09	14	++++	0.45	4.1
HF	0.00	12	15	++++	0.60	4.3
	0.15	12	02	+++	0.45	3.4
	0.05	08	10	+++	0.47	3.5
	0.01	07	14	+++	0.49	3.8
	0.15+ DNA	09	16	++++	0.60	4.5
2S	0.00	10	17	++++	0.60	4.2
	0.15	07	06	+++	0.55	3.8
	0.05	09	15	++++	0.60	3.9
	0.01	06	16	++++	0.50	4.1
	0.15+ DNA	08	18	++++	0.70	4.5
4S	0.00	11	18	+++++	0.80	4.7
	0.15	09	15	++++	0.60	4.1
	0.05	08	16	++++	0.65	4.1
	0.01	06	19	+++++	0.70	4.3
	0.15+ DNA	06	18	+++++	0.70	5.0

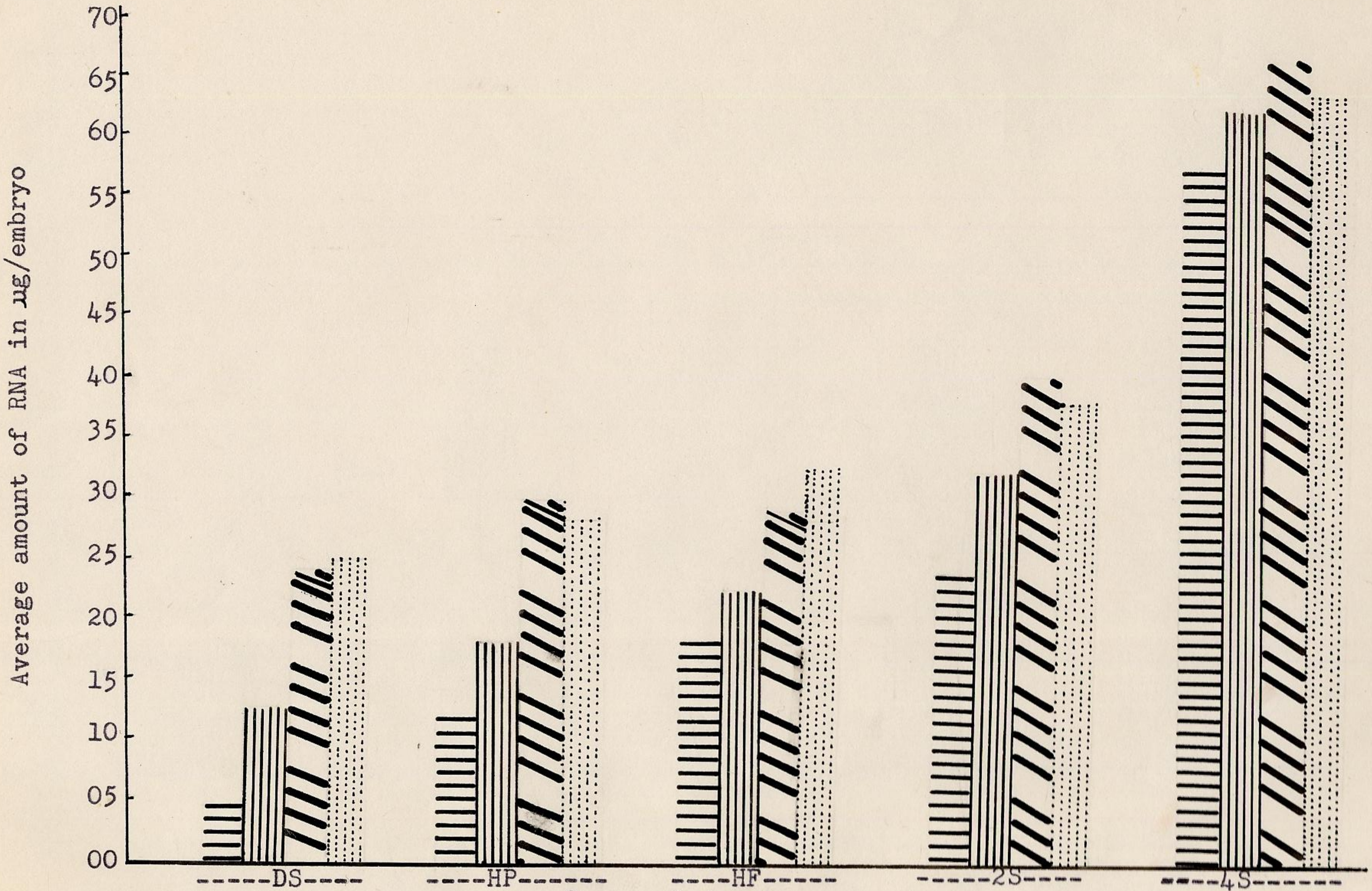
* + is equivalent to about 25 blood islands; ** unit of length is mm; *** dose in mg/ml

TABLE II

Average protein nitrogen (PN), RNA, and DNA of various embryonic stages cultured on AD-, AD+DNA-, and control-media, for about 20 hours

Stage	Dose*	Embryos used	RNA**	DNA**	PN**
DS	0.00	16	25.3	25.0	29.0
	0.15	14	04.5	05.4	07.3
	0.05	08	12.7	09.6	18.0
	0.01	08	20.2	23.5	25.0
	0.15+ DNA	10	24.2	28.0	33.0
HP	0.00	08	28.0	26.2	32.5
	0.15	09	12.4	15.1	14.7
	0.05	07	18.6	20.0	25.0
	0.01	--	---	---	---
	0.15+ DNA	07	30.0	35.7	29.5
HF	0.00	09	30.0	39.7	34.6
	0.15	11	18.4	23.0	24.6
	0.05	06	23.1	29.0	31.3
	0.01	--	---	---	---
	0.15+ DNA	05	29.4	37.6	36.2
2S	0.00	08	40.0	44.0	45.1
	0.15	06	26.5	30.6	28.6
	0.05	07	34.2	36.0	34.7
	0.01	06	38.0	37.1	42.0
	0.15+ DNA	07	41.2	42.5	48.0
4S	0.00	09	65.0	65.0	60.3
	0.15	08	60.0	55.0	62.5
	0.05	07	63.2	61.2	67.0
	0.01	--	---	---	---
	0.15+ DNA	07	68.1	75.0	70.0

* dose is in ug/ml; ** amount is in ug/embryo; --- not determined.



=treated with 0.15 µg/ml AD, =treated with 0.05 µg/ml AD,
=treated with 0.15 µg/ml AD + 100 µg/ml DNA , =control .

Illustration 1. Average amounts of RNA in chick embryos of different stages of development under different treatments.

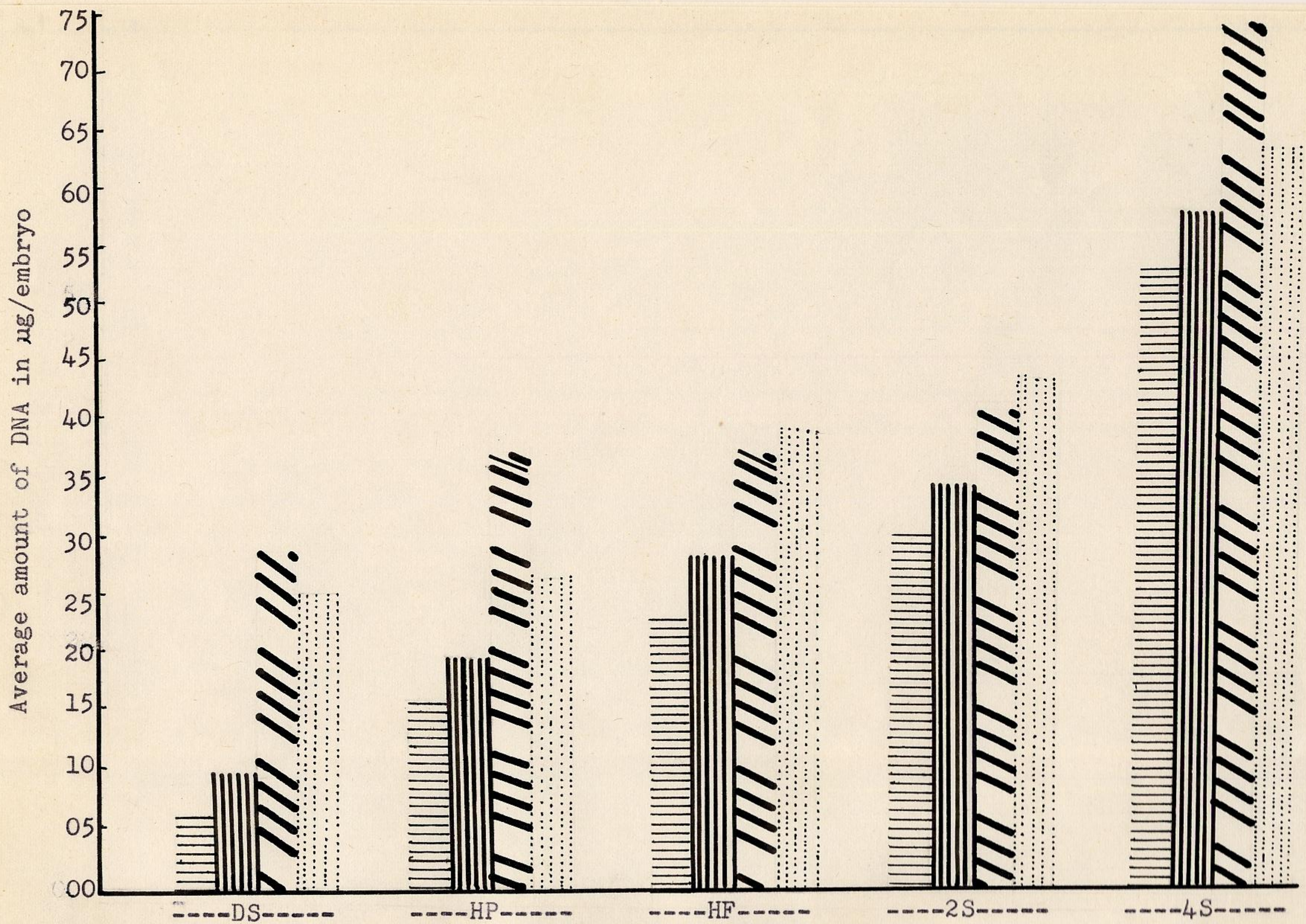


Illustration 2. Average amounts of DNA in chick embryos of different stages of development under different treatments.

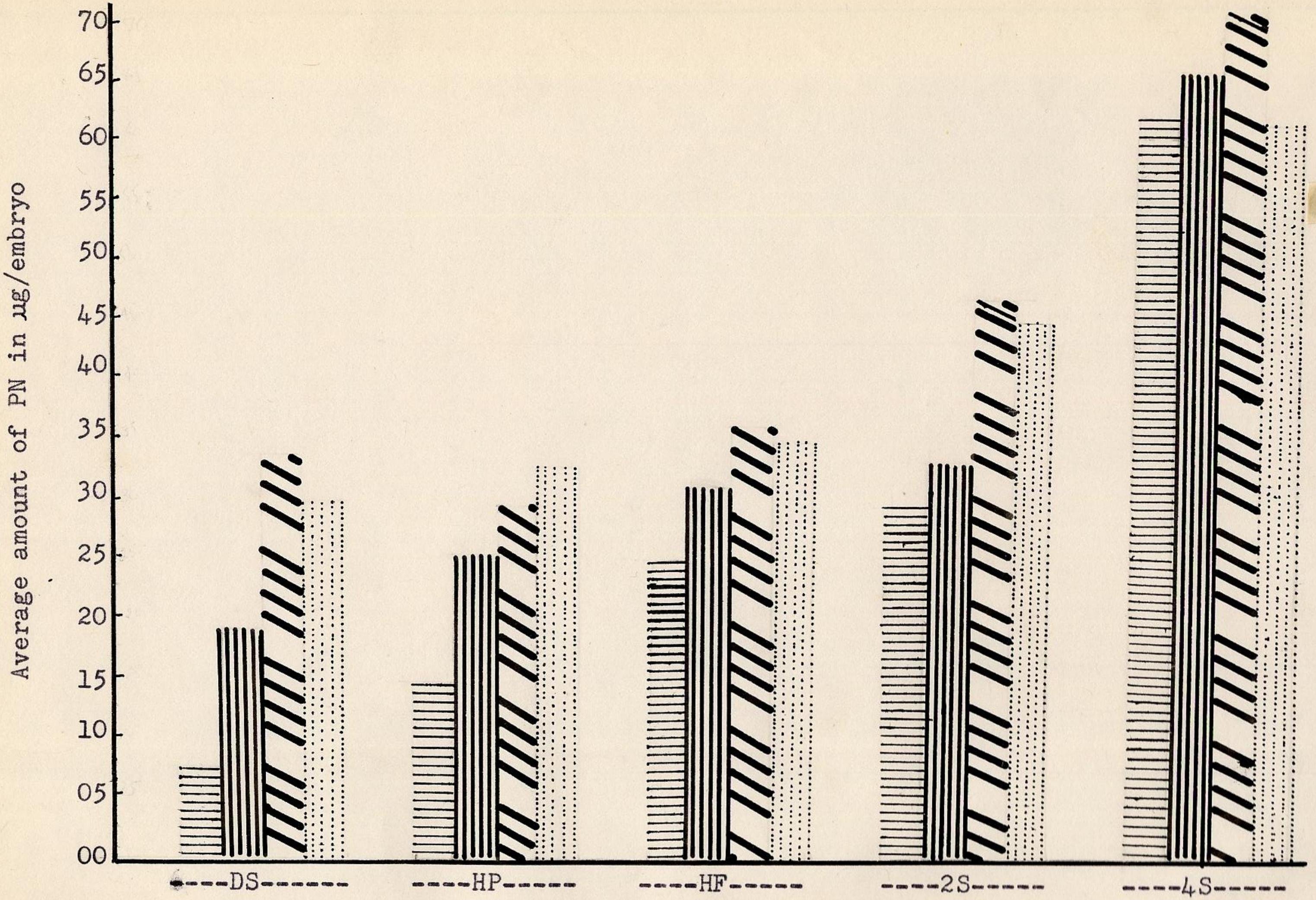


Illustration 3 .Average amounts of PN in chick embryos of different stages of development under different treatments.

PLATE 1

Explanation of Figures

1. Chick blastoderm explanted at the medium streak stage and cultured for 22 hours on a medium containing 0.15 ug/ml AD. X 25

2. Chick blastodern explanted at the definite streak stage and cultured for 24 hours on a medium containing 0.15 ug/ml AD. X 25

3. Chick blastoderm explanted at the definite streak stage, cultured for eight hours on a medium containing .15 ug/ml AD, and for 16 hours on a control medium. X 25

4. Chick blastoderm explanted at the definite streak stage and cultured for 22 hours on a control medium. X 25

5. Chick blastoderm explanted at the definite streak stage and culture for 22 hours on a medium with 0.05 ug/ml AD. X 25

6. Chick blastoderm explanted at head fold stage and cultured for 24 hours on a medium containing 0.05 ug/ml AD. X 25

7. Chick blastoderm explanted at the two-somite stage and cultured for 22 hours on a medium containing 0.15 ug/ml AD. X 25

8. Chick blastoderm explanted at the two-somite stage and cultured for 22 hours on a medium with 0.05 ug/ml AD. X 25

9. Chick blastoderm explanted at the two-somite stage and cultured for 20 hours on a control medium. X 25

10. Chick blastoderm explanted at the two-somite stage and cultured for 22 hours on a medium with 0.15 ug/ml AD + 100 ug/ml DNA. X 25

11. Chick blastoderm explanted at the four-somite stage and cultured for 24 hours on a medium with 0.15 ug/ml AD. X 25

12. Chick blastoderm explanted at the seven-somite stage and cultured for 22 hours on medium with 0.15 ug/ml AD. X 25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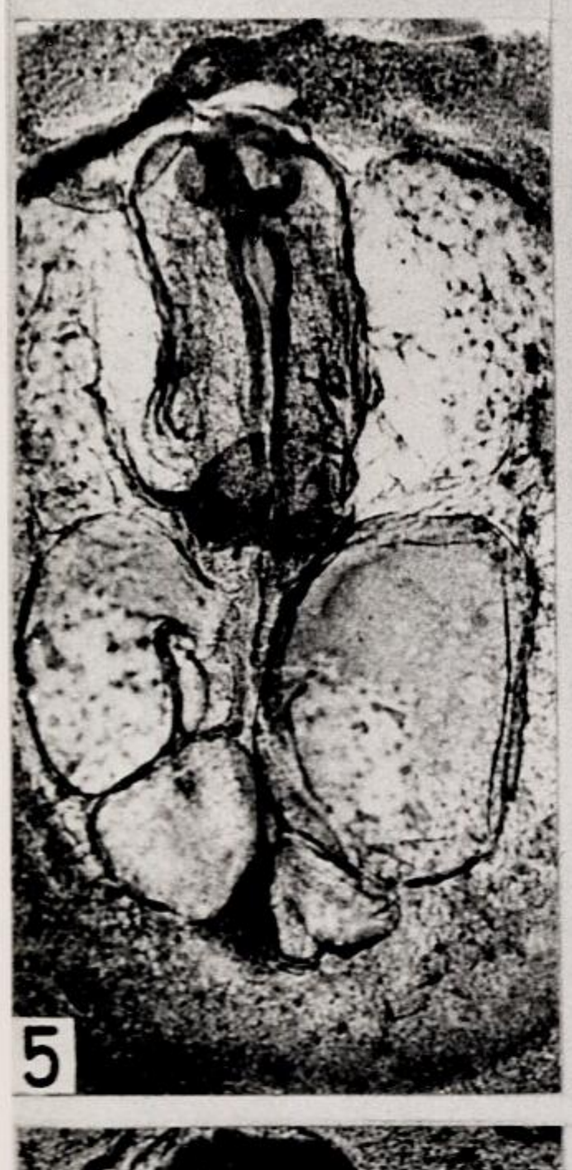
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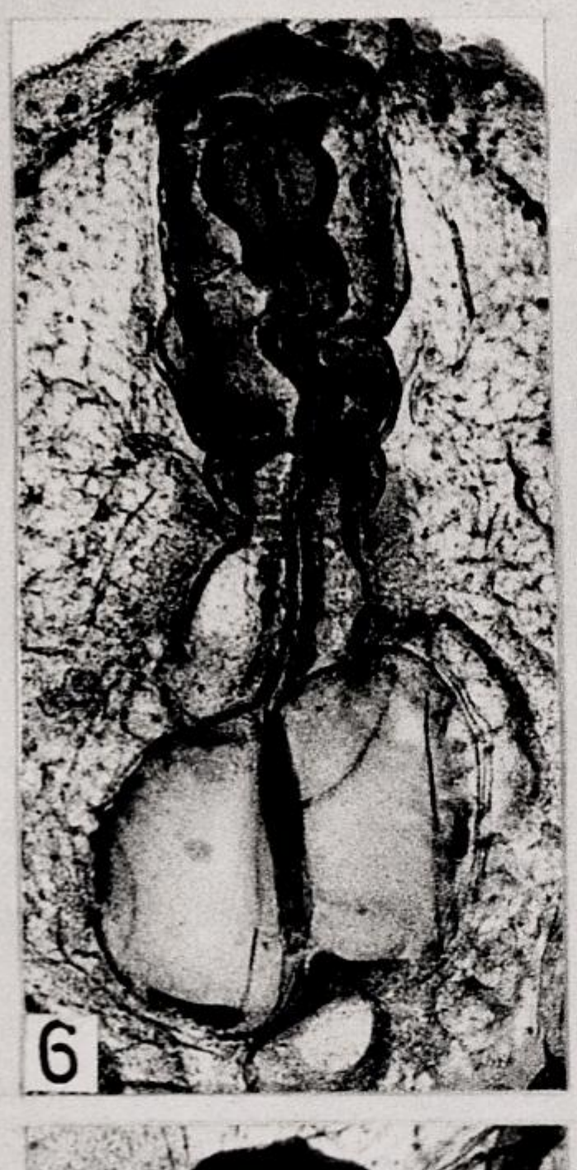
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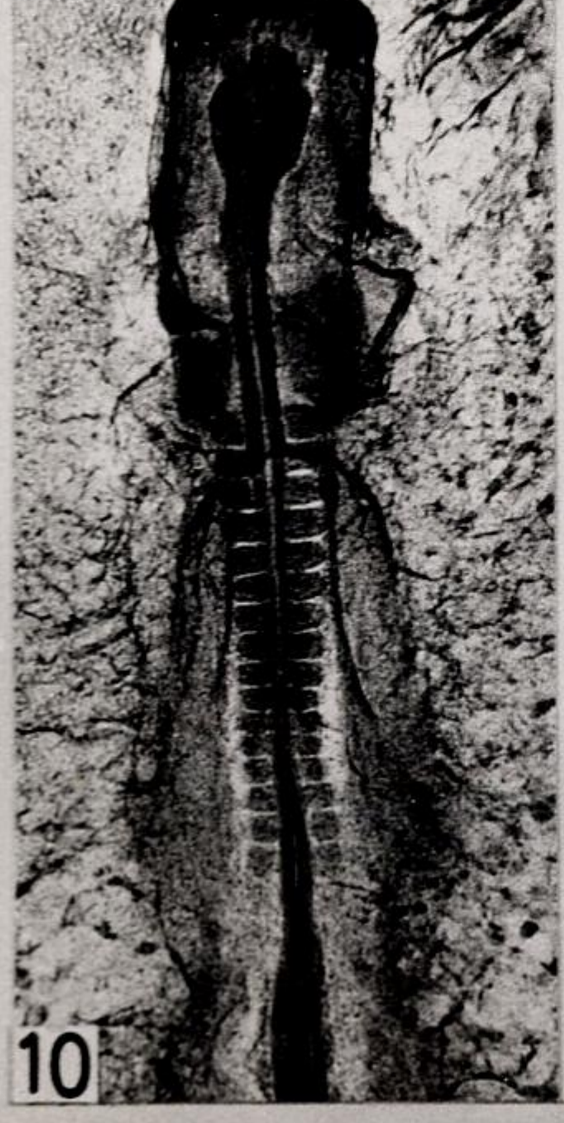
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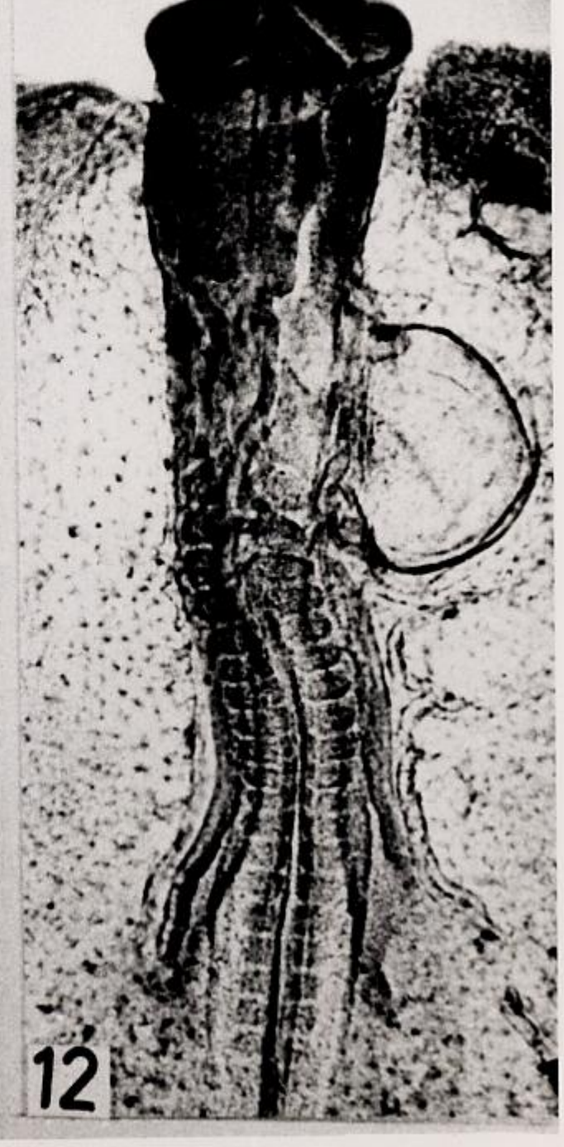
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PLATE 2
Explanation of Figures 13-24

13. Transverse section, through the heart, of a chick blastoderm explanted at the definite streak stage and cultured for 24 hours on medium with 0,15 ug/ml AD. X 100

14. Transverse section, through the heart, of a chick blastoderm explanted at the definite streak stage and cultured for eight hours on a medium with 0.15 ug/ml AD and for 16 hours on a control medium. X 128

15. Transverse section, through the heart, of a chick blastoderm explanted at the head process stage and cultured for 24 hours on a medium containing 0.15 ug/ml AD. X 128

16. Transverse section, through the heart, of a chick blastoderm explanted at the head fold stage and cultured for 24 hours on a medium containing 0.15 ug/ml AD. X 128

17. Transverse section, through the heart, of a chick blastoderm explanted at the two-somite stage and cultured for 24 hours on a medium with 0.15 ug/ml AD. X 128

18. Transverse section, through the heart, of a chick blastoderm explanted at definite streak stage and cultured for 20 hours on a control medium. X 128

19. Transverse section, through the anterior somites, of a chick blastoderm explanted at the definite streak stage and cultured for eight hours on a medium with 0.15 ug/ml AD and for 16 hours on a control medium. X 100

20. Transverse section, through the anterior somites, of a chick blastoderm explanted at head fold stage and cultured for 24 hours on a medium containing 0.15 ug/ml AD. X 100

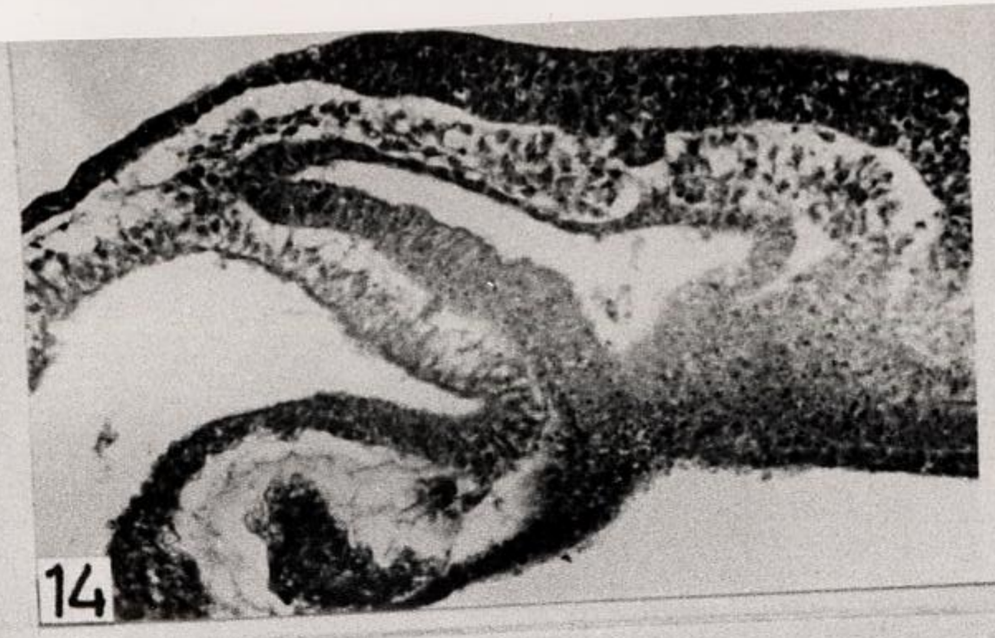
21. Transverse section, through the anterior somites, of a chick blastoderm explanted at the two-somite stage and cultured for 24 hours on a medium with 0.15 ug/ml AD. X 100

22. Transverse section, through the anterior somites, of a chick blastoderm explanted at definite streak stage and cultured for 22 hours on a control medium. X 128

23. Transverse section, just lateral to Hensen's node, of a chick blastoderm explanted at the two-somite stage and cultured 22 hours on a medium with 0.15 ug/ml AD. X 100

24. Transverse section, through Hensen's node, of a chick blastoderm explanted at the definite streak and cultured for 20 hours on a control medium. X 100


13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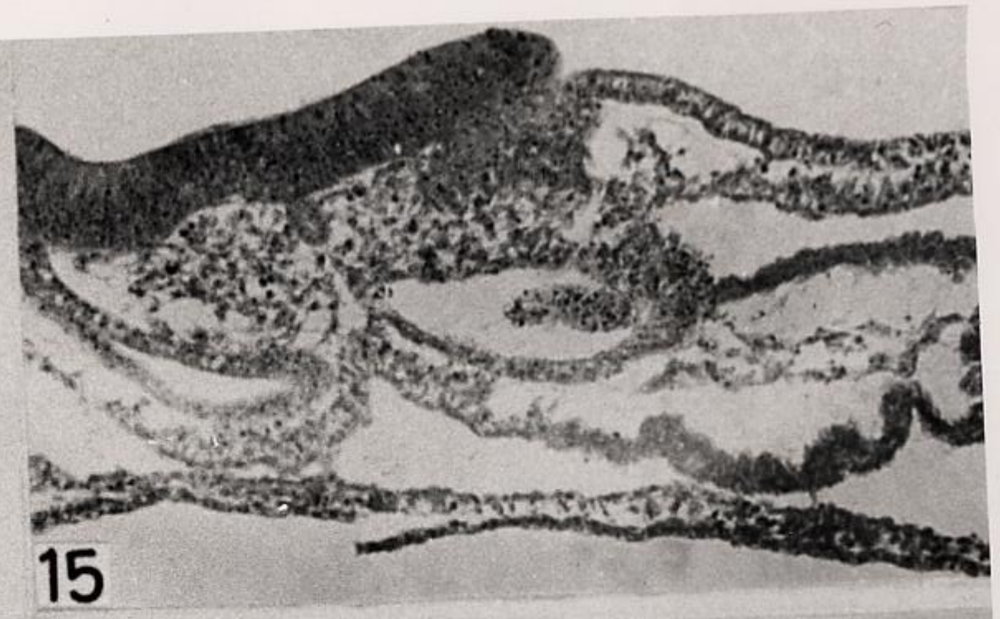
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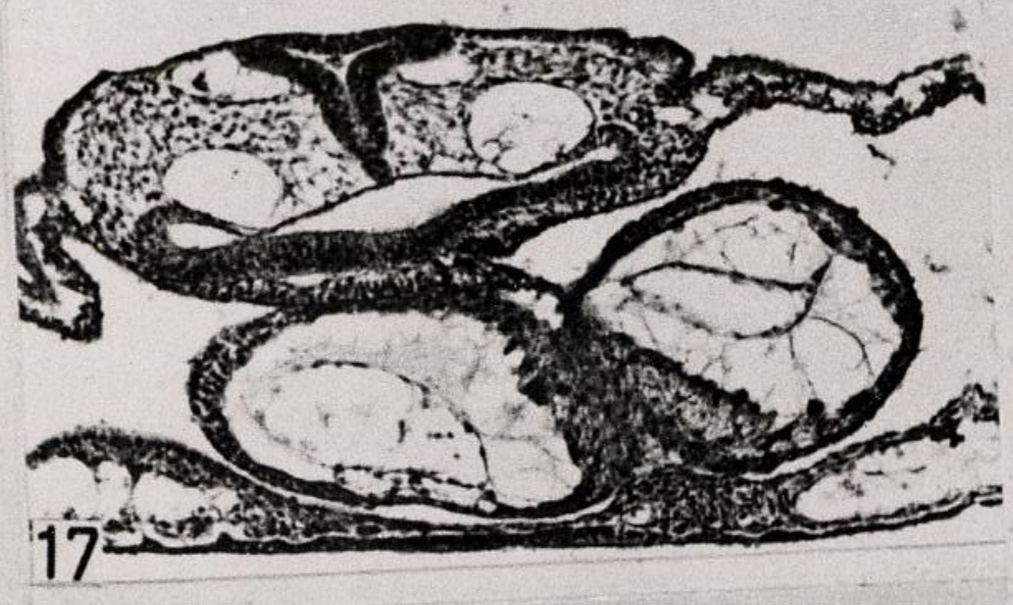
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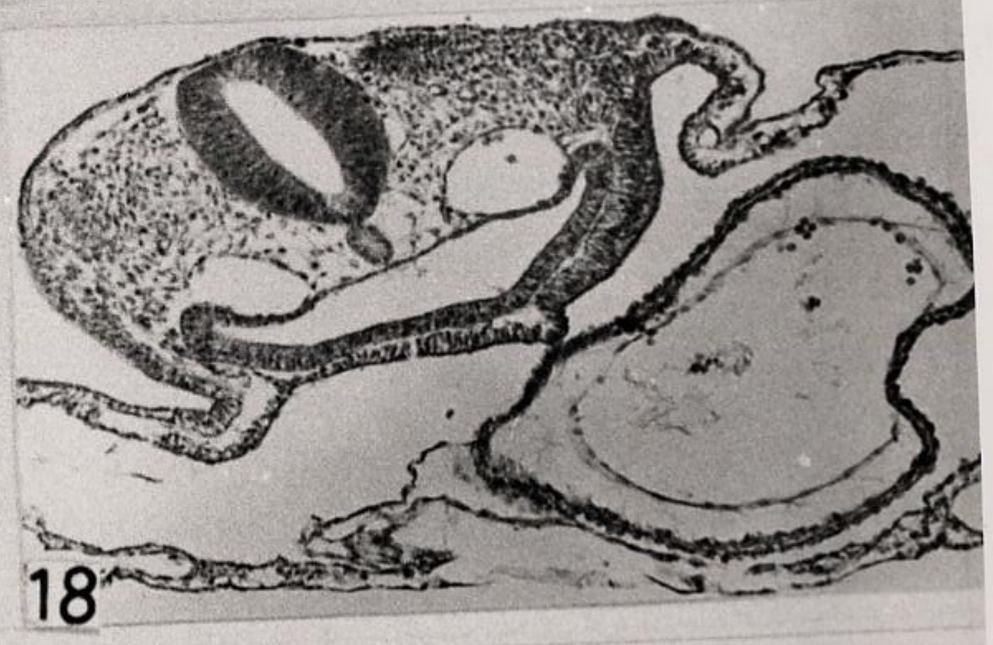
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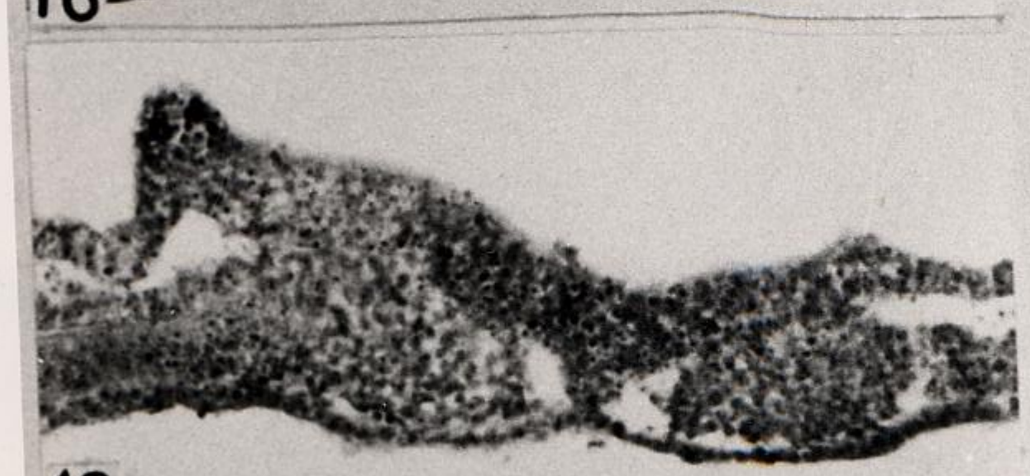
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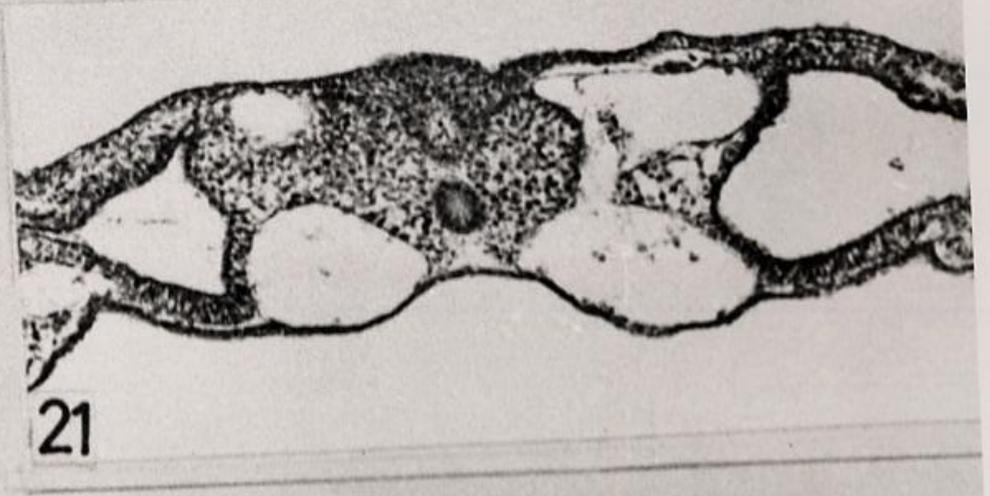
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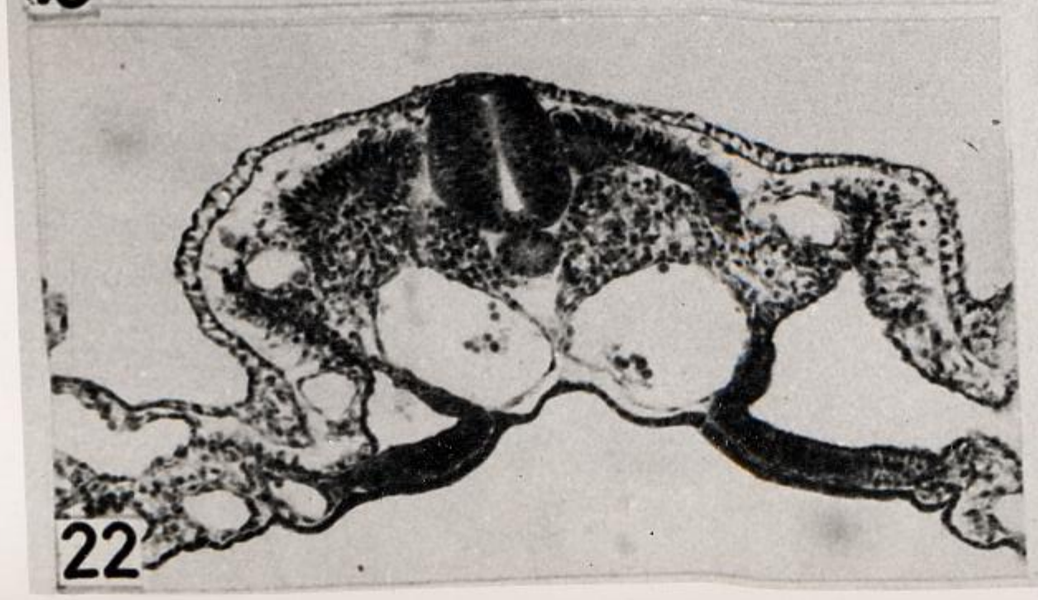
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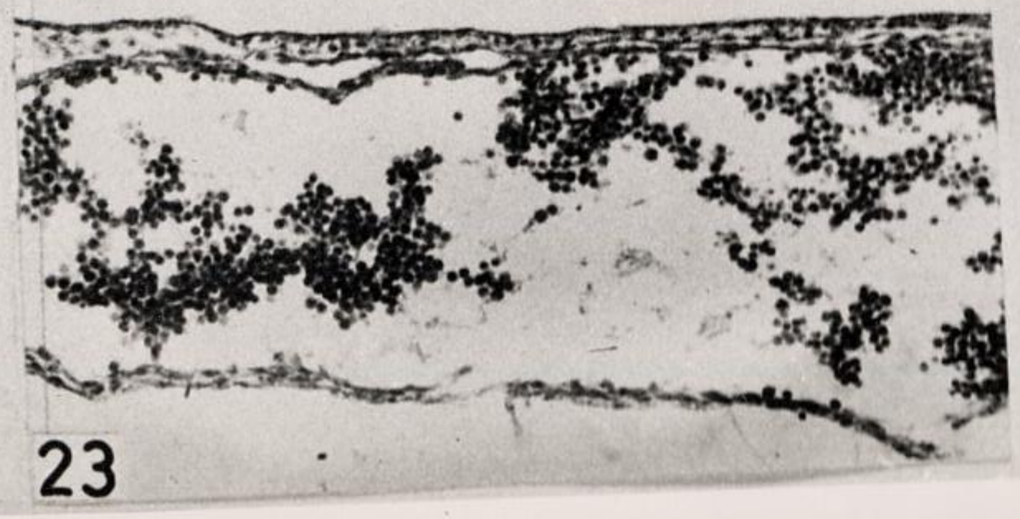
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