AUTORADIOGRAPHIC STUDIES OF THE UTILIZATION OF S^{35}-SULFATE BY THE CHICK EMBRYO*

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Received for publication September 28, 1956

INTRODUCTION

Most of the investigations thus far dealing with the autoradiographic visualization of S^{35} have been concerned with the uptake of this isotope in cartilage and chondroitin sulfate either in suckling or young adult animals (Dziewiatkowski, 1951, 1952, 1954; Boström *et al*, 1952; Campbell, 1951) or in late or advanced fetal stages (Dziewiatkowski, 1953; Boström and Odeblad, 1953; Friberg and Ringertz, 1954; Amprino, 1955). Insofar as is known, there is no report of an extensive study of sulfate metabolism in the chick embryo, using S^{35}-sulfate. Experiments were done to determine at which level of development the chick embryo begins selective utilization of S^{35}-sulfate and how the S^{35}-sulfate is subsequently redistributed. It was believed that this could be accomplished satisfactorily by injecting labelled sulfate into the egg albumen and then following the transfer of the S^{35}-sulfate to the embryo.

Materials and Methods

Fertile eggs from a flock of inbred White Leghorn hens were obtained locally. Upon receipt each egg was candled, weighed, and numbered serially. After equilibration to room temperature, each egg was injected with 0.1-ml. of solution containing 25 μc $\pm$ 20 per cent of sulfur-35 as carrier-free sulfate[1]. Injection was made into the dense albumen through the small end of the egg as described previously (Johnston and Comar, 1955). After a period of 12 to 14 hours, to allow for mixing, the eggs were sealed with cellulose tape and incubated in a small commercial incubator under standard conditions of temperature, humidity, and air movement. A counting standard was prepared from the dosing solution at the time of injection, equivalent to 0.1 per cent of the dose. Beginning with eggs incubated for 12 hours, embryos were removed and staged according to Hamilton (1952). Fixation was carried out in alcohol-acetic acid (1 part glacial acetic acid to 9 parts 95 per cent ethyl alcohol) and varied from a few minutes for the younger stages to several hours for the older. After paraffin embedding, sections were cut at scale settings of 5 and 7 microns, mostly 5 microns. Serial cross-sections

* This work was performed under Contract AT-(40-1)-1775 with the W. O. Atomic Energy Commission.

[1] Radioactive materials were obtained from the Oak Ridge National Laboratory on allocation from the W. O. Atomic Energy Commission.

were cut of the younger stages and serial sagittal sections of the older. Up to and including Stage 25, the entire series was mounted on glass slides using a minimum of albumen fixative and water. In the case of embryos older than Stage 25, only selected sections of the series were mounted. After drying, the activity of S^{35} in the sections was determined by counting. This was done in order to estimate exposure time. Autoradiograms were prepared by a modification (Lotz and Johnston, 1953) of the method of Doniach and Pelc (1950). After exposure, varying from 14 to 60 days, the autoradiograms were processed according to standard procedures and allowed to dry in the air. Some of the sections covered by their autoradiograms were stained with dilute acid Harris' hematoxylin or with dilute Giemsa and others with dilute toluidine blue (Hempelmann, 1940). Sections adjacent to those being used for autoradiograms were stained for mucopolysaccharides, employing the Bauer-Feulgen technique, and others were stained with toluidine blue (Hempelmann, 1940) to demonstrate the presence of chondroitin sulfate. The autoradiograms obtained were of sufficient density to be read subjectively and did not require grain-counting techniques.

Not all of the autoradiograms are presented in the figures which follow, but a sufficient number of them are given to illustrate their quality and to point up various points of interest. The autoradiograms are discussed sequentially according to the developmental stage of the embryo in terms of relative amounts of S^{35} in the various anatomical regions. Generally, only differences observed in an autoradiogram of any one section are compared.

OBSERVATIONS

In some instances several stages of development are grouped together, as little or no change in concentration of S^{35} could be detected between them. At this time, detailed description is given only through Stage 34. In the following observations, the term "chondroitin sulfate" pertains to that material which stains metachromatically with toluidine blue (Hempelmann, 1940).

Stages 3 and 3+: Intermediate Streak.—No particular difference in the distribution of S^{35} could be detected prior to Stage 3. However, in Stage 3+ in some embryos a definite concentration of the isotope could be seen in the primitive groove, primarily in the axial portion (Fig. 1).

Stages 4 through 8: Definitive Streak to 4 Somites.—In Stage 4, S^{35} is seen concentrated in the area of Hensen's node and in the hypoblast immediately adjacent to the node. The primitve groove and folds also show a concentration of the isotope and the floor of the groove possesses the most activity (Fig. 2).

In Stages 5 and 6, S^{35} is concentrated in the head process (notochord) as shown in Fig. 3. The concentration of S^{35} increases in the head process proceeding toward Hensen's node. This is seen in Fig. 4. The epiblast above the head process also shows some concentration of sulfate. In some cases a dense autoradiographic reaction was seen over Hensen's node, indicating a high concentration of S^{35} in this region (Fig. 5).

In Stage 8, the medullary plate and the epiblast of the lateral head fold are seen to have accumulated S^{35}. Occasionally some wandering cells (epiblastic in origin?) are seen with a rather high concentration of S^{35} which, from observations of the autoradiogram under high power, appears to be intracellular. The concentration of S^{35}

in the head process is pronounced and there is a tendency for the head process to round up (Fig. 6). At this time there is S^{35} in the walls of the amniocardiac vesicles and in some of the numerous scattered mesenchymal cells in the vesicles S^{35} appears to be localized intracellularly.

Stages 9 through 11.—The concentration of S^{35} is high in the notochord of Stage 9. In the neural groove the S^{35} concentration is greatest at the juncture of the groove with the adjacent mesenchyme in the region where the future limiting membrane of the cord will form (Fig. 7). Autoradiograms of the anterior trunk region show a concentration of S^{35} in the splanchnopleure of the anterior intestinal portal where it appears to be localized between the mesoderm and the endoderm (Fig. 8). The S^{35} seen in the splanchnopleure was found to coincide with a thin layer of chondroitin sulfate as determined by toluidine blue staining. The concentration of S^{35} is seen to be high at the junction of the ectoderm and the developing somitic mesoderm and at the junction of the neural tube and the adjacent mesoderm (Fig. 8).

In Stage 10, an accumulation of S^{35} occurs in the walls of the omphalomesenteric vein (Fig. 9). Some S^{35} appears to be distributed throughout the mesoderm with random loci of concentration and to be concentrated around the notochord (Fig. 9). The S^{35} associated with the notochord could be correlated with the formation of a thin ring of chondroitin sulfate formed around the chord. S^{35} also occurs in the area adjacent to the junction of the mesoderm and the ectoderm. Sections through the heart reveal a concentration of S^{35} in the subendocardial jelly and in the endocardial layer (Fig. 10). Punctiform localizations occur elsewhere in the splanchnic mesoderm and in the endoderm comprising the dorsal wall of the pharynx (Fig. 10).

In Stage 11, the distribution of S^{35} is essentially the same as before. However in the somitic region, the S^{35} is concentrated primarily at the periphery of the notochord (Fig. 11) with some intracellular localizations (Fig. 12). A marked accumulation of S^{35} occurs in the heart and is restricted for the most part to the endocardium and subendocardial jelly (Fig. 13). Examination of the autoradiogram under high power revealed that some of the S^{35} occurring in the endocardium is intracellular (Fig. 14). The S^{35} in the subendocardial jelly was found to coincide with a layer of chondroitin sulfate as demonstrated by toluidine blue staining.

Stages 12 and 13: 16 to 19 Somites.—The localization of S^{35} appears to be more precise than before. In the trunk region there is a marked concentration of sulfate at the periphery of the notochord and in the adjacent regions between the chord and the dorsal aortae (Fig. 15). The autoradiographic reaction over the endocardium and subendocardial jelly is pronounced, indicating a considerable S^{35} activity in this region. The S^{35} is concentrated more in the vicinity of the atrioventricular canal than elsewhere. The head mesenchyme now also shows a tendency to accumulate S^{35}, particularly in the area immediately adjacent to the forming auditory cup.

Stages 14 through 19: 22 to 36 Somites.—Even more S^{35} than in earlier stages of development appears to have accumulated around the notochord and this accumulation coincides with a ring of chondroitin sulfate (Fig. 16). The autoradiographic reaction of the endocardium and subendocardial jelly is pronounced indicating that the concentration of S^{35} in these regions is high (Fig. 17). Examination of the autoradiograms under the microscope, using high power, revealed that a considerable amount of the S^{35} is localized intracellularly in the endocardium. This would seem

to indicate that synthesis of the subendocardial jelly possibly occurs in the endocardial cells and that this material is subsequently passed out into the space which develops between the endocardium and the epimyocardial layer. A faint autoradiographic reaction over the mesenchyme beneath the thyroid diverticulum indicates a small concentration of S^{35} in this region. The head mesenchyme shows a general concentration of S^{35} with slight localizations in the areas immediately adjacent to the optic cup and the diencephalon. Posteriorly, an autoradiographic reaction is found over the mesenchyme surrounding the gut particularly in the region destined to become the stomach (Fig 18). S^{35} also is concentrated between the myotome and the ectoderm (Fig. 16).

Stages 20 through 25.—During this period there is additional concentration of S^{35} in the region of the notochord which coincides with the formation of the notochordal sheath and with the onset of primary chondrogenesis. Autoradiograms of the notochord and the primary cartilage surrounding the chord indicate an intracellular concentration of S^{35} in those cells which are differentiating into chondrocytes (Fig. 19). It is interesting to note that before there is any synthesis of matrix the S^{35} is localized in the cell. This would indicate that the chondroblast elaborates the matrix which fills the intercellular space. Although the notochord is considerably vacuolated some of its cells show a tendency to accumulate S^{35} and punctiform localizations may be seen near the center of the chord (Fig 20). These localizations were found to coincide with concentrations of chondroitin sulfate.

In the head region, in those areas which give rise to cartilage, the mesenchyme is accumulating S^{35} intracellularly (Fig. 21). This fact again points up the implication that the S^{35}-sulfate must first pass through a cell before it is incorporated into chondromucoid.

Stages 26 through 34.—Changes involving the redistribution and localization of S^{35} during this period primarily concern chondrogenesis. However it is interesting to observe that there is an accumulation of S^{35} in the mesoderm immediately beneath the epithelium of the gut (Fig. 22). This area later forms the basement membrane and the submucosal layer. Sulfur-35 also occurs just beneath the epithelium of the stomach where it forms a discrete layer (Fig. 23). This layer coincides with a thin layer of chondroitin sulfate, as demonstrated by toluidine blue staining. Sulfur-35 still may be found in the heart where it is concentrated primarily in the ventricle and truncus. The concentration of S^{35} in the endocardium and subendocardial jelly is not as definite as before and is possibly due to the intense chondrogenesis taking place in the skeleton particularly in the hind limbs.

Subsequent Stages.—No attempt will be made at this time to describe the changes which occur subsequent to Stage 34. However, it is interesting to observe that about Stage 38 the chick begins to form a layer of S^{35}-containing material in the stomach on the free surface of the epithelium (Fig. 24). This material stains negatively with toluidine blue but exhibits a positive reaction for mucoproteins. The autoradiograms of this layer suggest that it is being formed by secretion from the gastric epithelium and that S^{35} is being removed from the general body stream. It is deduced from the study of the autoradiograms that S^{35} is passing from the subjacent mesenchyme through the epithelium into the free surface layer as the latter develops.

DISCUSSION

The pattern of utilization of S^{35} by the chick embryo as determined in this study differs, in some degree, from that obtained by others. Feldman and Waddington (1955), using methionine-S^{35} to study S^{35} distribution, found that in Stages 4 and 5 the uptake of S^{35} was most pronounced in the ectoderm and that in the primitive streak the primitive ridges showed a higher concentration of the isotope than did the floor of the groove. Contrary to what is reported here they found that Hensen's node accumulated S^{35} at about the same rate as the mesoderm. Their findings show that the cells forming the head process, presumably derived from the Hensen's node, lose "their 'original' concentration as soon as they start the migration forwards from Hensen's node." This was interpreted as indicating the absence of any posterior-anterior gradient for the accumulation of S^{35}-methionine or of S^{35}-labelled materials derived from methionine. From observations made during this study it appears that a posterior-anterior gradient does exist in the head process with respect to the accumulation of S^{35}-sulfate or of S^{35}-labelled materials (Figs. 3 to 5). It is apparent that other differences between the uptakes of S^{35} given as labelled methionine and S^{35} given as labeled sulfate also occur. These differences probably reflect the metabolic pathways by which these substances, methionine and sulfate, are made available to the embryo. It is interesting to note that those regions of the embryo which later differentiate into supporting tissues exhibit a marked preference for S^{35}-sulfate or S^{35}-labelled substances derived from sulfate as early as the head process stage. However, the distribution of S^{35} derived from labelled methionine is primarily to the developing neural tissue and particularly to the brain (Feldman and Waddington, 1955). This marked uptake of S^{35}-methionine or of a labelled moiety of methionine, probably cysteine, by the neural tissue indicates that methionine is associated more closely with neurogenesis than with any other of the morphogenic processes (Feldman and Waddington, 1955). This is supported also by evidence obtained by others (Rapkine and Brachet, 1951; Lallier, 1951) suggesting the importance of the —SH group in neurulation. The fact that the floor of the primitive groove and later the Hensen's node and head process accumulate S^{35} derived from sulfate and not that derived from labelled methionine seemingly indicates that sulfate metabolism is in some way associated with those embryonic primordia which differentiate into connective or supporting tissues. This is seen later in development during the differentiation of chondroblasts from mesenchyme. In advanced stages, mesenchyme which is destined to become cartilage signals this fate by the accumulation of S^{35} or S^{35}-sulfate intracellularly. This selectivity occurs long before the chondroblast can be distinguished histologically. A similar selectivity and uptake of S^{35} by preconnective tissue areas of mesenchyme has been demonstrated in tissue culture (Mancini and Lusting,

1956). Once the differentiation into a chrondroblast has occurred these cells continue to utilize sulfate selectively and to accumulate it intracellularly before they begin to secrete chrondroitin sulfate. Also, since the labelled sulfate has been available to the embryo from "0" time it is believed that these cells are handling the labelled material in the same manner as the unlabelled and that it is only because of the special properties of the isotope that we can detect this early differentiation of the mesenchyme.

The occurrence of S^{35}-sulfate or of some derivative of S^{35}-sulfate in the splanchnopleure, amniocardiac vesicles, subendocardial jelly and endocardium is unusual. The sulfate found in the subendocardial jelly is associated with chrondroitin sulfate, as determined by toluidine blue staining (Hemplemann, 1940). From an examination of the autoradiograms of the heart region it was deduced that the subendocardial jelly is produced by the endocardium. The role of the intima of the aorta in the production of chrondroitin sulfate has been demonstrated (Buck, 1955). Also, studies of others have shown that there is an incorporation of S^{35} into the chrondroitin sulfate in the media of the aorta of the rabbit and also into the jelly-like tissue associated with the heart valves (Odeblad and Boström, 1953).

Other studies in which an early differentiation in the selective utilization of other elements by the chick embryo are those of Smith and Gray (1948) for Cu^{64} and Hunt and Wolken (1948) for P^{32}.

SUMMARY

From studies of autoradiograms of various developmental stages of the chick embryo containing S^{35} given us sulfate it was determined that as early as Stages 3+ and 4 there is a selective utilization or accumulation of sulfate by the various parts. The earliest accumulation site is the axial portion of the primitive streak and the floor of the groove. Later S^{35} was found in the head process, Hensen's node, notochord, amniocardiac vesicle, wall of the omphalomesenteric vein, endocardium, subendocardial jelly, mesenchyme destined to become cartilage, basement membrane area of the gut, and a mucopolysaccharide layer formed on the free surface of the stomach.

The early notochordal localizations of S^{35} coincide with the region in which a thin ring of chondroitin sulfate is subsequently laid down. However, it is apparent that there is an intracellular accumulation of inorganic sulfate by the chondroitin-forming cells prior to the time they produce sufficient chondroitin sulfate to be demonstrable histochemically.

It was interesting to note that the endocardium appears to concentrate sulfate that later apparently finds its way into the subendocardial jelly.

The fact that those mesenchymal cells which later form chondroblasts begin to utilize sulfate selectively before histological differentiation is apparent was determined.

In addition, the presence of sulfate-containing substances in the forming basement membrane of the gut would seem to indicate that sulfate is important in the histological differentiation of this membrane.

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EXPLANATION OF PLATES

All figures are unretouched photomicrographs of autoradiograms. The photomicrographs were taken using apochromatic objectives and compensating oculars on Kodak micro-file.

PLATE 61

FIG. 1. Cross-section of the primitive groove of Stage 3. Note the concentration of grains at *x*. × 133 (approximately).

FIG. 2. Cross-section of primitive groove of Stage 4. Note the grain density in the axial part of the groove lying between *x* and *y*. × 133 (approximately).

FIG. 3. Cross-section through the head process and medullary plate of Stage 5 anterior to Hensen's node. The autoradiogram over the head process (*hp*) is considerably denser than that over the medullary plate (*mp*). × 133 (approximately).

FIG. 4. An autoradiogram of a section of Hensen's node at the juncture of the head process with the node of Stage 5. Note the concentration of grains in the area indicated by arrows. × 133 (approximately).

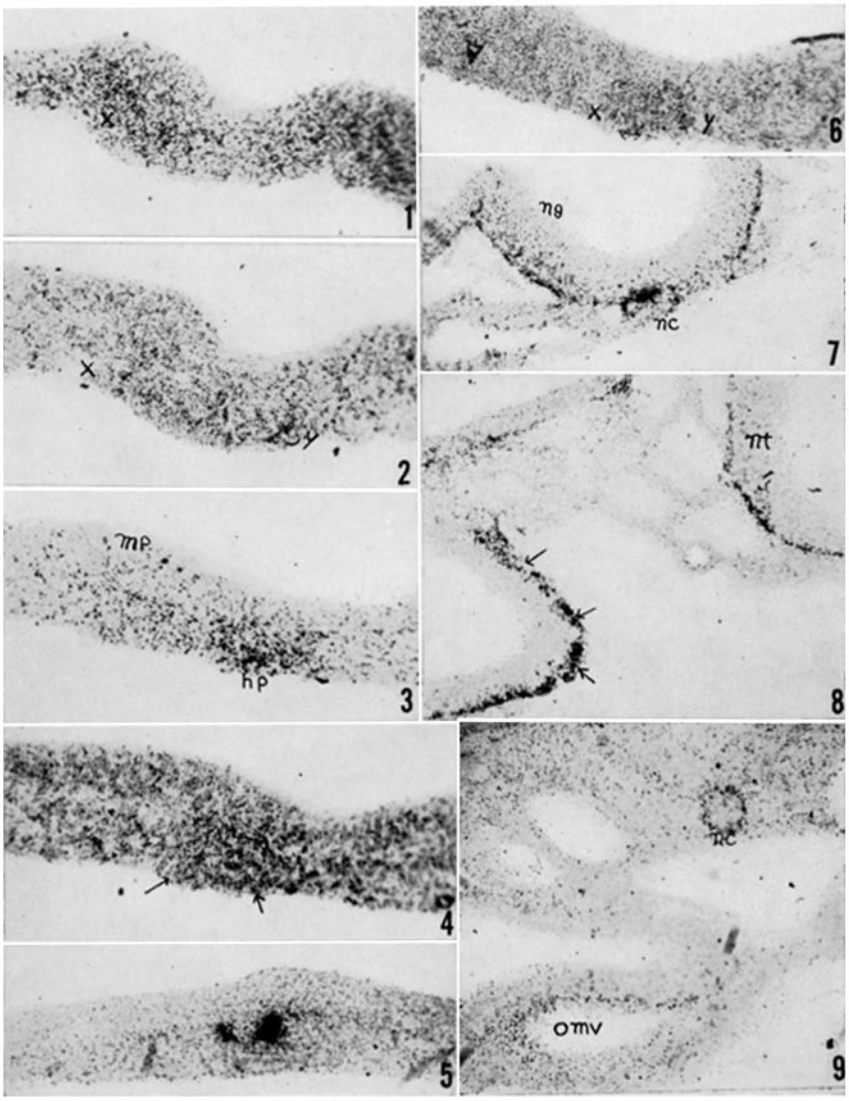
FIG. 5. A cross-section of Hensen's node posterior to that indicated in Fig. 4 of Stage 5. A marked concentration of grains occurs both to the right and left of the midline of the node. × 133 (approximately).

FIG. 6. Cross-section of the head process and medullary plate of Stage 8 anterior to Hensen's node An accumulation of grains may be seen above the head process lying between *x* and *y*. × 133 (approximately).

FIG. 7. Cross-section through the trunk region posterior to the anterior intestinal portal of Stage 9. A dense autoradiogram may be seen at the junction of the neural groove (*ng*) and somitic mesoderm and above the notochord (*nc*). × 133 (approximately).

FIG. 8. Cross-section through the anterior intestinal portal of Stage 9. Note the concentration of grains in the splanchnopleure (indicated by arrows) and at the junction of the neural tube (*nt*) with the somitic mesoderm. × 133 (approximately).

FIG. 9. Cross-section through the trunk and omphalomesenteric vein just anterior to the anterior intestinal portal of Stage 10. Note the grains lying in the omphalomesenteric vein (*omv*) and around the notochord (*nc*). × 133 (approximately).



(Johnston and Comar: S^{35}-sulfate in the chick)

PLATE 62

FIG. 10. Cross-section through the heart region of Stage 10. Note the concentration of grains in the subendocardial region and the punctiform localizations in the splanchnic mesoderm and endoderm as indicated by arrows. × 133 (approximately).

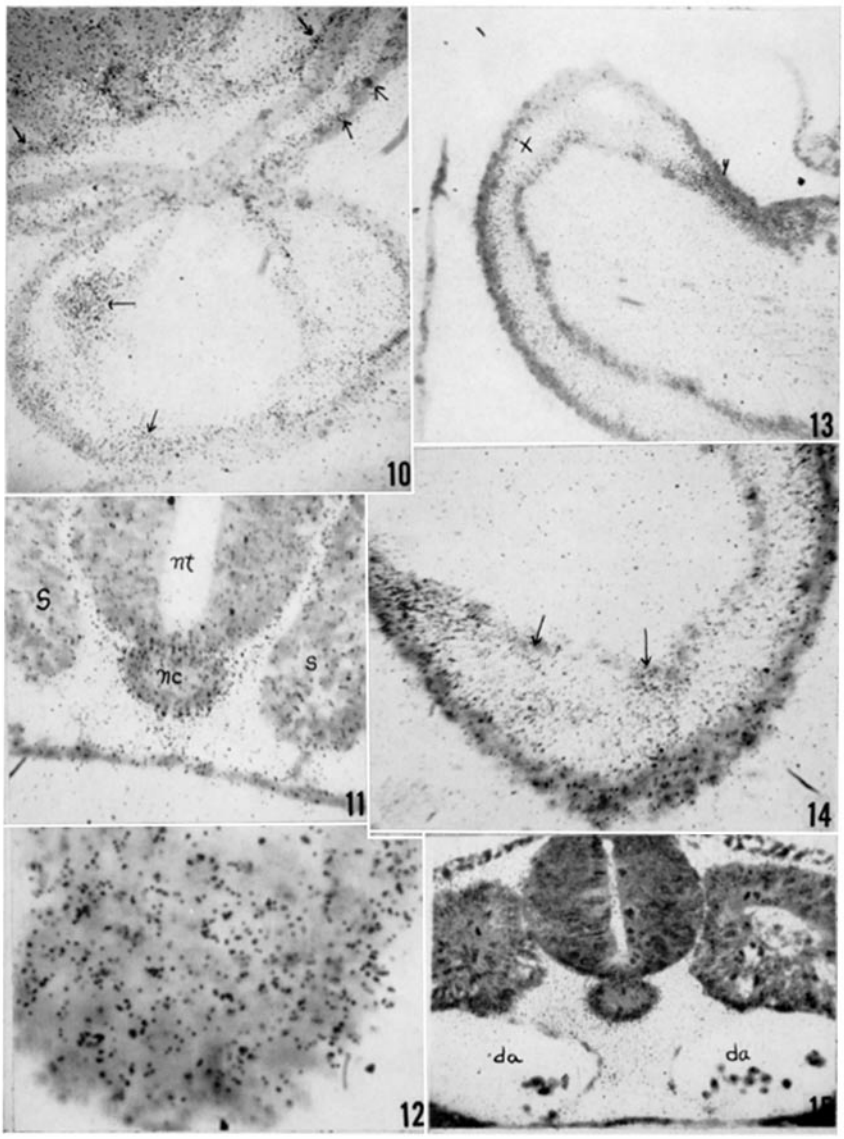
FIG. 11. Cross-section through the somite of the midtrunk area of Stage 11. A concentration of grains may be seen about the periphery of the notochord (*nc*) extending dorsally between the somites (*s*) and the neural tube (*nt*). × 287 (approximately).

FIG. 12. Cross-section of the notochord of Stage 11 taken through oil immersion. Note the tracks of grains which originated from S^{35} in the cells of the chord. × 647 (approximately).

FIG. 13. Cross-section of the ventricular portion of the heart of Stage 11. Note the concentration of grains in the subendocardial layer and in the endocardium. × 133 (approximately).

FIG. 14. Enlargement of a portion of Fig. 13 indicated by *x* and *y*. Several loci of sulfate concentration may be seen in the endocardial layer indicated by arrows.

FIG. 15. Somite region of Stage 13. Note that there is a concentration of grains about the notochord and in the perichordal space between the dorsal aortae (*da*). × 133 (approximately).



(Johnston and Comar: S^{35}-sulfate in the chick)

PLATE 63

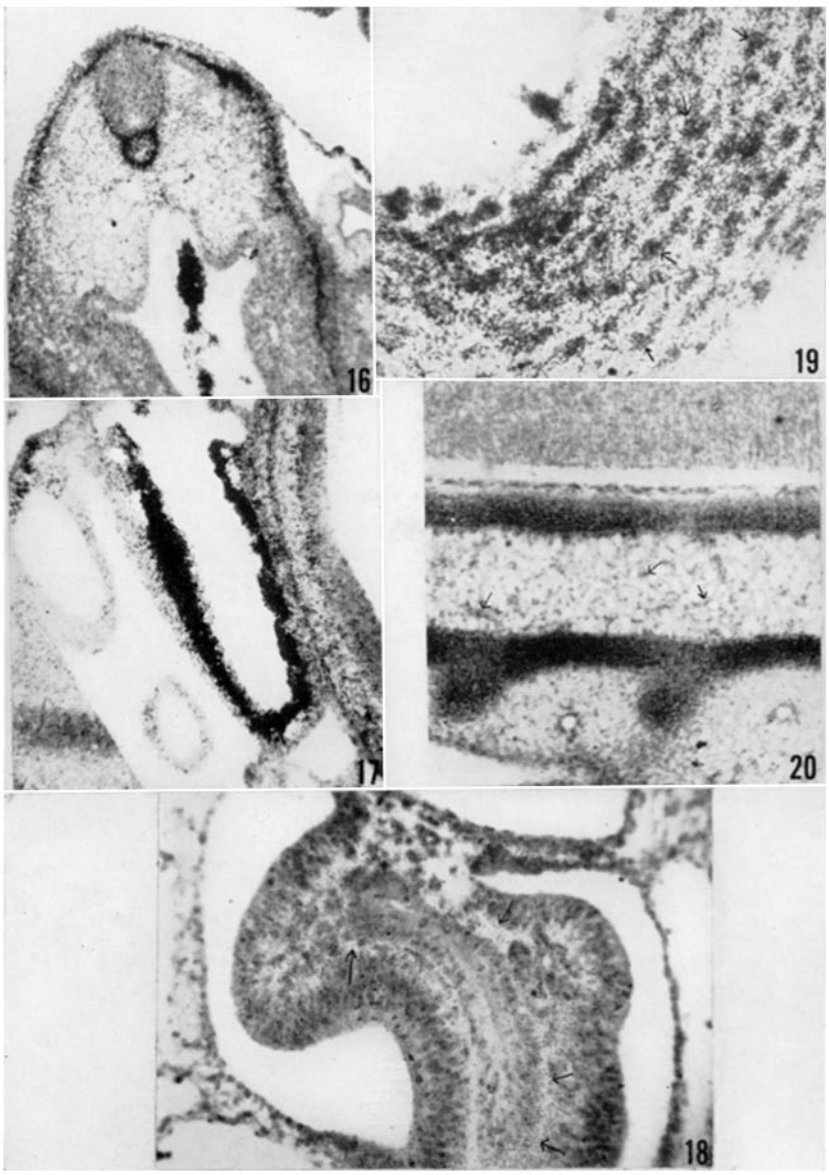
FIG. 16. Cross-section through the posterior trunk region of Stage 18. Note the intensity of the autoradiographic reaction about the notochord and between the ectoderm and the myotome. A high concentration of silver grains is seen above yolk material lying in the gut (cloaca). × 133 (approximately).

FIG. 17. Section through the ventricle of Stage 18. Note the dense concentration of grains over the subendocardial jelly area. × 133 (approximately).

FIG. 18. Section through the stomach region of Stage 19. A faint autoradiogram may be seen beneath the epithelium. Note areas indicated by arrows. × 287 (approximately).

FIG. 19. A portion of a cross-section of the notochordal sheath of Stage 25. Note the concentration of grains above the chondrocytes as indicated. × 287 (approximately).

FIG. 20. Longitudinal section of the notochord and forming sheath showing vacuolation of the chord, Stage 25. Dark areas of the notochord are caused by photographic grains. × 67 (approximately).



(Johnston and Comar: S^{35}-sulfate in the chick)

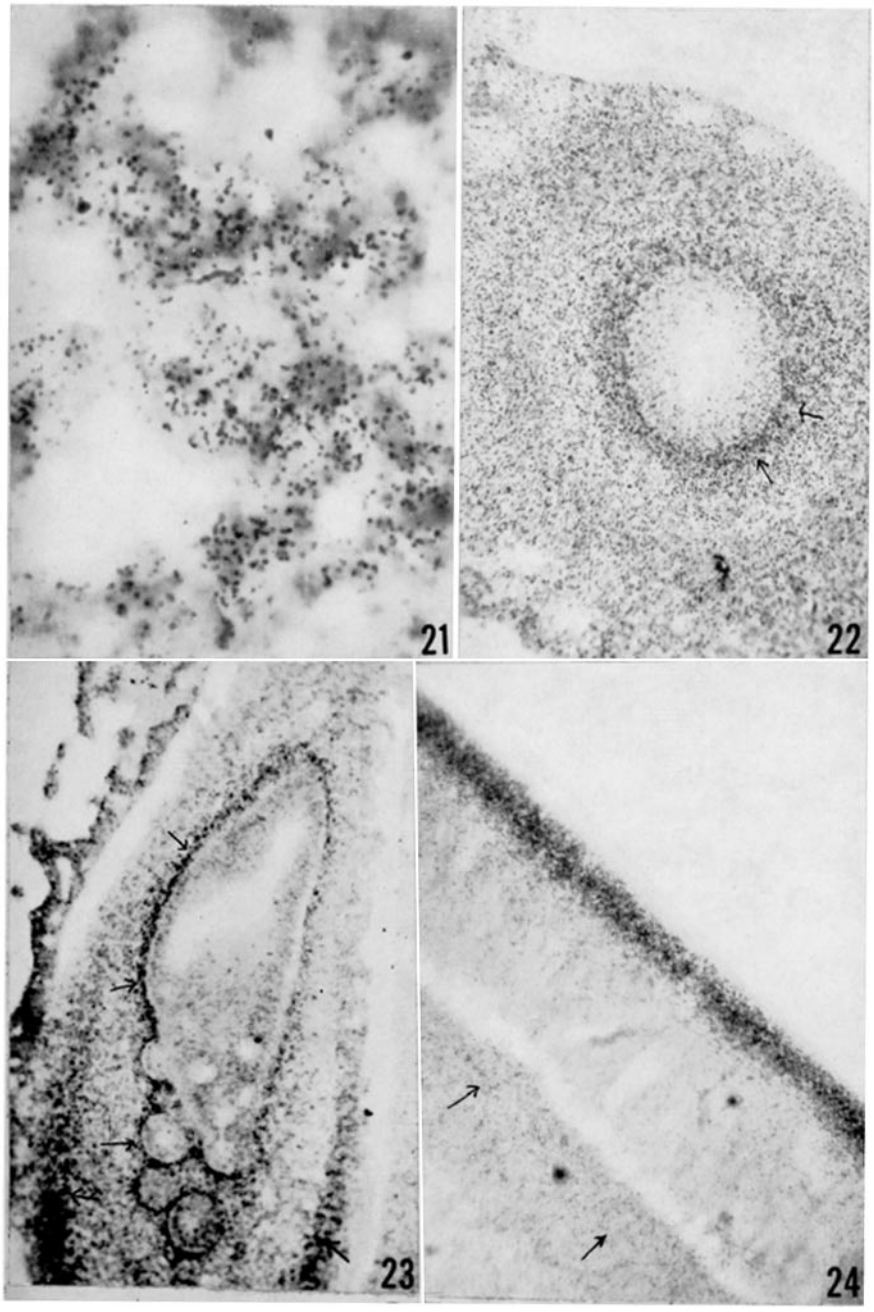
PLATE 64

FIG. 21. Several mesenchyme cells of the head region of Stage 25. These cells show intracellular localization of sulfur-35. Note the absence of grains in the intercellular spaces. × 814 (approximately).

FIG. 22. Cross-section of the gut of Stage 33. Note the concentration of grains immediately beneath the epithelium indicated by arrows. Some grains may be seen in the developing smooth musculature. × 167 (approximately).

FIG. 23. Partial longitudinal section of the stomach of Stage 34. Note the grains lying beneath the epithelium as indicated by arrows. A concentration of grains also occurs on the peritoneal surface. × 83 (approximately).

FIG. 24. A portion of the gastric epithelium of Stage 38. Note the dense layer of grains above the epithelium and the scattered grains throughout the epithelium. Note also the grains in the submucosal region as indicated by arrows. × 167 (approximately).



(Johnston and Comar: S^{35}-sulfate in the chick)