SULFATE-SULFUR METABOLISM IN THE RAT FETUS AS INDICATED BY SULFUR-35

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PLATES 10 AND 11

(Received for publication, May 11, 1953)

It has been reported by Layton *et al.* (1) that a transfer of sulfate-sulfur from the maternal organism to the fetus may occur in the rat. On the other hand, Hanahan, Everett, and Davis (2) found no sulfur-35 in embryos of pregnant rats which had been given estrone sulfate-S^{35} subcutaneously. However, they demonstrated that homogenates of various rat tissues were capable of hydrolyzing estrone sulfate and that nearly 70 per cent of the sulfur-35 was excreted as inorganic sulfate. In view of this, one would expect that the sulfate-sulfur of the conjugated estrogen would have been distributed in the tissues of the rat, qualitatively at least, as is sulfate-sulfur when given as sodium sulfate. Indeed, Lewison *et al.* (3) have reported that the distribution of sulfur-35 in the tissues of young adult rats following administration of estrone sulfate-S^{35} is similar to the distribution after administration of sodium sulfate-S^{35}.

Because of this apparent discrepancy between the observations of Layton *et al.* and Hanahan *et al.*, it seemed desirable to report some of our observations on the distribution of sulfate-sulfur in the tissues and embryos of pregnant rats at different stages of gestation.

Methods

The estrus cycle in each of 10 female albino rats (Sherman strain) was determined on the basis of vaginal smears taken at 10 a.m. and 4 p.m. of each day for a period of 3 weeks. The smears were evaluated according to the description of Long and Evans (4). This information was then used in estimating the age of the embryos after mating.

At the desired stage of gestation, each female rat was given intraperitoneally $10.1 \pm 0.2 \times 10^6$ C.P.M. of carrier-free sulfur-35 as sodium sulfate[1] in water. The animals were kept afterwards in metabolism cages, without food but with free access to water, for the following 24 hours so as to collect the urine and feces. They were then sacrificed by exsanguination under deep ether anesthesia. The blood was drawn directly from the heart. The embryos and samples of the maternal tissues were dissected out immediately thereafter and were mostly stored in a "deep freeze" at −25°C. until analyzed. Some of the embryos, however, were placed in fixative immediately after removal from each female. The weights of the embryos at the time of dissection served as a check on their previously estimated age (5).

[1] The sulfur-35 used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission.

Some of the tissues from two embryos of the oldest litter in the series were dissected out for separate analysis. Representative embryos from this and all other litters in the series were analyzed *in toto*.

Two non-pregnant female rats of the same age and strain were given the same amount of S^{35}-labelled sodium sulfate as the pregnant rats. They, too, were sacrificed 24 hours later and their tissues were analyzed like those of the pregnant rats.

The total sulfur-35 concentration in the urines was determined by the use of the reagent of Denis (6). For the analysis, the dilution of the urine samples was of such an extent that it was necessary to add carrier sulfate to insure nearly quantitative isolation of the sulfur-35. The carrier sulfate was added as 5 ml. of 0.05 N sodium sulfate solution prior to oxidation of the sample. The sulfur-35 was isolated as barium sulfate.

Weighed samples of the tissues, the whole embryos, and the feces were each digested on a steam-bath in pyrex tubes with 10 volumes of 10 per cent sodium hydroxide for 8 hours. After cooling, the digests were suitably diluted with water. Aliquots were transferred to Parr bomb crucibles. The liquid was removed by evaporation under an infrared lamp and the residue was then pulverized and mixed with the ignition mixture (potassium perchlorate, sodium peroxide, benzoic acid, and glucose). Fusion was effected in an electrically operated Parr bomb. After solution of the melt in water, 5 ml. of 0.05 N sodium sulfate solution were added. The pH of the solution was adjusted to 2–3 with concentrated hydrochloric acid. The sulfate was then precipitated as barium sulfate from the boiling solution by the slow addition of 5 ml. of 10 per cent barium chloride.

Some representative embryos were also homogenized in water. Aliquots of the homogenates were digested on a steam-bath in pyrex tubes with 1 volume of 10 per cent sodium hydroxide for 8 hours. After cooling and dilution, samples were evaporated in Parr bomb crucibles and then oxidized as above. Other aliquots of the homogenates were mixed with an equal volume of 10 per cent trichloroacectic acid and centrifuged. Samples of the supernatant fluids, neutralized with 10 per cent sodium hydroxide, were evaporated in Parr bomb crucibles. The resultant residues were oxidized as described above for the tissues.

The barium sulfate precipitates were isolated by filtration onto tared disks of Schleicher-Schull, No. 589, red ribbon, filter paper, using a stainless steel Tracerlab filtering funnel. During the course of the filtration, the pressure in the system was not allowed to fall below 35 cm. of mercury. The paper disks with the barium sulfate samples were dried and weighed. To prevent the barium sulfate deposits from flaking in subsequent handling, a drop of 1 per cent collodion in acetone was placed upon each quadrant of the circular layer. The barium sulfate samples were mounted on brass disks and held in place with brass rings. The mounted samples were counted with an end-window G-M tube. The thickness of the end-window was 1.50 mg. per $cm.^2$ and the distance of the barium sulfate layer from the window was about 2 mm. All values were corrected for decay and self-absorption.

At least one embryo from each female was fixed for 6 days at 25°C. in a 3.7 per cent solution of formaldehyde (1 volume of 37 per cent U.S.P. formaldehyde, Merck and Co., Inc., Rahway, New Jersey, was diluted with 9 volumes of distilled water). At least one other embryo from each female was fixed for 6 days at 25°C. in a 3.7 per cent solution of formaldehyde saturated with barium hydroxide. After dehydration in upgraded concentrations of ethanol, starting with 30 per cent ethanol, the embryos were cleared in xylol and embedded in paraffin. Starting, usually, from the side of an embryo, sections were cut at 7μ until the middle of the embryo was reached. From six to twelve representative sections from different levels of each embryo were taken for examination. The sections were mounted on microscope slides with egg albumin. The paraffin was removed with xylol and the sections were then covered in the dark with 3 by 1 inch strips of Kodak contrast process ortho film as previously described (7). After an exposure of 3 weeks' duration, the film was developed for 5 minutes in Eastman Kodak Co.

developer, D-19. Subsequently, the sections were stained for 30 seconds with a 0.1 per cent solution of toluidine blue in 30 per cent ethanol.

TABLE I

Sulfur-35 Concentration in Tissues of Adult Rats 24 Hours after Administration of Labelled Sodium Sulfate

The concentration is given per gram of wet tissue. Each rat received 10.1 ± 0.2 × 10^6 C.P.M. of carrier-free sulfur-35 intraperitoneally as an aqueous solution of sodium sulfate 24 hours before sacrifice. All values for sulfur-35 are corrected for decay and self-absorption.

Tissue	Pregnant females	Non-pregnant females	Embryos*
	C.P.M. × 10^3	C.P.M. × 10^3	C.P.M. × 10^3
Intestinal tract	47.60±4.20‡	47.57	6.30
Cartilage§	10.50±0.83	11.70	315.00
Uterus	8.54±1.02	9.19	—
Bone and marrow‖	7.55±0.49	7.68	—
Kidneys	6.53±0.52	6.42	6.05
Blood	5.01±0.95	3.25	—
Spleen	4.79±0.37	4.20	—
Liver	3.63±0.44	3.67	3.20
Lungs	3.51±0.25	3.30	3.85
Skin	2.38±0.18	2.77	5.82
Heart	1.68±0.20	1.63	4.20
Muscle	0.88±0.12	0.65	12.36
Brain	0.75±0.05	0.70	2.64
Hair	0.67±0.08	1.23	—
Urine	5915±176	6320	—
Feces	1064±165	565	—
No. of rats	10	2	2
Body weight, *gm.*	244 (196–269)	205 (198–213)	3.63 (3.60–3.67)

* The embryos were from the same litter.

‡ Probable error of the mean value is given.

§ The sternum of the adult animals and the humeri of the embryos were used.

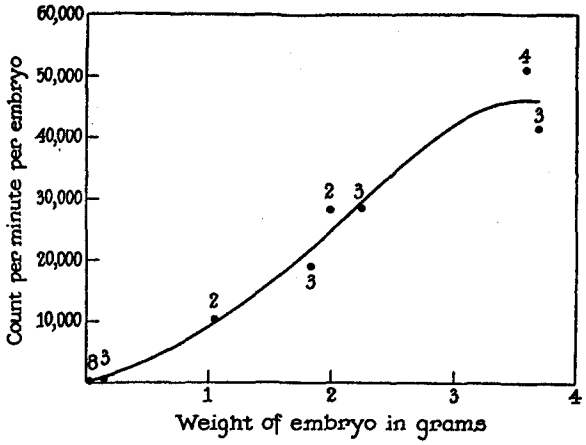
‖ The cylindrical portion of the humerus was analyzed.

RESULTS

The distribution of sulfur-35 in the tissues of the pregnant and non-pregnant female rats as well as in some tissues of two embryos removed near term is given in Table I. These data show that in the adult rats the highest concentration of sulfur-35, 24 hours after its administration as sodium sulfate, was found in the intestinal tracts and contents. Then followed, in order of decreasing concentration, sternal cartilage, uterus, bone and marrow, kidneys, and blood. In the spleen, liver, and lungs, the concentration of the isotope was nearly the

same as in the blood, whereas in the skin, heart, skeletal muscle, brain, and hair it was lower. No striking difference was apparent in the distribution of the sulfur-35 in the tissues of the pregnant and non-pregnant rats.

The excretion of the sulfur-35 by the pregnant rats, in the urine and feces combined, was also approximately the same as by the non-pregnant rats. Nearly 70 per cent of the dose was excreted within 24 hours; about 60 per cent of the dose was found in the urine.



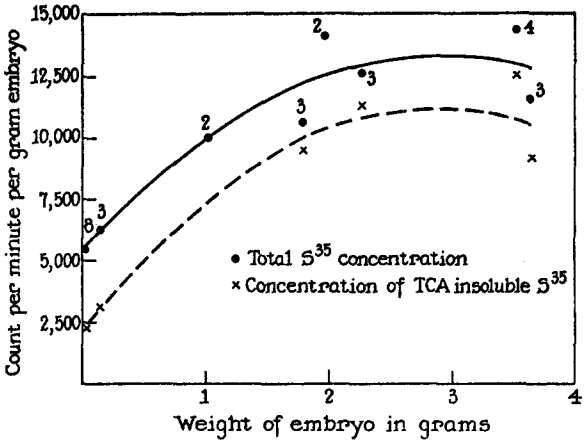
TEXT-FIG. 1. Relationship between the content of sulfur-35 in an embryo and its weight (age). The embryos were removed 24 hours after the intraperitoneal administration of carrier-free sulfur-35 ($10.1 \pm 0.2 \times 10^6$ C.P.M.) as sodium sulfate in water to pregnant rats. The number at each point is the number of embryos used to ascertain the value of the point. The curve was drawn by inspection.

The concentration of sulfur-35 in the kidneys, liver, and lungs of the embryos near term was found to be about the same as in the corresponding organs of the pregnant rats (Table I). It was lower in the embryonic intestinal tract than in the adult intestinal tract. In the other tissues which were analyzed, the sulfur-35 concentration was definitely higher than in the corresponding maternal tissues. The sulfur-35 concentration in the humerus (largely cartilage) was found to be about 30 times higher than in the maternal sternum. About 15 times more sulfur-35 was found per gram of embryonic biceps femoris than in the same muscle of the pregnant rats. Per unit weight, more of the isotope was also found in the embryonic brain and skin than in the brain and skin of the adult rats.

When the sulfur-35 content in the whole embryos was determined, it was found, Text-fig. 1, that the older embryos had accumulated more of the sulfur-35 than the younger embryos in the same length of time. The embryos showing the highest content of the isotope were approximately 20 days old and were

representative of 8 embryos in that particular litter. Eight times the highest value plotted in Text-fig. 1 accounts only for about 4 per cent of the sulfur-35 which had been given to the pregnant rat.

If, instead of considering the amount of sulfur-35 per embryo, one plots the sulfur-35 per gram of embryo against the weight (age) of the embryo (Text-fig. 2) it will be seen that up to an embryonic weight of about 2 gm. (about the 19th day of development), there is a progressive increase in the concentration of the sulfur-35. Beyond the 19th day of development to nearly term, the sulfur-35 per gram of embryo was not found to be strikingly diffeernt.



TEXT-FIG. 2. Relationship between the concentration of sulfur-35 per gram of embryo and its weight (age). The embryos were removed 24 hours after intraperitoneal injection of carrier-free sulfur-35 ($10.1 \pm 0.2 \times 10^6$ C.P.M.) as sodium sulfate in water to the pregnant rats. The number at each point is the number of embryos analyzed to obtain the value of the point. The curves were drawn by inspection. TCA is used as an abbreviation for trichloroacetic acid; for further details consult the text.

Of further interest is the fact that a large fraction of the sulfur-35 in the embryos was found to be insoluble in 5 per cent trichloroacetic acid. The magnitude of this fraction varied with the age of the embryo. Whereas, in the youngest embryos examined, those about 10 days old, this fraction accounted for approximately 40 per cent of the total, nearly 90 per cent of the sulfur-35 in the embryos near term was insoluble in the 5 per cent solution of trichloroacetic acid. This suggested the possibility that some of the isotope was in chondroitin sulfate. Indeed, the radioautographs of sections from embryos fixed in a solution of formaldehyde of pH 3.8–4.0 give strong support to this possibility. Reproductions of representative radioautographs of embryos thus fixed are given as Fig. 1 a, 2 a, 3 a, 4 a, and 5 a. Even a cursory examination of the radioautographs reveals that there was a relatively high concentration of sulfur-35 in

the embryonic skeleton. The skeletons of the embryos were largely cartilaginous and, therefore, probably contained much chondroitin sulfate. As can be seen from the reproductions of the sections, the skeleton was intensely stained, metachromatically, with toluidine blue.

On closer inspection, it can also be seen from Figs. 3 a, 4 a, and 5 a that throughout the regions of the skeleton where calcification had occurred less sulfur-35 was present than in regions which were still cartilaginous.

Radioautographs produced by an exposure of the same film for the same length of time to sections of the embryos fixed in a solution of formaldehyde saturated with barium hydroxide were of an over-all intensity approximating that given by the liver and lungs in Fig. 5 a. This indicates that the concentration of sulfur-35 in the inorganic sulfate and any other sulfur-containing component which is insoluble in the presence of barium ions, at a pH of about 12, was probably low as compared with the concentration of the sulfur-35 in the chondroitin sulfate of the skeleton.

Especially of interest were the radioautographs of the youngest embryos, together with the uteri in which they were developing. In Fig. 1 a, for example, which is a reproduction of a radioautograph of a section from an embryo about 10 days old, one sees that the embryo proper, *A*, did give a reaction, but this is not as intense as that given by the surrounding tissue. More sulfur-35 appears to have been retained in the decidual cells, the trophoblast, and even in the uterine wall than in the embryo. At a later stage in gestation, about the 14th day (Fig. 2 a) though the uterus gave a good autographic reaction, it is clearly apparent that the concentration of the sulfur-35 in the skeleton of the embryo was much higher.

In the reproduction, Fig. 5 a, of a radioautograph, yielded by a section from one of the oldest embryos in the series, about 20 days old, an arrow points to two black dots. Two small spots which corresponded exactly to the two dots in the radioautograph are likewise pointed to by an arrow on the reproduction of the section, Fig. 5. These two small spots in the section stained metachromatically with toluidine blue[2] and when magnified, as in Fig. 6, it can be seen that they were material free in the lumen of the intestine. The two dots in the radioautograph are reproduced at the same magnification in Fig. 6 a. It is readily seen that they correspond. The fact that the latter were produced by metachromatically staining material with toluidine blue suggests that even at about the 20th day of development *in utero* the embryonic intestinal wall was secreting a mucopolysaccharide which contains sulfate. An examination of all the sections from embryos 20 to 21 days old revealed that in 10 out of 40 such preparations material staining metachromatically was present free in the

[2] As the result of a question by Dr. Richard C. Greulich of McGill University, Montreal, our attention was brought to focus on the study of the material in the intestine of the embryos which gave the radioautographic reaction.

intestinal lumen. In each instance, corresponding dots were also found in the radioautographs. Although metachromatically stained material was found free in the intestinal lumen in sections from embryos down to the 18th day of development, only in the case of the embryos 19 days old or more was it possible to be certain that they corresponded in position to that of a definite reaction on the film.

The radioautographs of embryos at different stages of development were not prepared at the same time. No comparison, therefore, as regards the relative concentration of sulfur-35 in the tissues from one embryo to another is valid on the basis of the radioautographs.

DISCUSSION

A tabulation of the tissues from the adult female rats according to a descending concentration of sulfur-35 (Table I) agrees for the most part with the tabulation by Lewison *et al.* of the concentrations found in the tissues of their rats 12 hours after the subcutaneous injection of S^{35}-labelled sodium sulfate (3). Singher and Marinelli (8) also found that 14 to 16 hours after intraperitoneal injection of S^{35}-labelled sodium sulfate to a young adult rat, the sulfur-35 concentration in the kidneys > spleen > liver > brain. They found, however, a much higher concentration of the isotope in the hair than in the liver.

The excretion of the sulfur-35 in the urine and feces by the rats studied by Lewison *et al.* was found to be much higher than the average values noted in Table I. Their values of well over 90 per cent of the dose excreted in the urine and feces in 24 hours do not agree with our value of about 70 per cent, which is similar to the value previously reported (9).

Layton *et al.* (1) in the analysis of embryonic tissues washed them prior to assay. Despite this and the fact that the tissues were removed 7 days after the administration of the labelled sulfate-sulfur intramuscularly to the pregnant rat, the relative order of concentration in embryonic heart, kidney, muscle, and entire bone was found to be similar to that given for these embryonic tissues in Table I.

In the rat there is a transfer of sulfate-sulfur from the maternal organism to the fetus. The radioautographs in conjunction with the determinations of the sulfur-35 which is insoluble in 5 per cent trichloroacetic acid provide evidence that the sulfate-sulfur, which the embryos obtain from the maternal organism, is largely utilized in tissue synthesis. Of the tissues which utilize the sulfate-sulfur, the cartilage is the most prominent per unit weight. It is strongly suggested that the sulfate-sulfur in the embryo is one of the components in the synthesis of chondroitin sulfate of skeletal cartilage.

The embryonic skeletal cartilage, however, is not the only tissue in which the sulfur-35 was found to have been retained in a form insoluble in an acidic solution of formaldehyde. As judged by the radioautographs, Figs. 1 a, 2 a, 3 a,

4 a, and 5 a, a much lower but still discernible amount was found in almost all of the tissues.

Since the embryos apparently utilize the sulfate-sulfur for building tissue, particularly cartilage, it follows that as the embryo grows the daily uptake of the sulfate-sulfur increases. The period of gestation covered in the present study includes the period of the most rapid increase in weight of the rat embryo. It is not surprising, therefore, that in similar intervals of time, older embryos were found to have retained more sulfur-35 given as sulfate-sulfur than younger embryos (Text-fig. 1).

The meaning of the observation that the total amount of sulfur-35 per gram of embryo is roughly the same from 19th day of gestation to term can only be surmised at present. It is possible that the findings reflect the increase in mass of tissues which retained the sulfur-35 and a nearly proportional increase of other tissue. It does not appear to be a reflection of a limited availability to the embryos of sulfur-35 from the maternal organism. Approximately 25 per cent of the dose given was still present in the maternal rats at the time the embryos were removed; some of it was in the blood.

It is particularly interesting that even some secretion of a sulfur containing mucopolysaccharide was probably taking place in the embryos a few days before birth. This material, observed free in the lumen of the intestine, stained with toluidine blue in a manner characteristic of the acid mucopolysaccharides. Since in addition it elicited an autographic reaction on photographic film, it contained sulfur-35, probably as bound sulfate. Mucoitin sulfuric acid(s) have long been known to be present in the mucus secreted by the gastric mucosa (10). However, the material observed free in the intestinal lumen, and apparently made up in part of sulfate-sulfur, has not been proved to have derived from the cells lining the intestinal wall.

SUMMARY

Twenty-four hours after the intraperitoneal injection of sodium sulfate-S^{35} into pregnant rats, sulfur-35 was found in the embryos. The amount of the sulfur-35 retained by the embryos was directly related to their degree of development *in utero*.

A large fraction of the sulfur-35 found in the embryos was insoluble in 5 per cent trichloroacetic acid. At the 9th to 10th day of development, about 40 per cent of the sulfur-35 was present in this fraction. In 20-day-old embryos this fraction accounted for nearly 90 per cent of the total.

Radioautographs of sections of embryos fixed in a solution of formaldehyde revealed that the sulfur-35 was most highly concentrated in the cartilaginous portion of the skeleton. All other tissues gave much weaker autographic reactions, comparable with the over-all reaction obtained when sections from embryos fixed in a solution of formaldehyde saturated with barium hydroxide were used.

By analysis for the sulfur-35 content of individual tissues the concentration of the sulfur-35 in humeri from 20-day-old embryos was found to be about 30 times that in the maternal sternum. The concentration of the isotope in the skeletal muscle, brain, heart, and skin of the same embryos was also higher than in the corresponding maternal tissues. On the other hand, the concentration of the sulfur-35 in the maternal gastrointestinal tract plus contents was higher than in the gastrointestinal tract and contents of the embryos.

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EXPLANATION OF PLATES

PLATE 10

The sections reproduced in Figs. 1, 2, 3, 4, 5, and 6 are in all instances from specimens that had been fixed for 6 days in a 3.7 per cent solution of formaldehyde at 25°C. The specimens were removed from gravid female rats 24 hours after the latter had an intraperitoneal injection of $10.1 \pm 0.2 \times 10^6$ C.P.M. of carrier-free sulfur-35 as sodium sulfate in water. After their radioautographs were prepared, the sections were stained for 30 seconds with a 0.1 per cent solution of toluidine blue in 30 per cent ethanol.

FIG. 1. Photograph of a section of a rat embryo (*A*) *in utero* at about the 9th to 10th day of gestation. × 13.6.

FIG. 1 a. Photograph of the radioautograph produced by the section, Fig. 1. It can be seen that a slight reaction was given by the embryo (*A*). A more intense reaction, however, was given by the decidual cells, the trophoblast, the line of erosion, the mesometrium, and the uterine wall. × 13.6.

FIG. 2. Photograph of a section of a rat embryo *in utero* at about the 14th day of gestation. × 2.

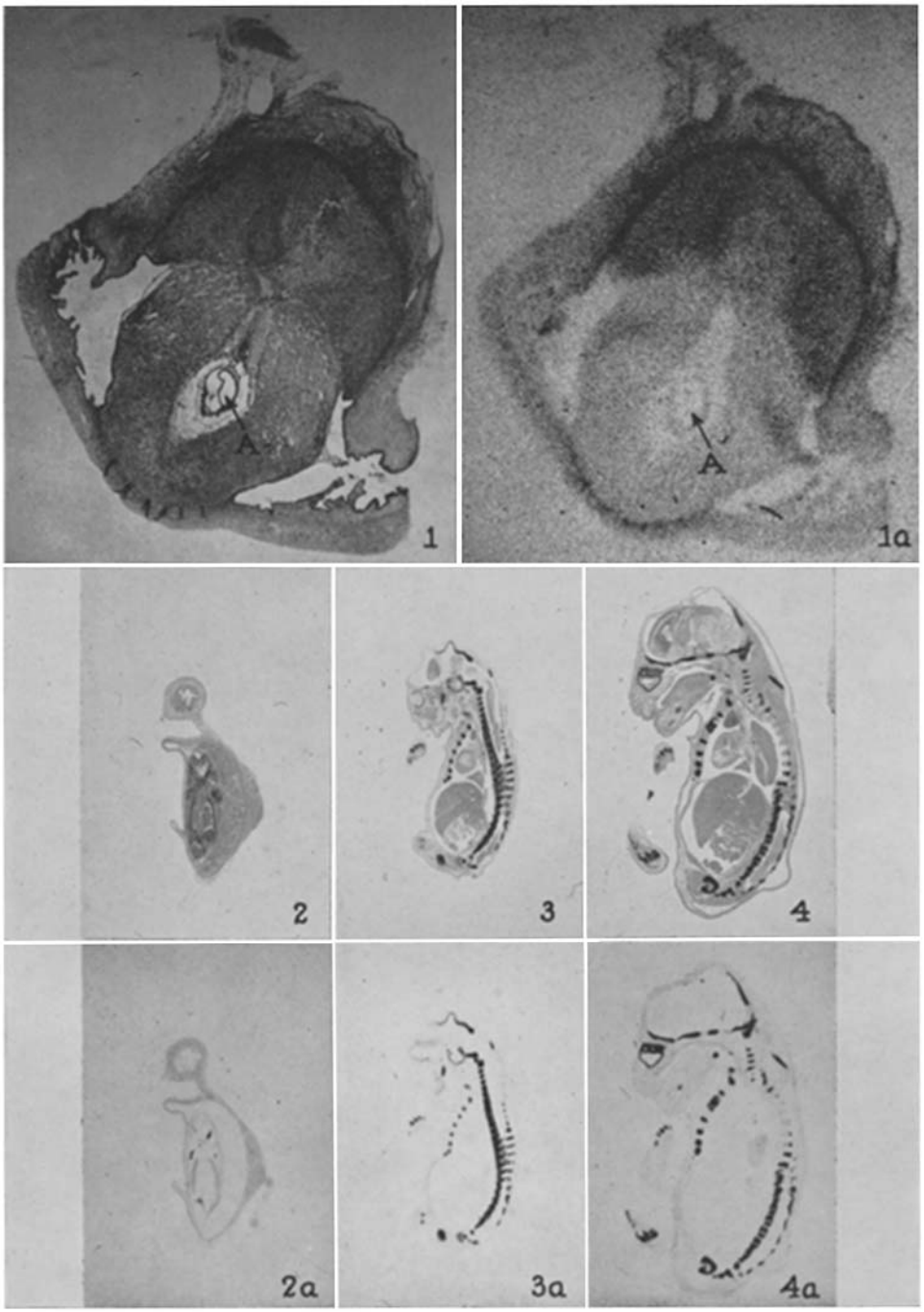
FIG. 2 a. Photograph of the radioautograph produced by the section, Fig. 2. The embryonic skeleton gave a more intense reaction than any of the other tissues. × 2.

FIG. 3. Photograph of a section from an embryo about 18 days old. × 2.

FIG. 3 a. Photograph of the radioautograph produced by the section, Fig. 3. It can be seen that the skeleton gave a more intense reaction than the other tissues. × 3.

FIG. 4. Photograph of a section from an embryo 19 days old. × 2.

FIG. 4 a. Photograph of the radioautograph produced by the section, Fig. 4. Again, it is apparent that the skeleton gave a more intense reaction than the other tissues. It can also be seen that calcified and calcifying regions of the skeleton give a less intense reaction than the cartilage in the skeleton. × 2.



(Dziewiatkowski: Sulfate-sulfur metabolism in rat fetus)

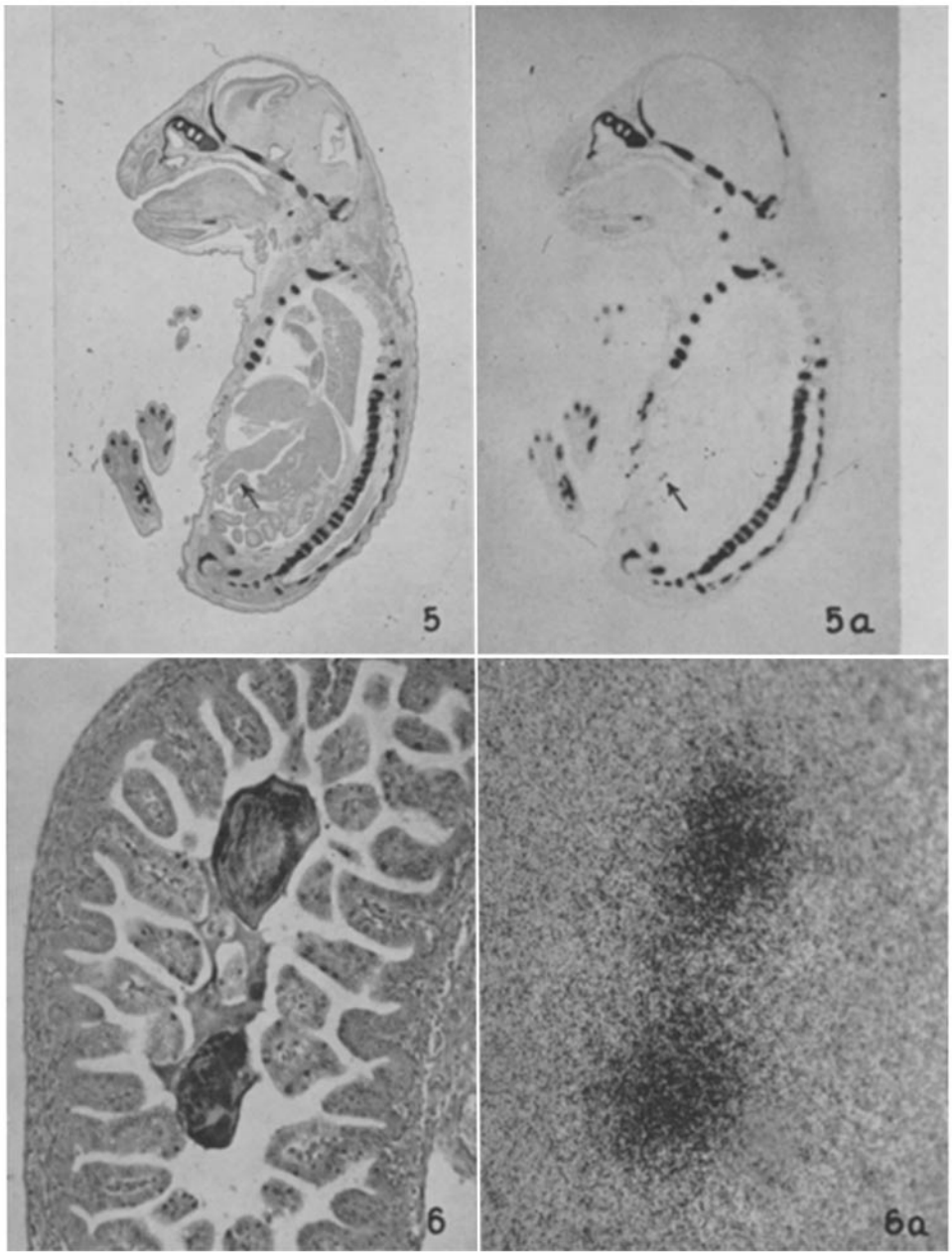
PLATE 11

FIG. 5. Photograph of a section from an embryo about 20 days old. The arrow points to metachromatically stained material which was found free in the intestinal lumen. × 2.7.

FIG. 5 a. Photograph of the radioautograph produced by the section, Fig. 5. The reaction of the skeleton on the photographic film was more intense than that of the other tissues. Regions of calcification can again be made out as having given a less itense reaction than the cartilage in the skeletons. The arrow points to the two dots which correspond to loci similarly indicated in Fig. 5. × 2.7.

FIG. 6. Photograph at greater magnification of the area indicated by the arrow in Fig. 5. The metachromatically stained material is seen as free in the intestinal lumen. × 113.

FIG. 6 a. Photograph at greater magnification of the area indicated by the arrow in Fig. 5 a. The correspondence in location is good between the relatively high concentration of silver grains in two loci and the metachromatically stained material in Fig. 6. × 113.



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