THE ORIGIN OF THE PRIMORDIAL GERM-CELL OF THE CHICK STUDIED WITH TRITIATED THYMIDINE

by

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To all of those cited or inferred and most of all to the atmosphere so conducive to the pursuit of knowledge, which I have had the privilege to have been a part of, this thesis is dedicated.

Kit G. Johnson, April 1962.

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INTRODUCTION

The desire for knowledge of the origin of life and of life itself led Aristotle to open the hen's egg hoping that by getting closer to life's origin, as he understood it, he could detect its secrets. We would not expect to find the origin of life in an egg today, but biologists have continued to be fascinated by the transmission of life, from the vital link carried by one nucleic acid to the packaged life potential of an egg. This investigation was undertaken in an attempt to provide better understanding of one step in the transmission of life, the origin of the primordial germ-cells.

PART I

The Problem

Because of its availability and ease of study, the chick embryo's developmental morphology has probably received more thorough study than that of any other. However, despite excellent exhaustive work by many competent investigators the origin of the gametes has remained obscured by voluminous conflicting evidence. In order better to understand the historical perspectives of this evidence and the problem as a whole, a brief description of the primordial germ-cells and their most widely accepted life history will be given, followed by a historical presentation, and a discussion of the conflicts and problems as they exist now.

A. The primordial germ-cell

1. Morphology

The most distinguishing characteristic of the primordial germ-cells is their large size with a diameter ranging from 14 to 22 microns. They are usually spherical and early in their life contain a great deal of yolk copiously distributed throughout the cytoplasm in the form of globules. By 2 days of age these globules seem to coalesce into 2 to 4 large yolk spheres and are slowly absorbed by

4 to 6 days. Another early prominent characteristic of early stages is the pale centrosphere which may be 3 to 4 x 6 microns in diameter and is often located at the opposite side from the nucleus. The Golgi apparatus is large but of variable prominence while the mitochondria are not significantly different from those of somatic cells. The large spherical nucleus is very striking, with a diameter of 8 to 10 microns before the early somite stage and a diameter of 10 to 12 microns after this. Its clear vesicular character is easily spotted and the reticular chromatin is often divided into two granular clumps. At the age of the primordial germ-cells in this study, they are identifiable as being 3 to 4 times as large as the somatic cells with diffuse cytoplasm containing a clear reticulated nucleus which is about the same size as a whole somatic cell, and with one or two large intranuclear chromatin clumps (12,25,63) (Illus. 1 and 2).

2. Natural history

When the chick embryo is 18 hours old, a primitive head fold is present with ectoderm, mesoderm and entoderm. This is surrounded by an area pellucida which has ectoderm and entoderm with a small central area of mesoderm supplemented by scattered mesenchymal cells. Surrounding the area pellucida is the area opaca composed of ectoderm and entoderm which is several cells thick (40,76). At this

time in a region anterior and lateral to the head fold and marginal to the area pellucida, called the germinal crescent, the primordial germ-cells begin to bud off of the area opaca's entoderm into the space between it and the ectoderm, where they wander with the few mesenchymal cells present (Illus. 1). By 33 hours of age the area opaca has been extensively vascularized, contains many blood islands, and the circulation with the embryo proper is established. Now by the amoeboid-like movement characteristic of many primitive cells, the primordial germ-cells enter the capillaries and the circulation, which carries them throughout the embryo. At the age of 45 hours, when the embryo has 21 to 25 somites, the primordial germ-cells begin to leave the circulation and to localize in the splanchnic mesoderm in the region of the future gonadal ridges. This completes the most controversial phase in the development of the primordial germ-cells (14, 31, 60).

At 4½ days of age the gonadal ridges are easily recognized on the medial aspects of the mesonephric ridges by their distinct columnar germinal epithelium which has the primordial germ-cells scattered in it and the underlying stroma (Illus. 2); however, in both sexes, which cannot yet be differentiated, the left gonadal ridge is about 2 to 5 times larger than the right. The primary proliferation of the germinal epithelium which carries many of the

primordial germ-cells deep into the stroma occurs at 6½ days. Another 1/2 day of development in the male allows these migrating cells to be recognized as sexual cords which are the precursors of the seminiferous tubules; they remain disorganized in the female. From the 8 to 11 days the primordial germ-cells of female's left gonadal ridge only undergo rapid mitosis with a secondary proliferation of the germinal epithelium on the ninth day which resolves as the follicles of the stroma, while the primordial germ-cells have become recognizable as the definitive oogonia, and the right gonadal ridge atrophies. The sexual cords of the male continue to organize until the 13th to the 15th day when their primordial germ-cells also undergo rapid mitosis and evolve as spermatids. This second phase in the evolution of gametes from the germinal epithelium has generally been agreed upon, except for the role played by the primordial germ-cells (60).

B. Prior research

1. Observational

As observation is the cornerstone of science, similarly observations have contributed most to our knowledge of the primordial germ-cells; therefore, we will discuss the findings of observations before progressing into experimental results. It is unfortunate for this review that almost all of the earliest observations on the

primordial germ-cell have been published in German. However; there were significant contributions by French investigators around the turn of the century. In 1886 Laulanie noted the early gonads of the chick embryo appeared to be bisexual, or at least their destined sex could not be determined (39). Prenant followed in 1889 with a complete study of the young chick embryo's gonads, describing their evolution from the germinal ridge and reporting primordial germ-cells located in the very early mesenchyme (53). Hoffmann raised the question of the possible extra-embryonic origin of the primordial germ-cells by reporting their presence in many locations in the 23 somite embryo and not being able to find intermediate stages in the gonadal areas, in 1893. Therefore, they must have an exogenous origin (32).

In 1904 d'Hollander stated that he could see the precursors to the gametes arising from the local germinal epithelium and the controversy was crystallized for posterity (33).

Supporting the exogenous theory of origin in 1913 were Firket's original concepts of amoeboid movement by the primordial germ-cells and a dual origin of the gametes. He believed that many of the primordial germ-cells degenerate while others form gametes and a parallel group of gametes is formed from some of the indistinguishable

germinal epithelial cells (17). He also noted in 1914 that the left gonadal ridge invariably had more primordial germ-cells than the right and postulated a chemotactic differential (18).

Swift, in three comprehensive articles in 1914, 1915 and 1916, presented his excellent study of the primordial germ-cells, which in detail traced them from an origin in the germinal crescent to the definitive gametes. Observing the left side to have more than the right, he too supported chemotaxis but did not believe the germinal epithelium contributed to the formation of gametes (63,64,65). Defretin in 1924 supported Swift's findings (7). Woodger in 1925 felt that the Golgi apparatus, a criterion employed by Swift, was not sufficiently distinct to use as a marker in tracing the primordial germ-cell (75).

In 1926 Richards, Hulpiea and Goldsmith said that there was "not the slightest doubt" that the primordial germ-cells alone formed the gametes, while the germinal epithelium formed the supporting elements of the follicle (57). Goldsmith reiterated this opinion in 1928 with one of the best morphological descriptions of the primordial germ-cells (25).

The possibility of another site of origin for the primordial germ-cells was raised by Matsumoto in 1932 when he reportedly saw them at the posterior margin of the

blastoderm in the primitive streak (45). Later Rawles supported this question in 1936 by reporting primordial germ-cells present in a graft from the nodal region taken before the primordial germ-cells should have migrated (54).

Witschi in 1935 thought that the primordial germ-cells were evenly distributed between right and left gonads until the third day; then on the fourth they started migrating to the left, because of an "attraction". He also failed to see any germinal cortex at any time in the right ovary (74).

But the last of the good descriptive studies, that by Essenberg and Garwacki in 1938, concluded that the primordial germ-cells were of endogenous origin. Their basis for this decision was the finding of pregamete "sex-cells" in the region of the germinal epithelium before the circulation was established, which would make these new cells the true primordial germ-cells, while they believe the classical primordial germ-cells degenerate and are functionless (12).

2. Experimental

Such divergent interpretations by observers of approximately the same material would naturally lead investigators toward an experiment designed to settle this intriguing question. The first experiment directed toward this end, by Ragan in 1916, was classical and perhaps

should have been definitive. He found that by excising the germinal crescent he created sterile gonads, which still developed interstitial cells. However, he was unable to draw any conclusions from similar "sterile" embryos to which he transplanted germinal crescents. Despite apparently thorough work he was unable to maintain an operated embryo beyond 21 days and doubted if his germinal crescent transplants took (55).

A problem and analogy which was to continually recur is the origin of the new or transformed gametes in mature chickens which undergo spontaneous true reversal of sex. Pearl and Boring first raised this question in 1918 and thought the new seminiferous tubules of prior hens appeared to arise from development of the old second proliferation "sex cords" of the ovary (50). Benoit concurred with their findings in 1923 (3) and again in 1924 Gatenby (24) and Fell (15) independently looked to sex reversal in adult chickens for a clue. They both felt that in metamorphasing hens the new male gametes were derived from the peritoneal cells covering the gonads and that this supports the germinal crescent theory of origin, but cell rests of exogenous primordial germ-cells seem just as likely.

Seeking clues from the possible hormonal or inductive influences of developing gonads upon each other and their developing primordial germ-cells, Greenwood in 1925

and Willier in 1927 grafted gonads into chorio-allantoic membranes. Using differentiated gonads grafted into hosts greater than 7 days old, Greenwood concluded no interaction occurred (28), and Willier concurred from his findings upon grafting 4 to 6-day old gonads into "indifferent stage" embryos (68). In 1926 Willier was unable to reach any conclusions regarding the interaction of 19-hour old area pellucida grafts onto 9-hour old chicks because of the great post-operative deformity in the gonadal region present after 9 days growth (67).

Brode in 1928 (6) and Domm in 1929 (8) opined that retention of the primordial germ-cells in the right gonad was necessary for it to be fertile after removal of the left. Destruction of the germinal crescent by Benoit in 1930 with ultraviolet light without subsequent appearance of the primordial germ-cells in the badly deformed gonads led him to conclude he had destroyed the primordial germ-cells in the germinal crescent (4); however, the probability of endogenous primordial germ-cells arising from the germinal epithelium of such apparently aberrant gonads and embryos as remained may be just as unlikely.

By grafting a whole blastoderm onto another embryo's chorio-allantoic membrane and finding a sterile parasite developing, Willier concluded in 1931 that exogenous primordial germ-cells are necessary (69). An interesting

observation by Domm in the same year was that the left ovary of the hatched female chick was capable of forming a testis if the cortex alone is destroyed, a finding which, he thought, supported an endogenous origin of the gametes (9), but again primordial germ-cells cell rests cannot be ruled out.

Willier performed a series of chorio-allantoic grafting experiments in 1933 and stated that the primordial germ-cells remaining in the germinal crescent failed to differentiate but merely continued to multiply, while those in contact with coelomic epithelium not only differentiated, but also induced gonadal formation. He also noted that there was great variability in the viability and sterility of gonadal grafts, with both viability and fertility increasing with increased morphological differentiation at the time of transplant, but neither could be counted on at any age (70,71). In 1937 he reported a great deal of difficulty in obtaining viable transplants, but noted that all sterile transplanted gonads were male, while transplanted primordial germ-cells seemed to be capable of developing into either sex (72).

A rather confusing paper by Essenberg in 1937 reports his attempt to destroy the primordial germ-cells by 600 roentgens of total embryo irradiation. At 3 days of age this dose was lethal to 96% and in those sectioned

immediately post irradiation no migrating primordial germ-cells were present. The chicks which managed to survive to six days of age with normal appearing gonads had primordial germ-cells present, while those with abnormal appearing gonads did not have primordial germ-cells present. By tenuous reasoning this led him to conclude that the primordial germ-cells were of endogenous origin (11).

In 1938 Hooker and Cunningham used the retroperitoneal regeneration of testes in castrated cocks to support the postulate of two sites of origin of gametes in fowl, the exogenous primordial germ-cells and the germinal (coelomic) epithelium (34).

In a rather detailed and convincing paper in 1939, Essenberg and Svejda reported the destruction of the germinal crescent with the persistence of the appearance of primordial germ-cells in the gonads. They also noted the usual high operative mortality: the hatching of only 6 of 77 eggs merely opened, 3 of 54 with a small germinal crescent incision only, and none in which any operative procedure took place (13).

The last experimental attempt to elucidate the history of the primordial germ-cells, which is not nearly as enlightening as the first, is Willier's 1950 transplantation of the pregonadal and gonadal areas before and after the primordial germ-cells should have arrived. Of his 86

chorio-allantoic grafts only 10 took, all of which were sterile testes. Despite this he concluded that the primordial germ-cells are sexually bi-potential (73).

C. Present status

Although the preceding brief resume' of the research upon the primordial germ-cells may have left the reader with an opinion about their probable life history, it was designed to create confusion because this is exactly where a perusal of the original articles would lead one. Evidence of the confusion reigning among the principal workers in this field is the 50 divergent articles published by 37 of them and reviewed above. Each aspect of every major question raised has excellent evidence to support it. Moreover many of them are also supported by reports in which the investigator is clearly biased and drawing significant conclusions from tenuous evidence, subjective interpretations, or no data. The crucial problems evolving from these antithesis are obvious, but we will briefly formulate them together with a summary of the principal evidence.

1. Origin of the true primordial germ-cell

The fundamental question is the origin of the germ cells, or where do the true primordial germ-cells come from, and its natural resolution from the above data is

into two possible answers: either endogenous or exogenous.

a. Endogenous

The theory of endogenous origin is based upon observations of primordial germ-cells in the pre-gonadal region before the circulation is established (12), and the chronological observations of germinal epithelial cells (33) forming gametes. The non-observational bases of support are the experiments of gonadal regeneration from apparently undifferentiated peritoneal cells (9,15,24), the experimental destruction of the germinal crescent with persistence of the primordial germ-cells and the questionable interpretations of x-ray destruction experiments (13),(11).

b. Exogenous

The evidence for an exogenous origin of the primordial germ-cells divides itself into three groups. First, there is the evidence which indicates the primordial germ-cells are not endogenous. This consists of the lack of observed evolution of gametes from endogenous cells, with the primordial germ-cells suddenly appearing as identifiable cells (32,53). It is also contributed to by the growth of sterile embryos from chorio-allantoic grafts of blastoderms (69).

Second, the evidence which indicates the germinal crescent as the source of the primordial germ-cells is

based primarily upon the observations of cells, morphologically similar to the primordial germ-cells of the gonads, in the germinal crescent (7,25,57,63,64,65). This is supported by the experiments in which sterile gonads are found after destruction of the germinal crescent; although the embryonic deformity is great enough to make any interpretations questionable (4,55). Experiments which indicate that transplanted germinal crescent primordial germ-cells are capable of inducing fertile gonad formation from coelomic epithelium lend further support (70,71).

Lastly are the observations of the primordial germ-cells at sites other than the germinal crescent of the pre-gonalad region prior to the establishment of the circulation (45,54).

c. Dual

Of course a compromise observation of the primordial germ-cells arising both endogenously and exogenously is worth considering (17). But, for the interest of those who prefer counting to reading and evaluating, and laying odds from the number of proponents of the two major alternatives, the exogenous theory wins by a margin of 12 to 8.

2. Role of the "classical" primordial germ-cell

The second fundamental question incorporates and expands upon the first: what is the role of the "classical" exogenous primordial germ-cells?

a. Becomes definitive gamete

This too has numerous proponents of several theories, but the most massive evidence seems to have been accrued in favor of their becoming the definitive gametes. This is supported by observational chronological studies of embryos (7,25,57,63,64,65) and experimental work of two types. The first is destruction of the germinal crescent with the resultant sterile gonads, which often are badly deformed (4,55). The second is the destruction of the left gonad before the primordial germ-cells have migrated, which results in the formation of a fertile right gonad, while if this is done after they have migrated the remaining gonad is sterile (3,6,8).

b. Induce gonadal formation

Another major theory for the role played by the "classical" primordial germ-cells is that they are vital for induction of gonadal formation but do not become gametes themselves. This is arrived at observationally by reasoning that they arrive at the gonads presumably for a purpose but are not seen to differentiate directly into gametes. Experimentally it is supported by the induction of gonads from pregonadal coelomic epithelium following pregonadal region destruction, and the induction of gonads from chorio-allantoic transplanted coelomic epithelium by transplanted primordial germ-cells of the germinal

crescent (70,71). Of course the experimental evidence from these two major theses can support each other.

c. Pluripotent stem cell

A third theory, not wholly incompatible with the first, is that the "classical" primordial germ-cells are no more than pluripotential stem cells which can differentiate into many specialized cells depending upon induction around them, and do differentiate into gametes in the gonads. This may be in part supported by the evidence that grafts of gonads and primordial germ-cells of the area pellucida have no inducing effect upon each other as they each differentiate independently (28,67,68); however, it is also claimed that the primordial germ-cells from the area pellucida will not differentiate without contacting coelomic epithelium or gonads (70,71), which would indicate an induction factor requiring close proximity.

d. Functionless

Of course the fourth theory, that the "classical" primordial germ-cells are functionless and degenerate, is supported by the observations of the same group which proposes an endogenous origin of the primordial germ-cells (12,33). This theory completes the sphere of ideas, evidence and pseudo-evidence surrounding the primordial germ-cells and gives some idea of the difficulty prior

investigators have had in achieving decisive and objective results regarding them.

PART II

Initial Investigations

The information presented in the initial section of this thesis should leave the reader with some idea of the relatively simple alternative pathways proposed for the primordial germ-cells and, with some reflection, he can design one or more experimental procedures which might shed light on the matter. However, the many frustrated attempts by competent investigators, and the conflicting opinions derived from them should serve as an omen of the technical difficulties encountered in charting the course of the elusive primordial germ-cell.

A. Transplants of whole chicks for propagation

1. Hypothesis

Without the benefit of the great deal of detailed knowledge gathered by prior investigators, summarized above, and with only a hazy idea of the postulated natural history of the primordial germ-cells, three and one-half years ago the initial investigations in this series were undertaken. Testing the initial hypothesis required the maturation and reproduction of a chick which had been grafted into the developing membrane of another variety, thereby replacing the original embryo before the circulation

of either was established and the primordial germ-cells had migrated. Thus, if the germinal crescent theory of the origin of the primordial germ-cells were true, the progeny of the transplanted embryos would have been of the host egg variety rather than of the grafted chick species. Sterile gonads would have indicated either failure of the primordial germ-cells to migrate or failure of the gonads to develop due to operative stress.

2. Data

For testing of the above hypothesis 197 eggs were observed or operated on between 11/20/58 and 5/30/59 at approximately 30 to 45 hours of incubated age (Table 1). The great variation in age was due to varied developmental rate in the convection-draft incubator used. Eighty-nine of the eggs were morphologically matured between the early headfold and the 18 somite stages; in these an attempt was made to prepare them as either a host or a donor. Only 11 transplants were judged satisfactory for incubation. Of these, five had some further development of the area opaca and the remaining six had no further development.

Seven control embryos had a small incision made on one side between the area pellucida and the area opaca. Two showed very slight further development and the remaining five did not progress beyond the stage of operation.

3. Discussion

The greatest single detrimental factor seemed to be the immediate widening of any incision in the vitelline membrane and the underlying embryonic membranes because of the internal pressure of the yolk sphere. An attempt was made to reduce this factor by initial aspiration of 1 to 2 cc of yolk, but this gap still occurred, preventing apposition of the donor embryo with host membranes. Even with the inclusion of the largest possible area of the donor's area pellucida, and with destruction of the minimal amount of hosts' tissues using either glass needles or electrocautery, this difficulty could not be overcome.

Other investigations employing less radical procedures at similar ages have not hatched any chicks (41, 42,46,61), although Martinovitch and Pavlovic have reported hatching five chicks, one living over 60 days, after transplanting only the forebrain region (44). The details of their precise technique are enlarged upon in a report by Martinovitch (43) and do not significantly differ from my technique. After hypophysectomy in 691 similarly aged embryos, Fugo successfully had 162 chick embryos survive for at least 12 days -- but none hatched (22). In the light of these reports and my experience, it seemed fruitless to continue following this course.

B. Transplants of primordial germ-cells for propagation

1. Hypothesis

The next attempted procedure, from 6/23/60 to 9/12/60, and one far less radical, was the transplanting of the germinal crescent area with its presumed primordial germ-cells of one variety into the undestroyed germinal crescent area of another variety; i.e., Black Barred Rock and Rhode Island Red. This time the host chick would be required to mature and propagate, and the only positive result required from this experiment would be the production of one offspring of the donor variety, which would indicate the primordial germ-cells had become gametes. Reproducing the host variety would be meaningless, but if the postulated hypothesis of the germinal crescent origin of the primordial germ-cells were true and enough progeny from chickens containing some of these transplanted cells could be produced the donor variety should show up.

2. Data

Therefore, using drawn glass needles as scalpels and drawn glass tubes as pipettes, (with an O.D. of less than 0.5 mm at the tip) the germinal crescent areas of either Black Barred Rock or Rhode Island Red chick embryos in the same developmental stages as previously used, (from the early headfold to no visible beating heart) were aspirated and transplanted into the germinal

crescent areas of similarly developed embryos of the other variety. About 4/5 of the transplants were judged to have been placed below the vitelline membrane and above the embryo's area pellucida ectoderm, while the remainder were inadvertently placed within the yolk mass, below the germinal crescent (Table 1). 374 eggs were used for 197 operations to produce 91 transplants suitable for incubation of which 45 showed no or only slight development, 46 developed to greater than 3 days of age, and 30 lived to 6 days or longer, but none lived longer than 12 days. In 17 eggs merely opened without operation, 3 had slight development, 7 had significant development, and 7 lived longer than 6 days. The ports in these eggs were sealed with Scotch Tape which allowed their development and death to be accurately observed so the embryo could be preserved within 1/2 day of death.

In an attempt to isolate for correction the major cause of this post-operative mortality, 22 bacterial cultures were taken from dead embryos. Fourteen plates from embryos which lived through the third day were negative while 6 had bacterial growth, and of two plates taken from 6 day-old dead embryos, one was positive and one was negative for bacterial growth. This was not judged to be sufficient evidence of contamination, considering the fate of unentered eggs containing dead embryos at incubation

temperatures and the well-known transmission of bacteria in chicken eggs.

3. Discussion

Although the crucial factor would seem to be damage to the embryonic membrane and indeed other investigators report survival is apparently correlated with damage, without citing figures (22,41), and maturation of chick embryo wound healing mechanism occurs only after the tenth incubated day (66); this is not supported by data from Tables 2 and 3 in which the transplants made during this and subsequent experiments did not have a significantly greater survival of the embryos which escaped rupture of their area pellucida by the operation. Neither was there a significantly greater survival due to experience in Tables 4 and 5 which compare this experimental group with latter groups; although, greater experience would seem to result in less damage from mechanical factors and more speed with concurrent less exposure to drying of the embryonic membranes, which has been implicated in mortality (62).

Fortunately in November of 1960, when it became clear that any experiment demanding both operation upon the embryo at such an early age, and then maturation of the chicken, required considerably more technical ability than had been demonstrated, another approach presented itself.

C. Tracing of primordial germ-cells with the sex-chromatin mass

1. Hypothesis

As the six day survival time, which was being regularly achieved, would be sufficient to determine the fate of transplanted primordial germ-cells if they could be adequately identified, the next trial was an attempt to use the sex-chromatin mass as a marker. Since Graham and Barr first noted the significance of the female sex-chromatin mass as a cytological sex differential (26), it has been extensively investigated with several staining procedures (58). In chickens Kosin and Ishizaki used Harris' hematoxylin stain to demonstrate that the Barr-body occurred about 10 times more frequently in females than males, despite the fact that the distribution of the major sex chromosomes is, in birds, different from that of mammals (38). In all Aves, the male has two Z's and the female only one Z (60). This cytological sex differential was present as early as two days of embryonic age (36). The Feulgen squash method used by Ohno, Kaplan and Kinosita confirmed these reports (47).

The hypothesis is that in approximately 1/2 of the transplants the donor primordial germ-cells should be of the opposite sex from the host and that this can be determined anytime after 3 days of embryonic age. All cytological sex differentiating is done on a statistical

basis, by determining the percent of sex-chromatin mass positive cells in males and females with the sexes falling into different population curves. First the two normal curves for percents of primordial germ-cells with sex-chromatin masses in male and female controls would have to be determined. Then an analysis would be made of embryos containing transplanted primordial germ-cells. Presuming that the germinal crescent theory of origin of these primordial germ-cells is true these experimental embryos should fall into the two normal distribution curves, but with a scattering of a significant number of embryos between them as a result of the opposite sexed transplanted primordial germ-cells. Failure of this to occur would indicate either that the transplants failed to take or that the primordial germ-cells do not originate in the germinal crescent.

2. Data

In anticipation of successfully developing a satisfactory method for differentiating the sex-chromatin masses of the primordial germ-cells in histological sections, 108 eggs were used to obtain 22 control embryos and to perform 59 operations which produced 28 incubatable transplants, of which 20 were fixed at the required four days of age or greater while 8 showed little or no further development after operation (Table 1). This work was for

naught.

3. Discussion

Obviously this experiment required the use of a stain which would allow rapid screening of large numbers of primordial germ-cells for assessment of the presence or absence of the sex-chromatin mass. It would also have to be consistantly productive in a large number of histological sections; however, almost all of the sex-chromatin mass staining techniques have been developed for smear or squash preparations, which are more easily differentiated. The sex chromatin mass is DNA, similar to other heterochromatic masses in the cell nuclei, and its visibility in most DNA staining methods is simply dependent upon the slower dedifferentiation of a larger mass, but this mass differential is much less significant for destaining in histological sections than in single cell thickness preparations. Therefore, the sex-chromatin mass is proportionately more difficult to demonstrate consistently.

After reviewing the techniques of several sex-chromatin mass stains (1,2,27) and unsuccessfully attempting to adapt the thionin stain (37) to control embryo sections, the Biebrich scarlet-fast green stain was selected for investigation (29). This stain could be readily used to sex human buccal, mouse vaginal, and chick buccal and cloacal smears; however, extensive work could not adapt

Illustrations 10 and 11 demonstrated a type of silver granule reduction occurring over single and groups of blood cells which was finally called artifactual. This decision was based upon several things. Two significant factors were the spotty occurrence of this phenomena upon a slide, but always affecting all of the blood cells where it affected any, and its being accompanied by a yellow tint of the film, which may have indicated chemical changes. These silver granules were smaller than the typical radiographic granules and accompanied by an unnatural light refraction when slightly out of focus above them, indicating possible physical changes in the gelatin layer over them.

Illustrations 12 and 13 demonstrate whole sections for orientation to the following material.

After only 22 days exposure embryo No. 6-28-E had a high background of silver reduction on all of the slides and it was darkly stained; however, there is little question but that the cells in illustration 14 are well tagged. These are a kidney tubular cell and an adjacent stromal cell. The tagging is certainly equivocal, if even worth noting, in the spinal cord neuron of illustration 15. While the probable random tagging in the gonad of illustration 16 can only be called artifactual after evaluating the background of the surrounding area microscopically.

A good stain and a low background after 47 days exposure make the small concentration of reduced silver granules over the nucleus of the possible primordial germ-cell in the gonad of embryo No. 7-26-J (Illus. 17) possibly of significance.

Embryo No. 8-9-A has a probable primordial germ-cell in illustration 18, but the tagging is not significant. The dissociation from a nucleus of the very likely looking group of silver granules in the gonad of illustration 19 removes it from probable tagging to artifact; this group, also, falls upon a line of reduced granules probably caused by static electricity. On all slides the gut mocosa of this embryo showed slightly greater tagging than the surrounding stroma; however this could not be convincingly illustrated (Illus. 20).

Two adjacent cells seen at low power (Illus. 21) in the gonadal ridge of embryo No. 8-9-D require careful microscopic evaluation. The cell in illustration 22 is probably a primordial germ-cell and the tagging appears increased; however, it is less likely that the poorly visualized cell in illustration 23 could be identified with certainty as a primordial germ-cell and the tagging appears more artifactual. The gut mucosa throughout this embryo's sections was well tagged (Illus. 24 to 26).

Embryo No. 8-9-G is significant for having two

it with any degree of reliability for sex-chromatin identification to histological sections of man, mouse or chicken. This stain also was to be dropped, this time in favor of trying Harris' hematoxylin, when a supposedly much superior method of tagging the transplanted primordial germ-cells was conceived.

PART III

Studies of the fate of transplanted germinal crescent cells tagged with tritiated thymidine

A. Theory

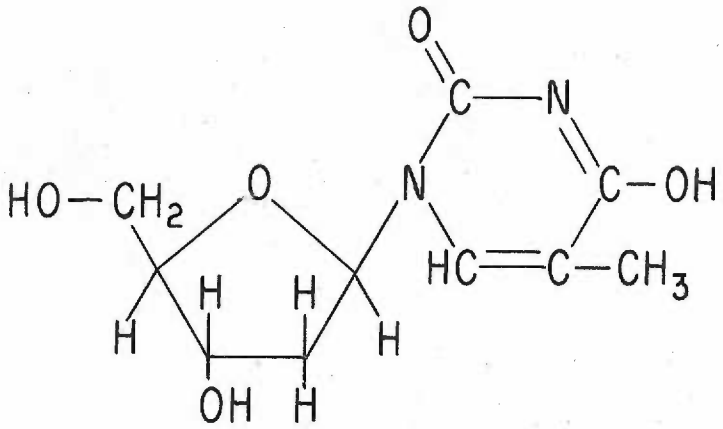
1. Tracing a primordial germ-cell

The final phase of this series, attempting to discover the origin of the primordial germ-cell, was started in the spring of 1961. The plan of following marked cells which was the basis for using the inadequately identifiable sex-chromatin mass still appeared feasible, providing suitable marked cells or a suitable marker for cells could be found. Success of this plan is dependent upon then finding the marked transplanted germinal crescent cells in the gonads as recognizable primordial germ-cells or as gametes at later stages.

Embryologists long have followed marked cells using inert materials which were later phagocytized by the cell, diffused into it, or marked its membrane; however, this work was done primarily with groups of cells which remained intact and in part held their marker by mass action. A marker for single migrating cells would have to be a permanent one which would not diffuse into surrounding media or be lost in transient.

2. Choice of tritiated thymidine

Tritiated thymidine seemed to fulfill the stated requirements. Thymidine, a deoxyriboside, is incorporated into, and comprises about 22% of developing DNA of mitosing



Thymidine

cells, with little sidetracking to other products and without readily re-entering the metabolic pool (16, 19,20,21, 23,56). Tritium has a half life of 12½ years and emits a Beta particle of very low energy, 0.018 milli-electron-volts, which only travels an average distance of 1 micron and a maximum distance of 6 microns in air. The combination of these attributes in tritiated thymidine (H^3TDN) produces a stable marker for the nuclei of cells incubated in the proper concentrations (10,23,35,48). While improved autoradiographic techniques allow accurate localization of these tagged cells with the stripping-film method (51,52, 56,59).

With these considerations in mind it was postulated

that developing germinal crescent cells could be tagged with tritiated thymidine by injections of the proper dosage into the yolk prior to incubation. These cells then could be transplanted into the germinal crescent areas of "cold" embryos at the latest possible age prior to the establishment of the circulation. If the primordial germ-cells were in the transplants they could be detected in the gonads by using the stripping-film technique for autoradiography.

B. Materials and methods

1. Tagging with tritiated thymidine

Under the supervision of Mr. John Brook tritiated thymidine obtained from Schwarz Bio-Research, Inc. with an activity of 1.88 counts per millemole was evaluated for the proper dose which would tag embryonic cells after intra-yolk injection. After injecting and autoradiographing embryos with dosages from 0.005 to 25 microcuries per egg it was determined that 5 microcuries per egg gave satisfactory tagging without visibly mutating or injuring the embryo. As a volume of 0.1 milliliter could easily be injected into the yolk without damage or overflow of albumen, and this volume could be accurately measured with a tuberculin syringe, a concentration of 50 microcuries per milliliter of tritiated thymidine was adopted.

Preliminary trials led to the conclusion that intra-yolk injections could always be made without direct obser-

vation by drilling a one milimeter hole with a diamond stylus in the shell at a point on the broad end where a line tangential to the surface would form a 45° angle with the longitudinal axis of the egg. With the broad end of the egg up for several minutes and cleansed with 70% ethanol, this hole was drilled. A 27 gauge needle was inserted perpendicular to the surface with the tip at the longitudinal axis and the injection was made. After copious rinsing with 70% ethanol a Scotch Tape patch was placed over the hole and the egg was incubated.

The incubator was one of the weaker links in the experimental design. It was a convection current type, with an open pan of water for humidity control. The thermometer was in the roof, when it registered the optimal 37.5°C, the incubating shelf temperature might be as high as 40°C. This was the major cause of variability of embryonic development compared to standard incubation age.

2. Transplanting

After 30 to 40 hours of incubation the "hot" and "cold" eggs were opened. The eggs were cleansed with 70% ethanol and sterile instruments were used, but no other special sterile techniques were employed. An ampule saw was used to cut a 1 x 1 centimeter hole in the shell toward the broad end, just above the equator. A glass needle was pushed through the egg's membrane toward the

broad pole to rupture the air pocket there and to allow the albumin and yolk to drop away from the port. Then the membrane was removed. The embryos were inspected and those having developed to between the head fold and the beating heart (16-20 somites) stages were operated upon. As much matching of donor and host development as possible was attempted for transplants; although, as length of embryonic exposure with consequential drying was kept to a minimum dissimilar development was often ignored so as not to have an egg open very long. Using fine drawn glass needles to incise the vitelline membrane, a micropipet with an end outside diameter of less than 0.5 milimeter, and just having been coated on the inside with the egg's fluid albumin, was inserted over the germinal crescent (Illus. 1) and this tissue was aspirated. The cell and yolk mass thus obtained amounted to between 0.02 to 0.06 milliliters. This was immediately transported to the prepared host and injected through an incision in the vitellin membrane. In about 65% of the eggs the injection was made above the area pellucida while the remainder were made beneath it. It was thought that cells between the area pellucida and the vitelline membrane would be in better position for taking, but it was noted that those under the area pellucida floated up against it. The host egg was then sealed with Scotch Tape and incubated port down. A smear was made of

the remaining area pellucida cells of the donor, using the same aspiration technique.

3. Fixing, sectioning, and mounting

After three days of incubation the viable embryos were fixed in 10% glacial acetic acid in absolute alcohol. Picric acid and similar fixatives are counterindicated as they may chemically reduce the stripping-film (51). The embryos were then dehydrated and embedded through standard procedures. As it had been estimated that a resolving power of 2 microns could be achieved (52,56) and the average distance the tritium-emitted beta particle travels is only 1 micron, sections of 5 microns were prepared. Thinner sections were technically feasible with the equipment available. Those sections containing the gonadal ridges were mounted on slides prepared with an albumin-ether solution and were then heat stabilized at 56°C.

4. Stripping and developing

The prepared slides were stripped with Kodak AR-10 film. Although the standard stripping techniques are well known (Appendix, I), there are several important details which experience indicates need emphasis. The first is the importance of room humidity, which can easily be controlled in most dark rooms by a running hot water tap. If the humidity is low, the gelatin film tends to curl and

become unmanageable as well as to produce a considerable amount of static electricity when being stripped from the glass plates. Too high humidity results in moist, weak film which sticks to the instruments and to itself and tears easily. The floating film strips must be allowed to expand maximally before application to the slides for the closest wrinkle-free approximation. The filmed slides, while being exposed, must be thoroughly dried for optimal adherence; this is easily accomplished by placing dry $CaSO_4$ crystals in each slide box. If the film is maximally expanded before application, overlaps around three edges of the slide, and is absolutely dried during exposure, it should not separate from or move on the slide during development or staining.

Kodak D-19 developer and Kodak Fixer were used for the standard developing technique (Appendix, II). In this process it is essential that the slides be gently treated and the wet time be of minimal duration to prevent loosening of the film on the slides. These slides were exposed for varying times from 15 to 112 days.

5. Staining

Various methods of staining, prior to and after filming autoradiographs, have been used (51,52), including the post development Gemsi stain technique used in the

Division of Experimental Medicine here.# None of these methods seemed to offer the combination of contrast, simplicity, speed and reliability that was desired. After a brief encounter with Erlich's hematoxylin, and a few random stains used in various labs, Harris' hematoxylin was adopted which gave extreme clarity of differentiation and was easy to use (Appendix, III).

One relatively important innovation in technique was taking the slides directly from the post development rinse to the stain. This bypassed a major cause of separation of the film from the slide, by leaving out one dehydration and rehydration procedure.

6. Photographing

An interesting technique was developed for the illustrations of the results for this paper. All of the illustrations of tagged cells are from double exposure negatives. This was necessary because the cell in question would be in focus at one focal plane and the reduced silver granules above it would be out of focus at a higher focal plane at this high magnification, approximately 970 times. Using an AO Spencer, Model 635, Photomicrographic Camera 35mm with the 97x oil immersion objective and a Photovolt meter, Model 501-M, reading of 20 x 100 Kodak

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#Personal instruction by Mr. John Brook.

High Contrast Copy film, tungsten ASE index equals 32, would be exposed to the focused cell for one-fifth second, then after refocusing on the silver granules it would be exposed for one-half second. This procedure was derived from an emperical series of trials varying one factor at a time. A standard 1:3 dilution development technique was employed with Kodak Microdol-X developer (Appendix, II).

C. Results

1. Controls and general

a. Controls

At the appropriate age three normal embryos and three embryos receiving untagged germinal crescent cells were sectioned, filmed, exposed, developed, and stained. Two additional controls were obtained from 10 embryos which received an injection of yolk only from tagged embryos. These controls did not have any significant tagging artifacts.

Smears of the area pellucida cells were made from eight donor embryos, all of which showed tagging; although quite variable in density (Illus. 3 to 7). One tagged chick was sectioned at 5½ days of age and a single section was applied as a control to the slides of 15 embryos. All of the sections were well tagged (Illus. 8 and 9).

b. Operations

The 28 gonadal regions prepared on slides and enumerated in Table 6 are the products of applying the methods above to 240 eggs, 1/2 of which were injected with tritiated thymidine. Operating on 196 of the eggs produced 88 satisfactory transplants for incubation. Thirty-four of these lived less than 4 days and 54 lived longer than 3 days (Table 1).

c. Effect of experience

A statistical analysis of Table 1 demonstrates an interesting interaction of experience, with its possible beneficial effects, and tritiated thymidine, with its possible harmful effects, upon early mortality. As was mentioned previously, the overall effects of experience and trauma are not significant; however, Table 7 indicates the non-parametric statistically significant effect of experience upon increased survival in embryos in which the area pellucida had been perforated, but presumably with less damage due to experience. In this group the injected tritiated thymidine would have maximum dispersion within the yolk and minimum detrimental effect. Table 8 on the other hand shows even a decreased survival, but not significant, for the last sub-group which did not have the area pellucida perforated. Here the effect of the tritiated thymidine confined between the vitalline membrane

and the ectoderm would be maximum; however, could experience improve upon essentially no damage to the living embryo from the start? The F-test of Table 9 indicates that the difference between these two groups is highly significant. Although this may be due to the effect of the tritiated thymidine it is more likely due to the fact that no significant improvement could be made in the group where the area pellucida was not injured, while much less damage occurred with perforation of the area pellucida in the last series and was significant in increasing the embryos' survival time.

2. Description and evaluation of findings

Nine of these 28 embryos have increased tagging of one or more cells. A great deal of time was involved in carefully examining at high magnification 203 slides from all of these embryos before satisfactory rigid standards for significant tagging were established. In this section an attempt will be made to judge only the validity of increased autoradiographic tagging over the nucleus of a cell or group of cells; an analysis of the causes other than artifactual will be left for the discussion. It should be emphasized that a decision regarding tagging and the nature of the cells can only be made with the microscope and the illustrations are merely that, illustrations.

probable tagged primordial germ-cells. The first occurs in the root of the mesentary while the second is at the germinal epithelium (Illus. 27 to 29). The gut of this embryo is also well tagged (Illus. 30 to 34).

Unquestionable tagging is demonstrated in embryo No. 8-9-H by two adjacent sections containing parts of the same endothelial cell of the aorta (Illus. 35 and 36). A well tagged cell is also present in the peri-spinal cord membrane of illustration 37. Two artifacts which should be seen to appreciate their similarity to tagging are illustrated in 38 and 39. In this embryo tagging is also present in the ventral plate of the spinal cord (Illus. 40 to 42), the notochord (Illus. 43 to 46), the yolk sac membranes (Illus. 47 to 49) and the gut (Illus. 50 and 51).

Embryo No. 8-9-J has several interesting contributions. Among these are two blood cells, one of which (Illus. 52) appears to have a well tagged nucleus while the other (Illus. 53) has cytoplasmic tagging. Another aortic endothelial cell is present in illustration 54 which, considering the low background, may be tagged. The spinal cord (Illus. 55) and notochord (Illus. 56 and 57) are tagged. Illustrations 58 and 59 demonstrate the gut opening up into the yolk sac, while illustrations 60 to 66 show tagged yolk sac membranes and gut.

A questionably artifactually tagged vascular

endothelial cell is present in a gonadal vessel of embryo No. 8-9-L (Illus. 67). This may be significant tagging considering the low background.

Embryo No. 9-6-K was only 3½ days old when fixed; therefore, any transplanted primordial germ-cells should be easily recognizable and probably not present in the germinal epithelium. The dark stain of illustration 68 makes interpretation difficult but the silver reduction over a cell of the germinal epithelium appears to be artifactual.

Illustration 69 is included merely for interest. It is the star resulting from an atomic nucleus disintegrating after being bombarded by a sub-atomic particle from outer space presumably.

Illustrations 70 to 72 are of various dividing cells and primordial germ-cells in embryos from 4½ to 5½ days old. This, it will be remembered, is at an age when the primordial germ-cells supposedly do not multiply.

D. Discussion

1. Primordial germ-cells

This experiment was designed to avoid the necessity of subjective decisions due to ambiguous material, which resulted in the different interpretations of prior histological and experimental studies with consequential conflicts. However, the crucial factor which the result of

this experiment depends upon is the validity of the tagging of the three primordial germ-cells cited in the results (Illus. 22,27 and 29). Despite the most honest attempt at objectivity, subjective factors influence this decision.

The primordial germ-cells in question do not exhibit the heavy tagging seen in some of the smears or sections of known tagged cells; however, a close scrutiny of the histological sections of known tagged cells will disclose many cells, which have as little or less tagging present. It must be remembered that the three primordial germ-cells noted have enough of their nucleus present to make them identifiable as primordial germ-cells, their average nuclear diameter is about 8 to 10 microns, the histological sections are 5 microns thick and the average distance a tritium beta particle travels in tissue is less than 1 micron. The combination of these factors means that any recognizable primordial germ-cells would have a great deal of its tagged DNA too far from the film for reduction to occur. This factor would also help explain why only two of the much smaller recognizable blood cells were well tagged; although many more must have been transplanted.

Although the primordial germ-cells are not supposed to divide prior to six days of age, illustrations 70 to 72 demonstrate primordial germ-cells in mitosis at $4\frac{1}{2}$ to $5\frac{1}{2}$ days of age; therefore, these primordial germ-cells may

have divided, which would lower their silver reduction potential.

The number of tagged primordial germ-cells transplanted in this experiment to the embryos from which sections were examined can not be estimated nor can the number which survived and found their way into the circulation. Smears taken by identical methods indicate that the morphological damage to the cells transplanted in this manner was not great; however, few of the many tagged hemapoietic cells transplanted seemed to produce recognizable progeny. Of course, divisional reduction of tagging must be considered, but mature fully tagged blood cells were also transported. The importance of finding so few of these innumerable transplanted blood cells is that it makes the significance of finding three tagged primordial germ-cells much greater, if not conclusive evidence of their origin as transplanted cells of the germinal crescent. The possibility of their origin as host germinal crescent cells receiving a high dose of transplanted tritiated thymidine should be also considered.

2. Single cells

a. Blood cells

The presence of only two tagged blood cells is discussed above; however, the nature of the tagging of these two cells deserves discussion. The tagging of the cell

in illustration 52 is seen to be entirely over the nucleus when examined with the oil-immersion lens. This would be expected in a transplanted red or white blood cell. However, the tagging of the cell in illustration 53 is entirely over the cytoplasm. This raises an interesting question, particularly since this phenomenon was observed in several other similar cells to a less marked extent. Is this phagocitized DNA from disintegrated cells?

b. Tissue cells

The questionable or probable artifactually tagged single cells do not warrant further discussion, but the unquestionably tagged single cells create an exciting problem. Did freely circulating undifferentiated extra-embryonic cells become differentiated by induction?

The kidney tubular cell shown in illustration 14 is the most striking example. This is a highly specialized cell, yet tubular cells are known to regenerate after destruction in various conditions which apparently do not leave any viable tubular cells, if the organism can be kept alive. Here we have the presence of an integrated tubular cell adjacent to an undifferentiated stromal cell. The probability that these two cells are twin daughter cells from an undifferentiated transplanted cell is almost overpowering. Is it possible that at the immediate post-mitotic phase a cell can be induced to become highly

specialized? Or can a specialized cell produce an undifferentiated cell?

Another example is the peri-spinal cord membrane cell in illustration 37. This cell is presumably a fibroblast; although, it may be a tissue macrophage, which would explain the silver granules over the cytoplasm. As a fibroblast or macrophage it would actually be in a rather direct line from any circulating multipotential cells in an embryo.

The aortic endothelial cells (Illus. 35, 36 and 54) are similar examples of relatively primitive cells which could be differentiated from transplanted circulating stem cells. In all of these tagged cells the possibility of host origin, with an unusual high uptake of tritiated thymidine can not be ruled out; although it is extremely unlikely.

3. Organs

The tagging of whole organs such as gut, yolk sac, spinal cord and notochord indicates selective localization of either transplanted stem cells or free tritiated thymidine.

a. Gut and yolk-sac

The increased tagging in the gut of 5 embryos (Illus. 20, 24 to 26, 30 to 34, 50 and 51, 61 to 66) associated

with the increased yolk sac tagging of two of these (Illus. 47 to 49,60) and the demonstration of gut mucosa opening through the yolk stalk into the yolk sac in illustrations 58 and 59, leads to the conclusion that free tritiated thymidine could be concentrated in the yolk sac and have its greatest effect upon adjacent cells. As the gut mucosal cells seem to have the greatest concentration of tagging the possibility of their functioning as active transport cells, with concurrent increased exposure to intra luminal tritiated thymidine, even at this early age is raised.

b. Spinal cord and notochord

The alleged increased tagging in the notochord and spinal cord are thornier problems. First, it should be noted that not all portions of the spinal cord show increased tagging, indeed it was found only in the ventral and to a less remarkable extent in the dorsal plate (Illus. 40 to 42,55). Careful evaluation of these regions indicates that the cell density here is not enough greater than the other portions of the spinal cord to explain the increased tagging density. Second, prior to the fourth day the elements of the spinal cord have about an equal growth rate, while our free tritiated thymidine concentration would be greatest on the third day and after that it would be rapidly depleted by multiplying cells; therefore,

cells formed on about the third to fourth days would have maximum tagging. After the fourth day the ventral (floor) and dorsal (roof) plates of the spinal cord do not grow; however, rapid multiplication and growth occurs in the basel and alar plates (60, Romanoff, pp. 282-302). These newly formed cells would have decreased tritiated thymidine concentration with each division, while the stalk roof and floor cells would appear to have increased tagging. This type of phenomena would be expected to occur in any organ which has increased mitotic activity during the period of high tritiated thymidine concentration and then remains relatively quiescent or proceeds to more specialized differentiation while the rest of the embryonic cells continue to multiply. And this exact situation occurs in the notochord, which has its greatest mitotic activity around 72 hours, then proceeds to rapid differentiation (Illus. 43 to 46,56 and 57) (60, Romanoff, pp. 924-928). Of course, once this cause of increased tagging is established its possible application to the other tagged cells discussed above must be considered. Indeed primordial germ-cells or any single cells arising during this period and then remaining mitotically inactive would be expected to have relatively significant tagging. However, this would not apply to the yolk sac or gut which continue to actively multiply (60, Romanoff, pp. 1050-1060, and 433-439).

4. Conclusions and criticisms

Tritiated thymidine has been used satisfactorily for tracing transplanted cells in an embryo. By this method it has been confirmed that the primordial germ-cells of the 6 day-old embryo are derived from the germinal crescent. The probability that circulating pluripotential stem cells become highly differentiated cells, through embryonic induction, is demonstrated.

The possibility of the gut mucosa, as early as 4 days old, being involved in active transport of yolk sac contents is raised. The phenomena of different tagging density due to relatively different growth rates is demonstrated in the ventral and dorsal plates of the spinal cord and in the notochord and yolk sac membrane.

This experiment would have been more meaningful if, at 10 to 12 days of embryonic age, differentiated progametes could have been demonstrated to have been transplanted.

Although the quality of radioautographs obtained for this study compare quite favorably with others derived by the stripping film technique, it is now apparent that the newly improved emulsion technique is much superior for detecting tagged cells, particularly in histological sections, and its use would undoubtedly produce more significant information.

E. Summary

The history and morphology of the primordial germ-cell of the chick embryo as conceived by prior researchers is presented along with the problems which remain unanswered.

A brief description of three futile attempts by this investigator to elucidate the fate of the primordial germ-cells is presented: (1) whole embryo transplants producing viable progeny, (2) primordial germ-cell transplants producing viable progeny, and (3) primordial germ-cell transplants being traced by their sex-chromatin masses.

Studies with transplanted cells tagged with tritiated thymidine demonstrate (1) the germinal crescent origin of the primordial germ-cell and (2) the high degree of differentiation possible by circulating stem cells.

Free transplanted tritiated thymidine dynamically demonstrates (1) higher exposure to yolk sac metabolites by gut mucosa due to active transport and (2) the differential growth rate of specific regions (e.g., ventral and dorsal spinal cord plates, notochord and yolk sac membrane).

The necessity of further investigations using improved techniques is stressed.

Illustration 1

Diagram of embryos at head-process and 3-somite stages, and picture of primordial germ-cells from Willier (72).

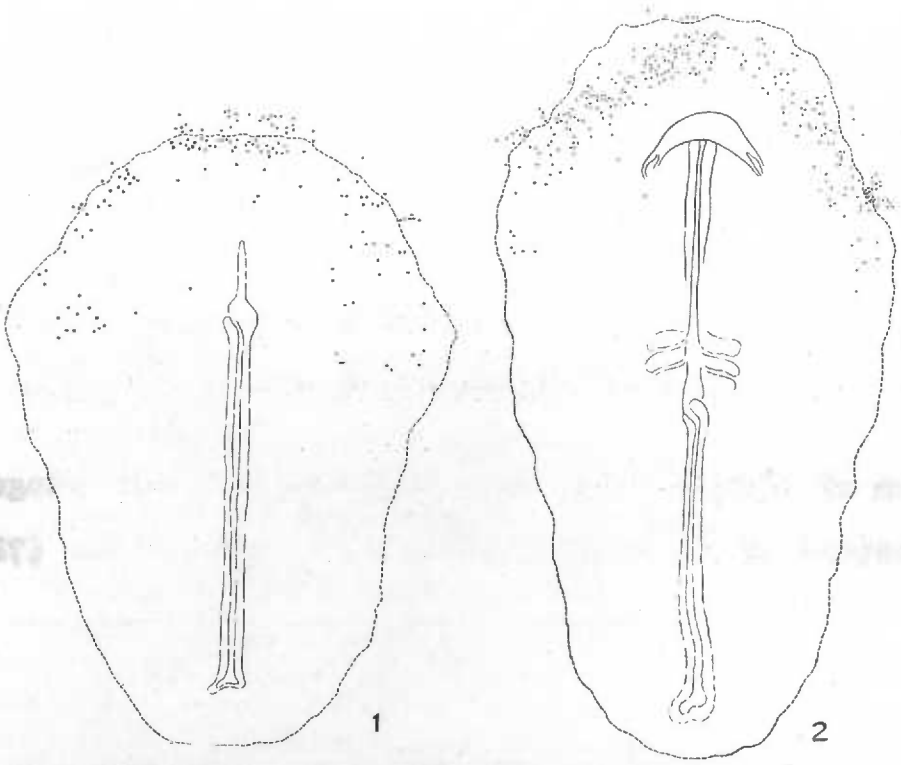

Fig. 1 Map showing distribution of the primordial germ cells in a head-process blastoderm. × 29.

Fig. 2 Map showing distribution of the primordial germ cells in a 3-somite blastoderm. × 27.

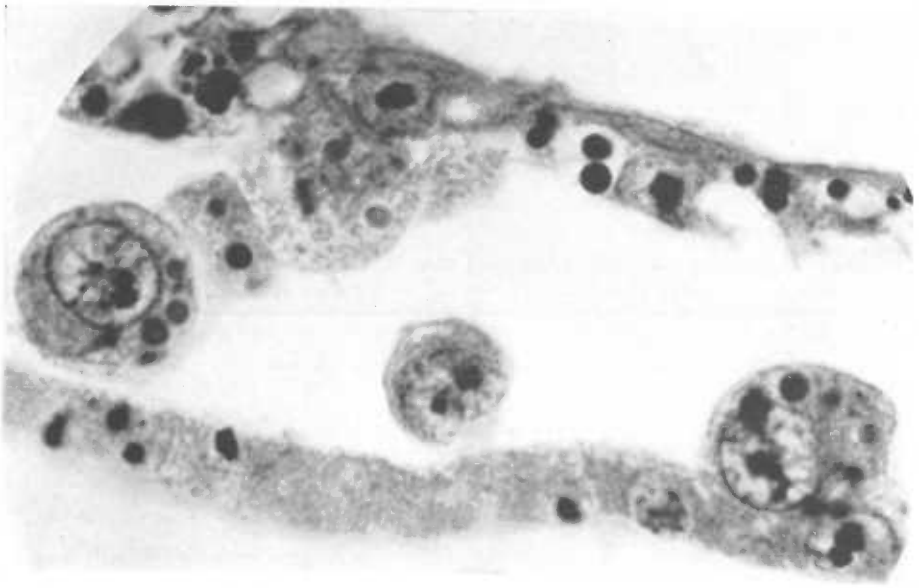

Fig. 3 Photograph of primordial germ cells situated between the ectoderm and entoderm of a 3-somite blastoderm. × 1500.

Illustration 2

Camera-lucida figures from Reagan (55) depicting primordial germ cells beneath germinal epithelium in 90 hour old embryo. G. epth. - germinal epithelium, Mst. - mesentery, Pr. O. - primitive ova (primordial germ-cell).

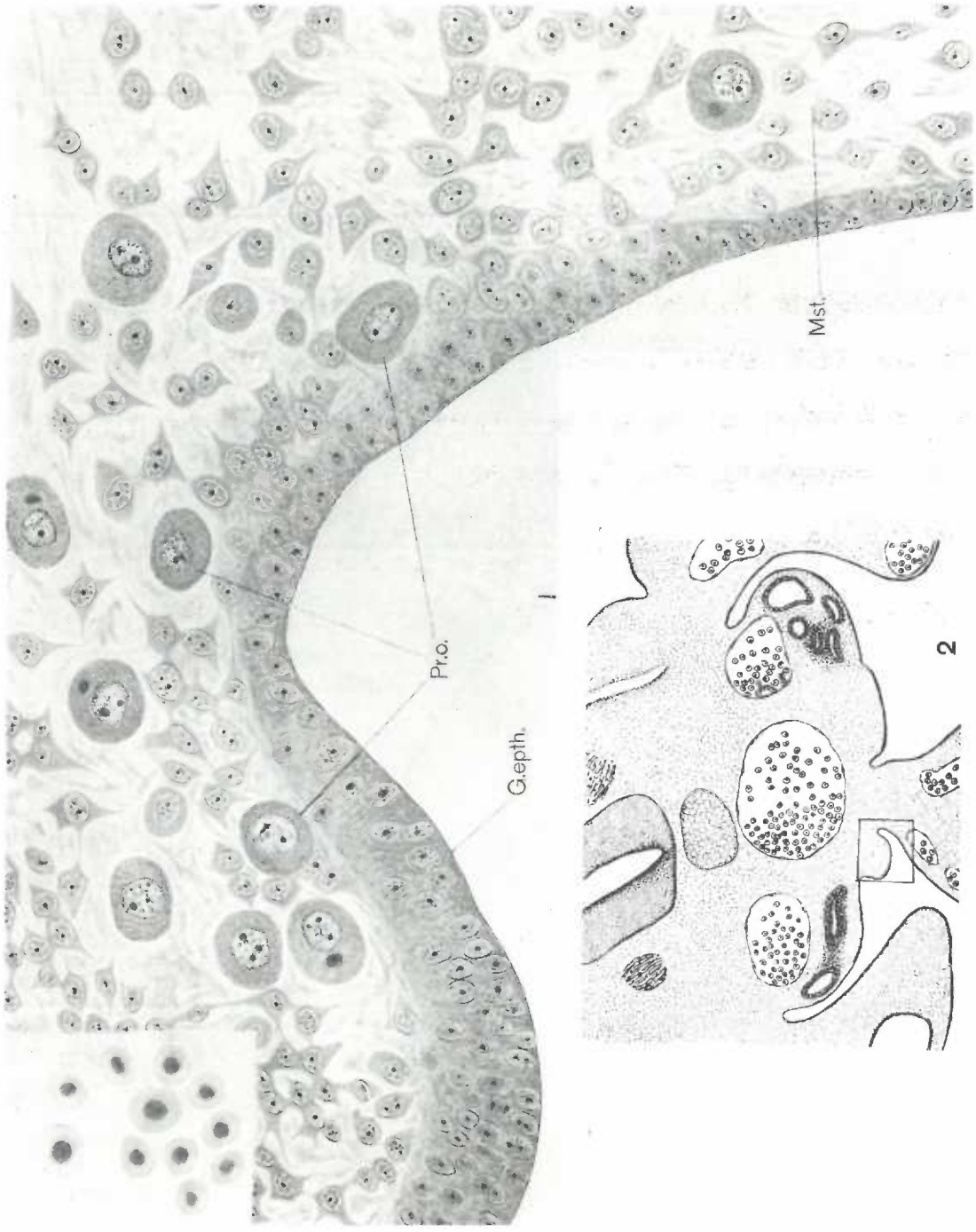
Mst.
Pr.o.
G.epth.
1
2

Illustration 3

Radioautograph of smear of tagged cells taken from embryo 6-24-1. Oil-immersion. All of the smears were made from tagged cells collected in exactly the same manner as cells were gathered for transplanting.

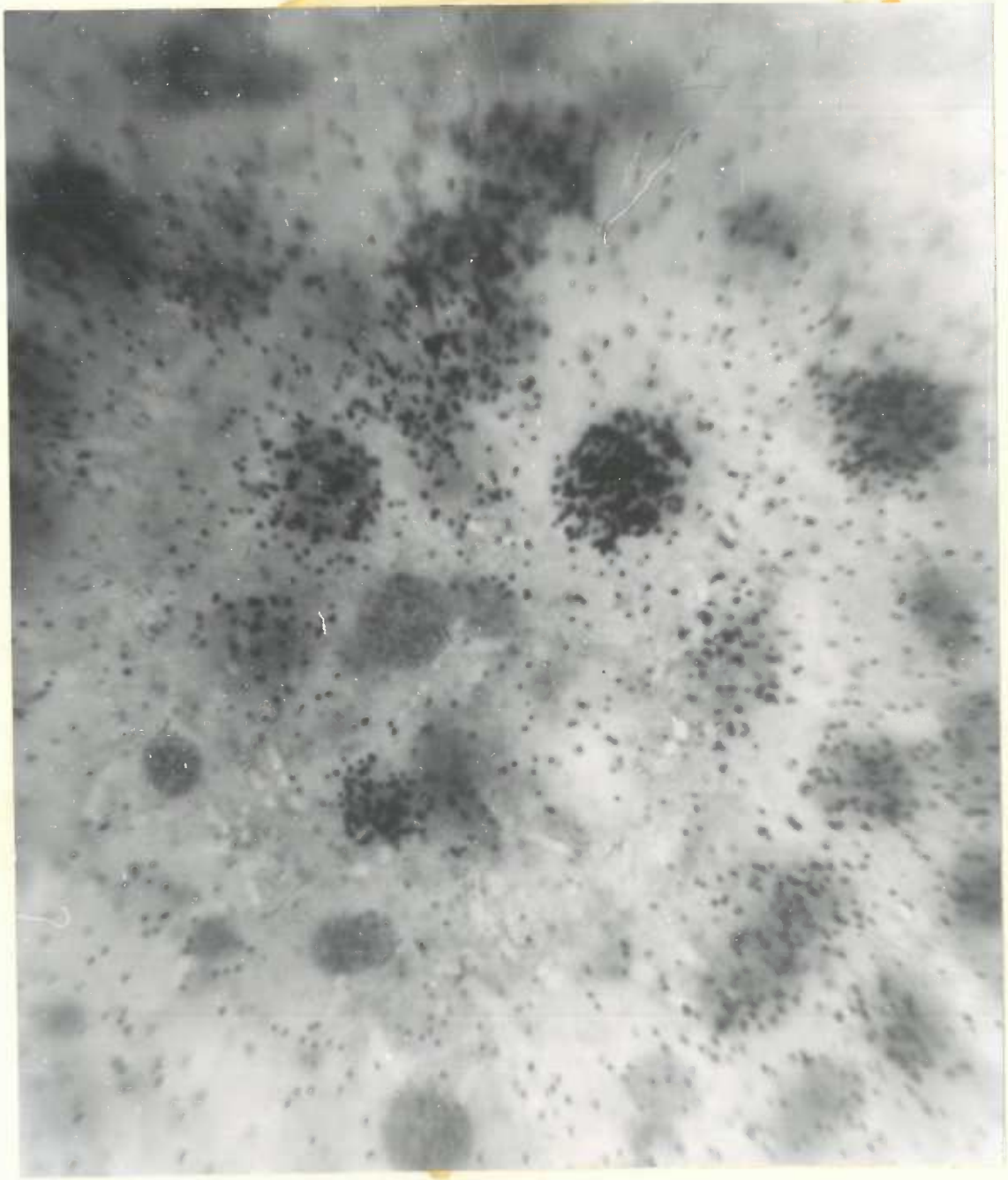

Illustration 4

Radioautograph of smear of tagged cells taken from embryo 6-28-7. High-dry.

Illustration 5

Radioautograph of smear of tagged cells taken from embryo 6-28-7. Oil-immersion.

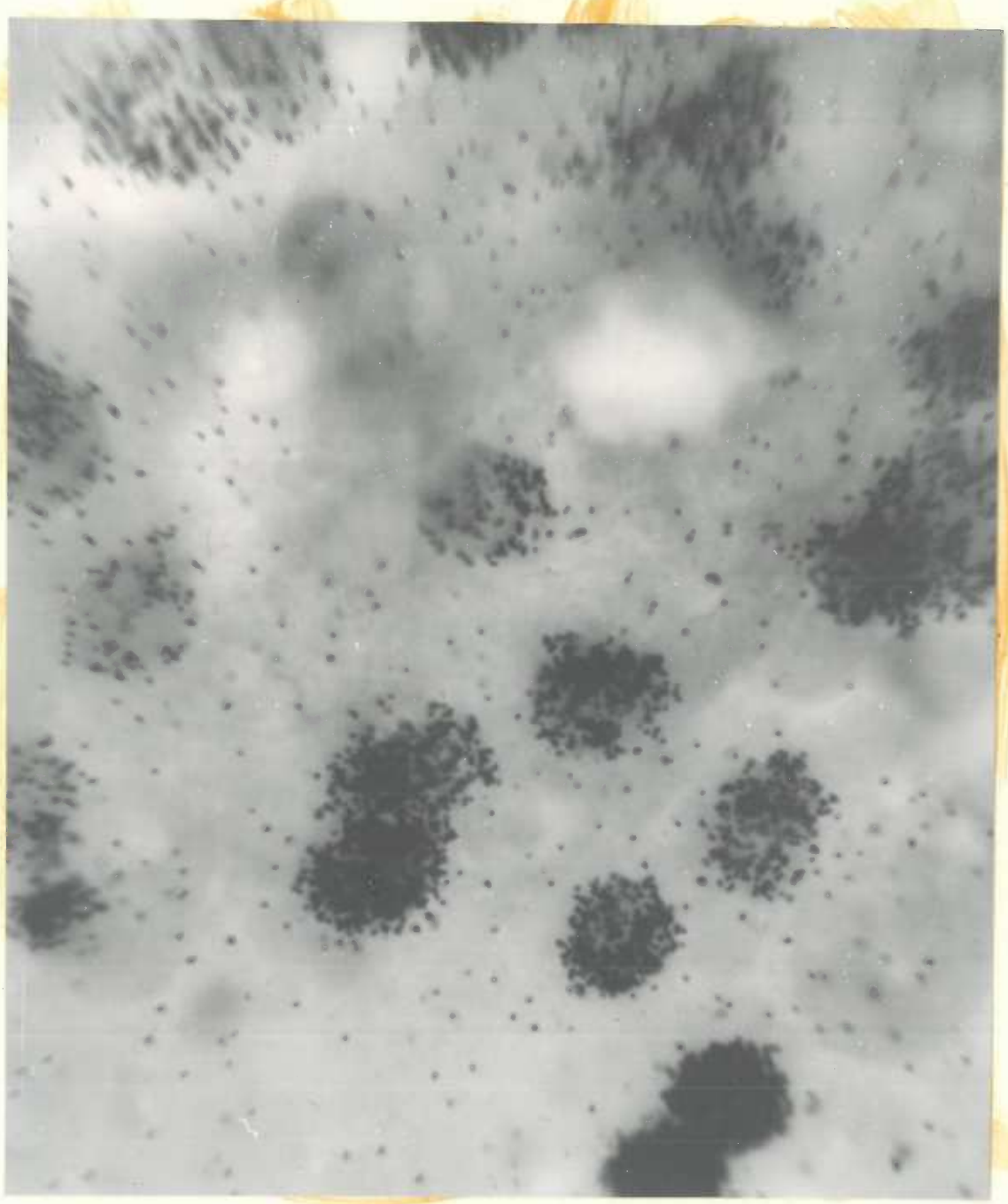

Illustration 6

Radioautograph of smear of tagged cells taken from embryo 6-28-4. High-dry.

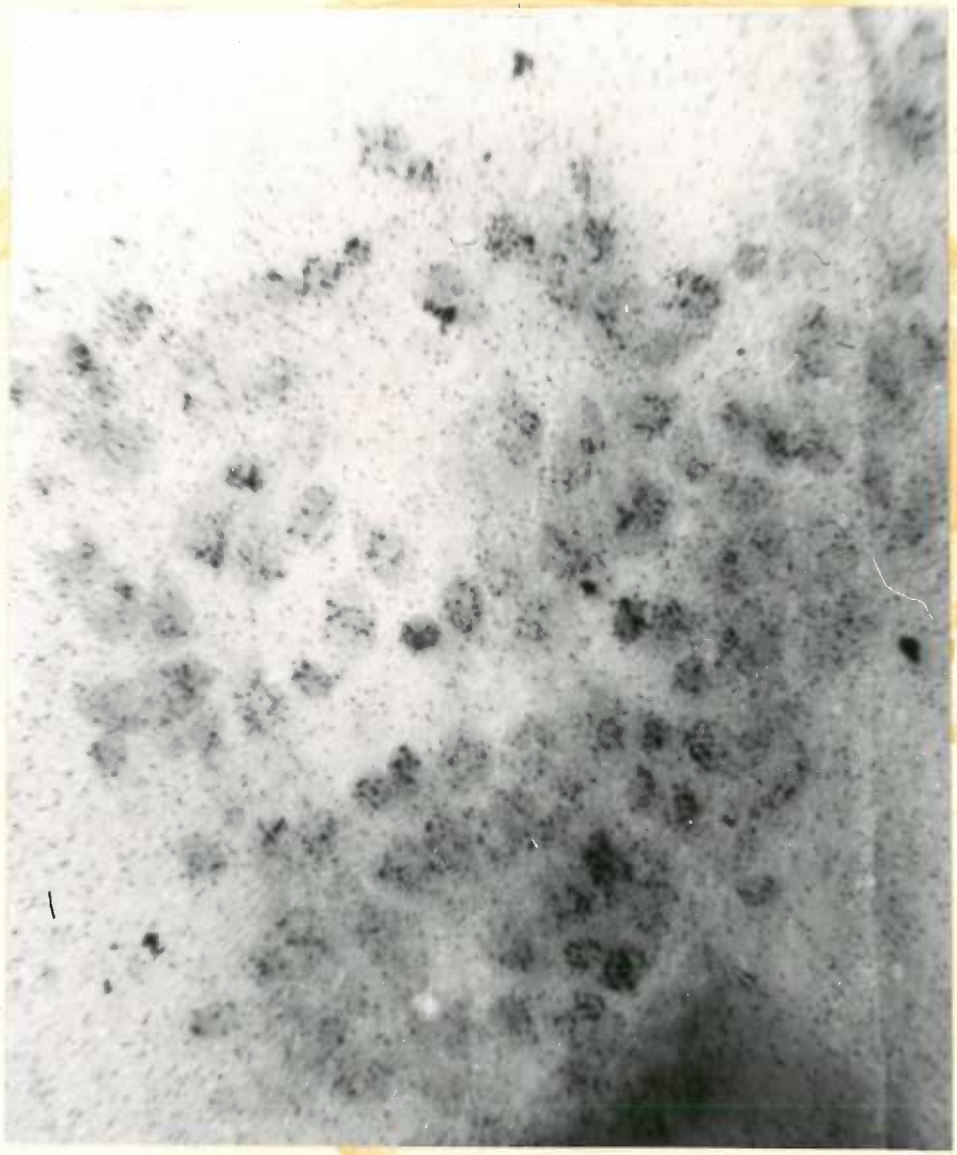

Illustration 7

Radioautograph of smear of tagged cells taken from embryo 6-28-4. Oil-immersion.

Illustration 8

Radioautograph of tagged blood cells in a histological section from an embryo (7-26) incubated for 6 days with 5 microcuries of tritiated thymidine. Oil-immersion. All histological sections are 5 microns thick.

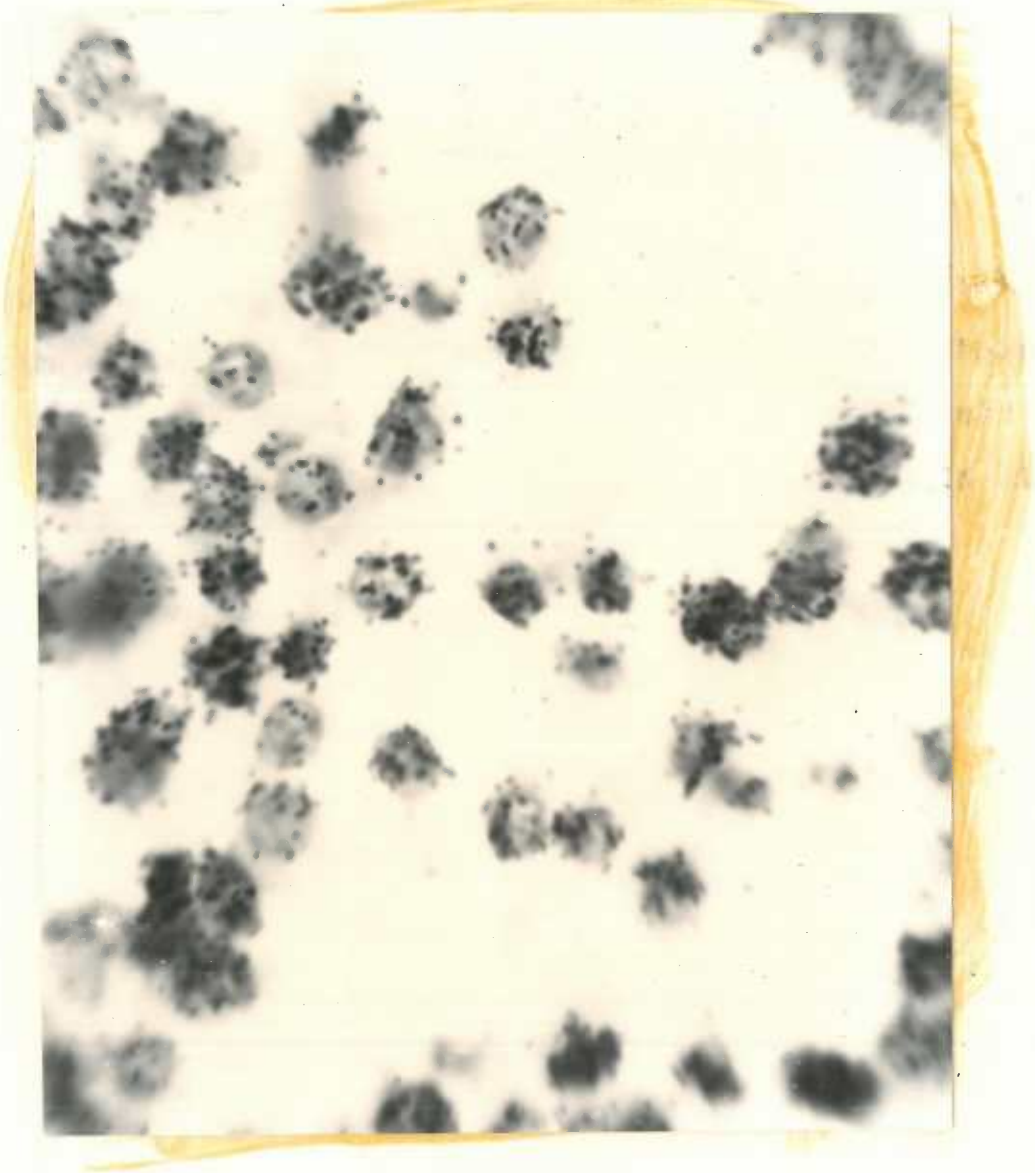

Illustration 9

Radioautograph of tagged kidney cells in a histological section from an embryo (7-26) incubated for 6 days with 5 microcuries of tritiated thymidine. Oil-immersion.

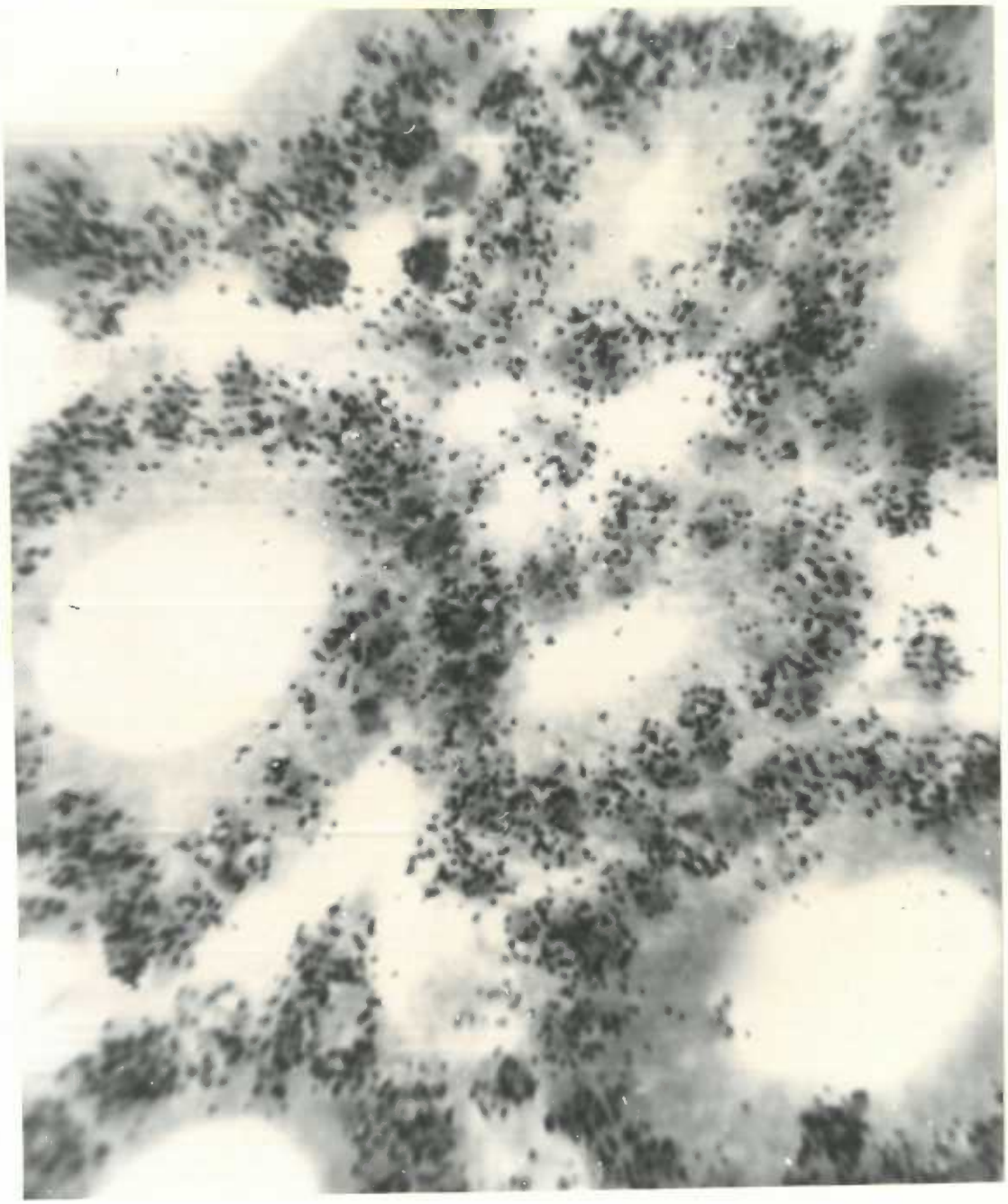

Illustration 10

Radioautograph with artifactual (chemical?) reduction over blood cells in a mesenteric artery of embryo 6-12-J. Oil-immersion. (16-6).

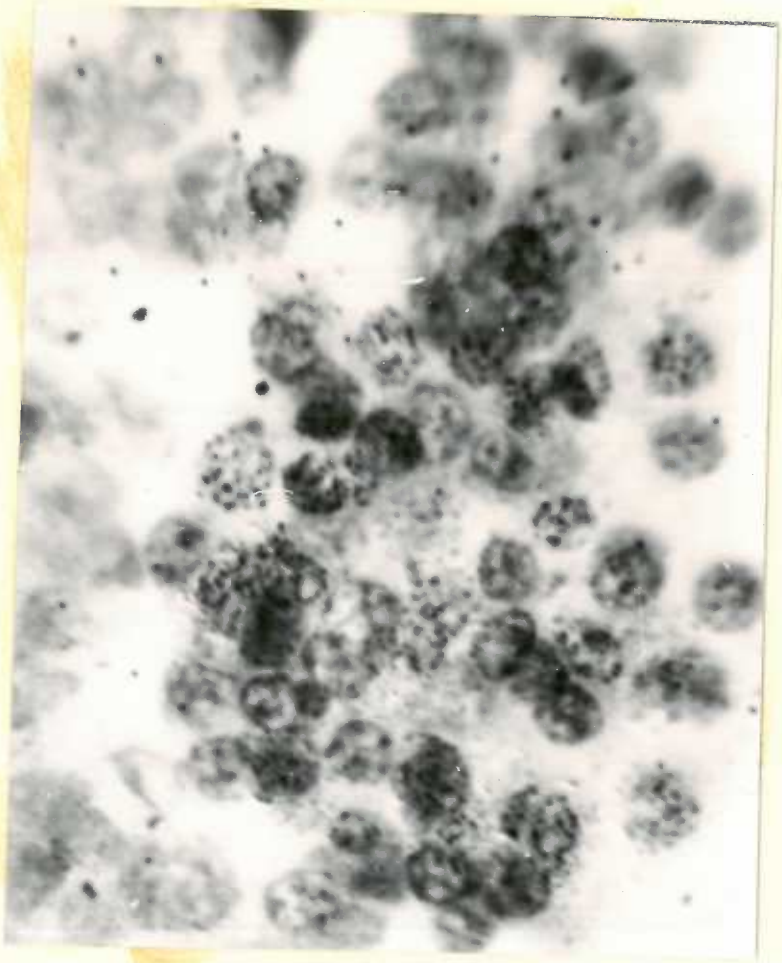

Illustration 11

Radioautograph with artifactual (chemical?) raduction over blood cells in the liver of embryo 6-12-J. Oil-immersion. (16-6).

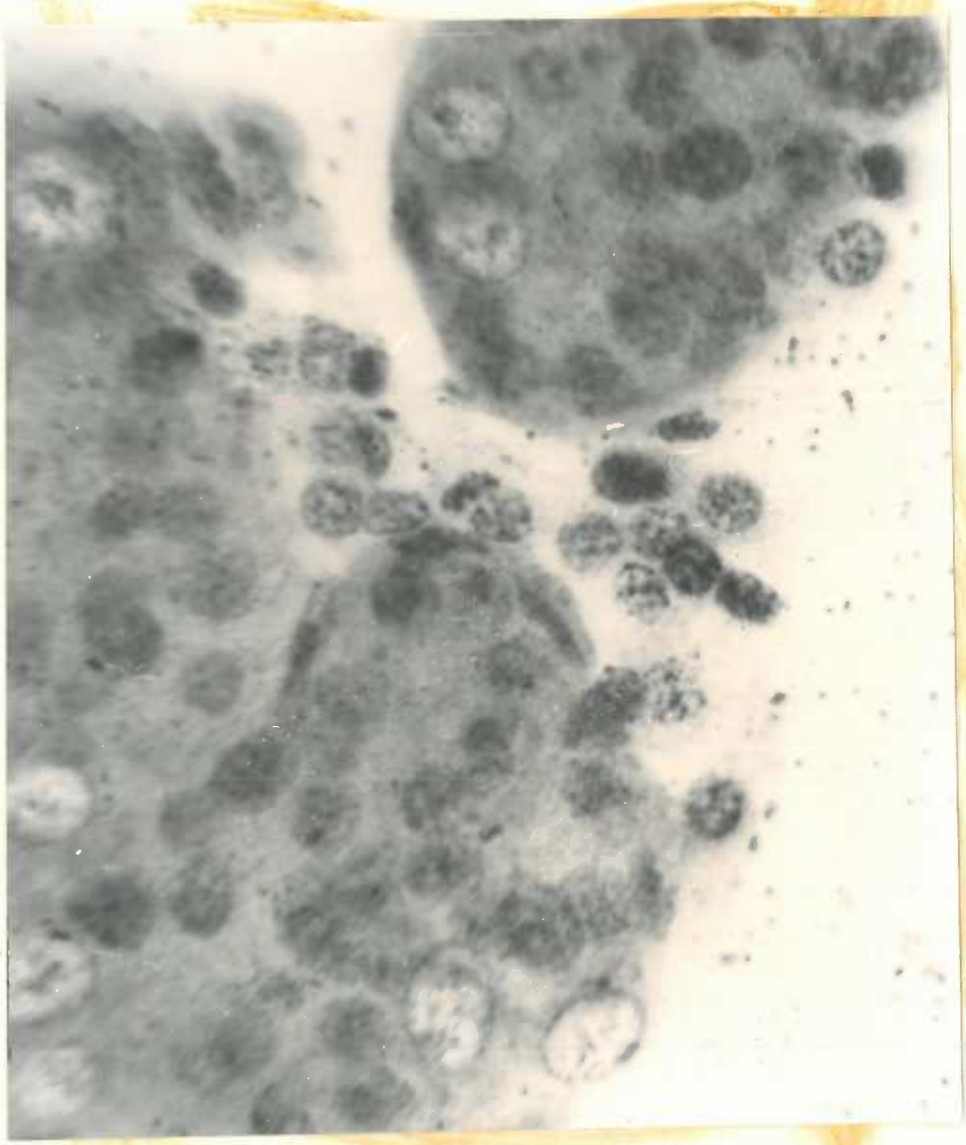

Illustration 12

Representative whole embryo 8-9-A to demonstrate spinal cord, notochord, aorta, kidneys, gonadal ridges, mesentary and gut. Compare to illustration 13. Scanning-power. (3-B-8).

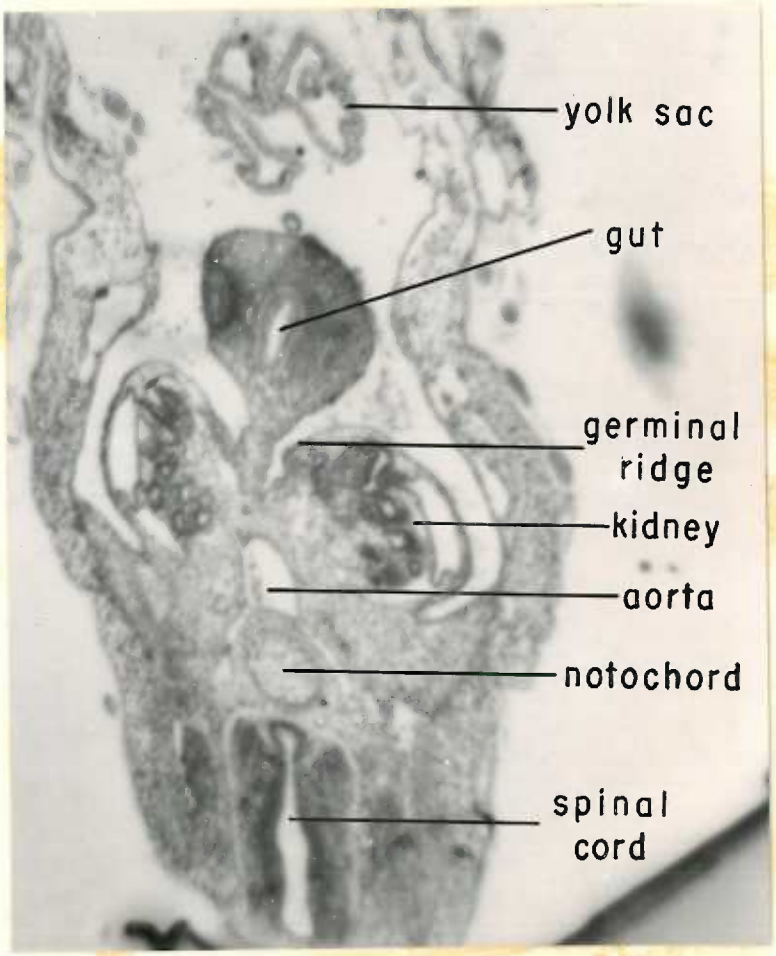
yolk sac
gut
germinal ridge
kidney
aorta
notochord
spinal cord

Illustration 13

Gonadal ridge region of embryo 8-9-A. See previous illustration 12. Low-power. (3-B-8).

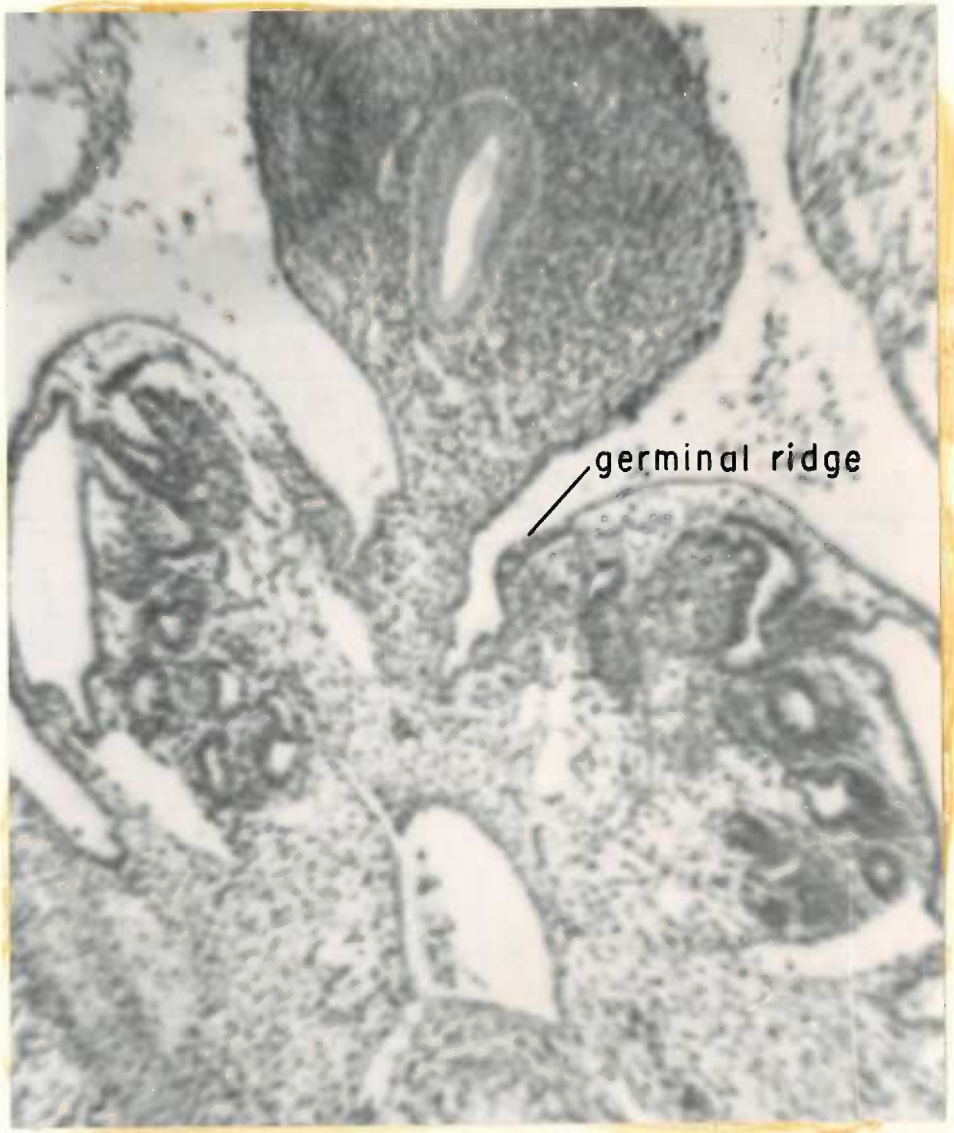
germinal ridge

Illustration 14

Radioautograph with well tagged transplanted cells in the kidney of embryo 6-28-E. Oil-immersion. (4-C-3, left kidney).

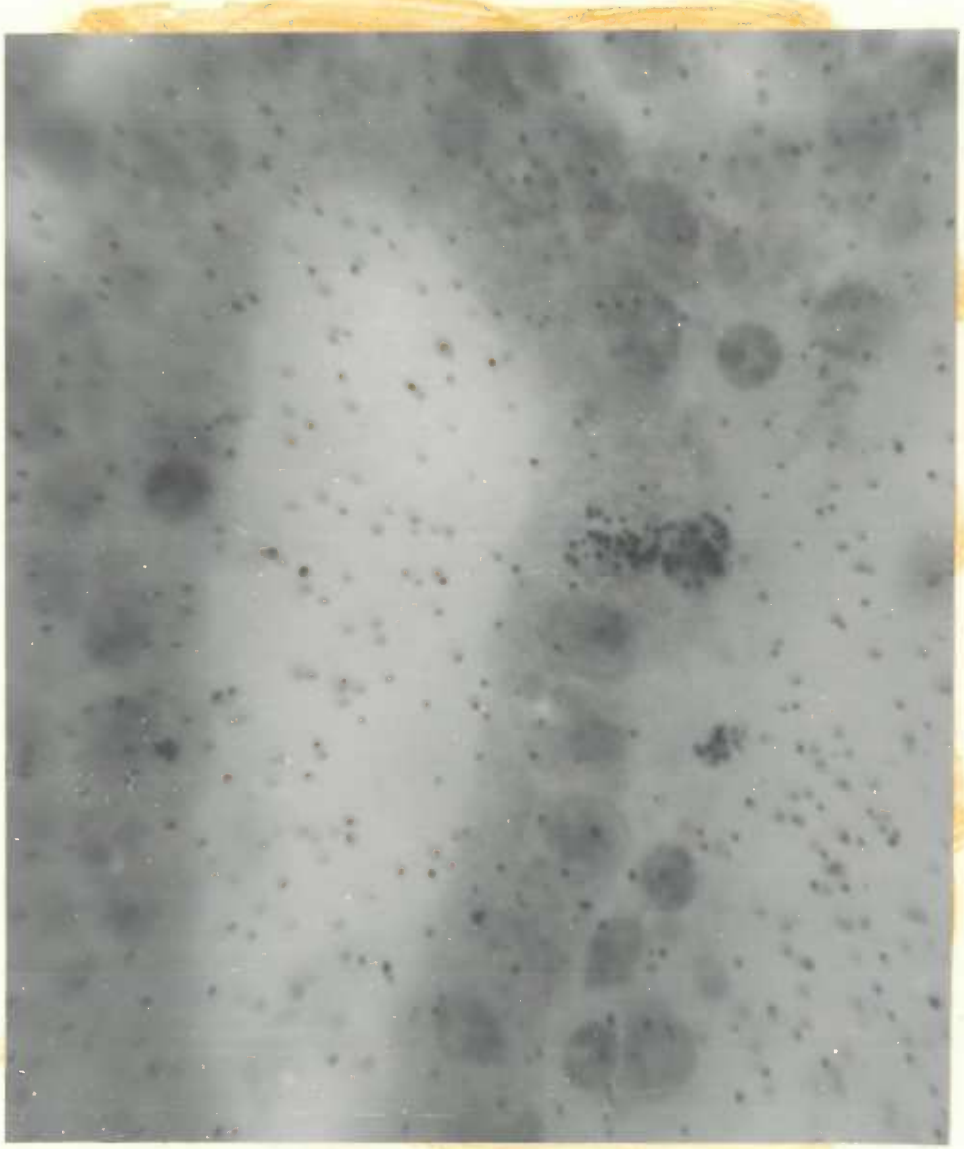

Illustration 15

Radioautograph with probable artifactual tagging over neuron in spinal cord of embryo 6-28-E. Oil-immersion. (6-C-10, left superior spinal cord).

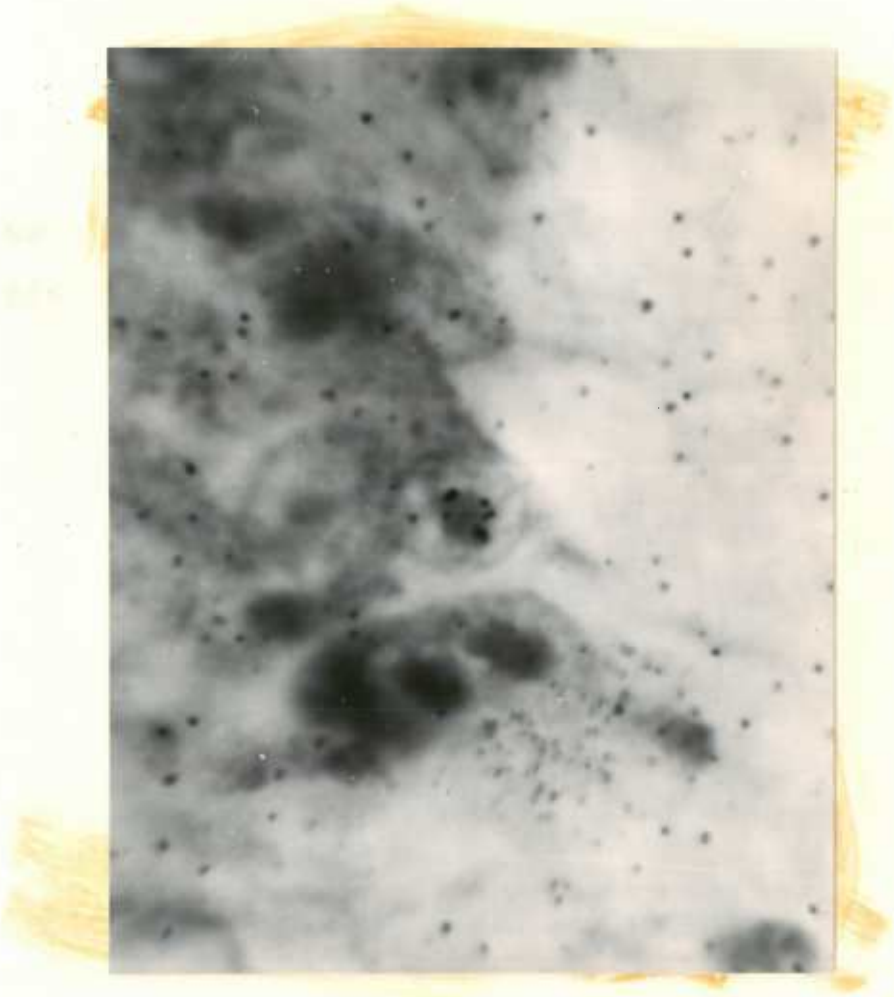

Illustration 16

Radioautograph with probable artifactual reduction over gonad of embryo 6-28-E. Oil-immersion. (6-E-9, right gonad).

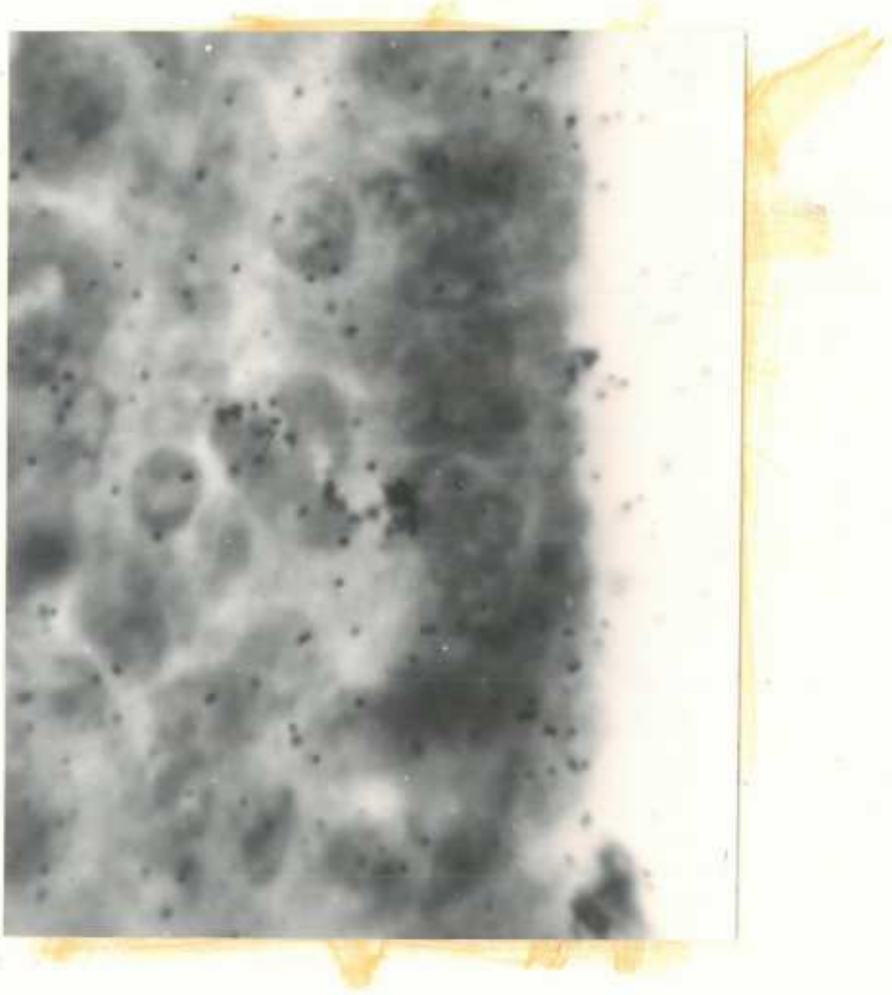

Illustration 17

Radioautograph with possible tagging of possible primordial germ-cell of embryo 7-26-J. Oil-immersion. (1-B-8, left gonad).

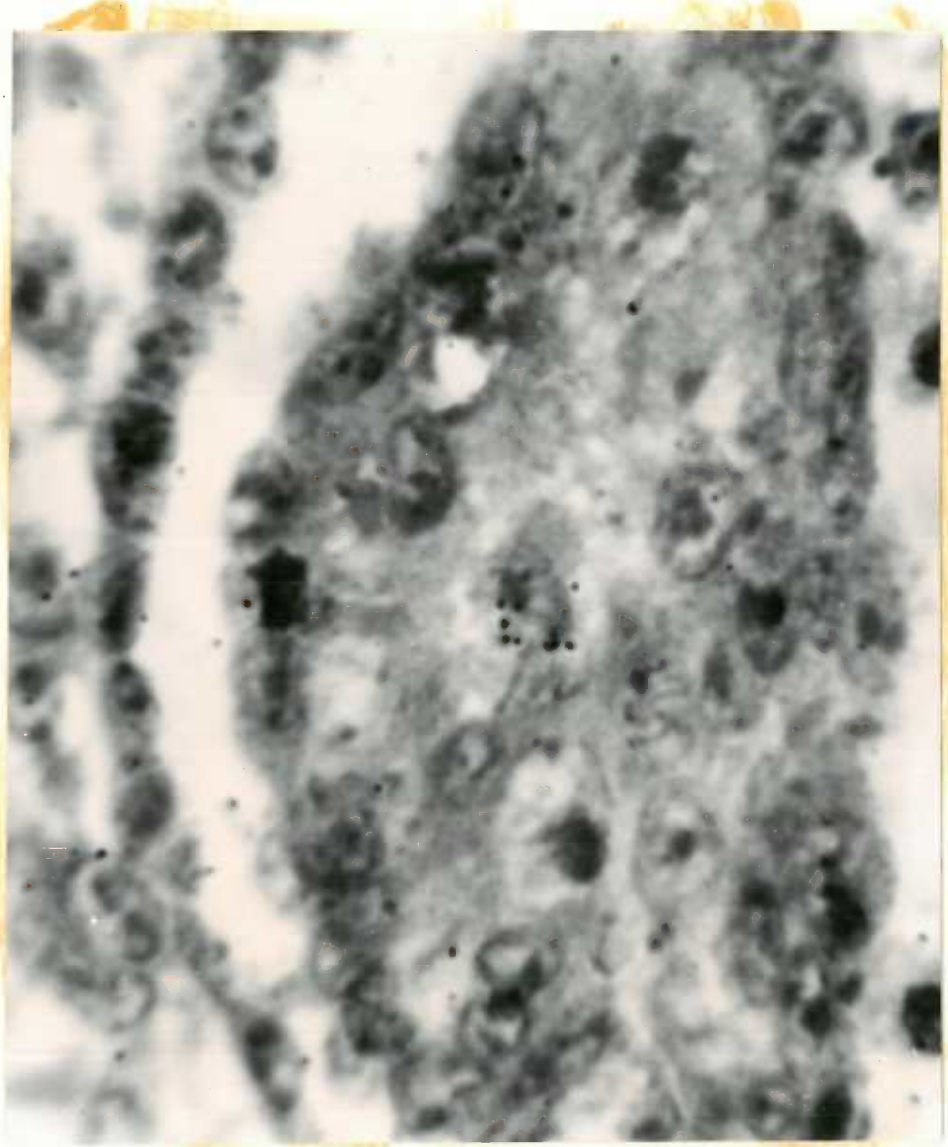

Illustration 18

Radioautograph with probable primordial germ-cell in gonad of embryo 8-9-A with probable insignificant tagging. Oil-immersion. (2-C-12, right gonad).

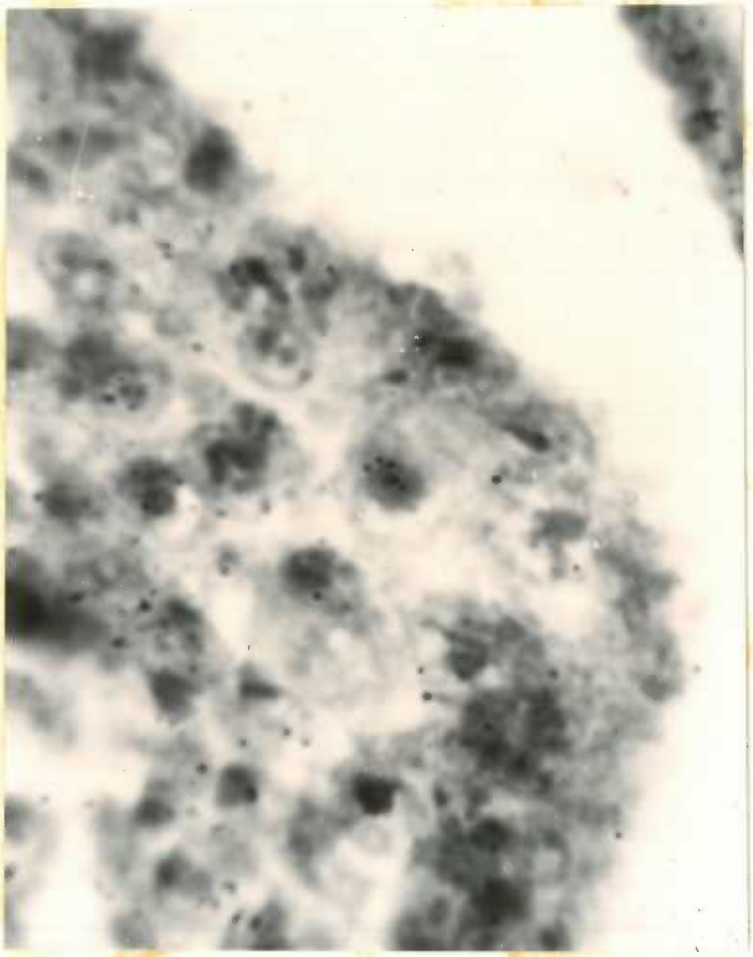

Illustration 19

Radioautograph with artifactual reduction over gonad of embryo 8-9-A. Oil-immersion. (4-C-6, right gonad).

Illustration 20

Radioautograph with moderate tagging of gut mucosa of embryo 8-9-A. Oil-immersion. (3-C-8).

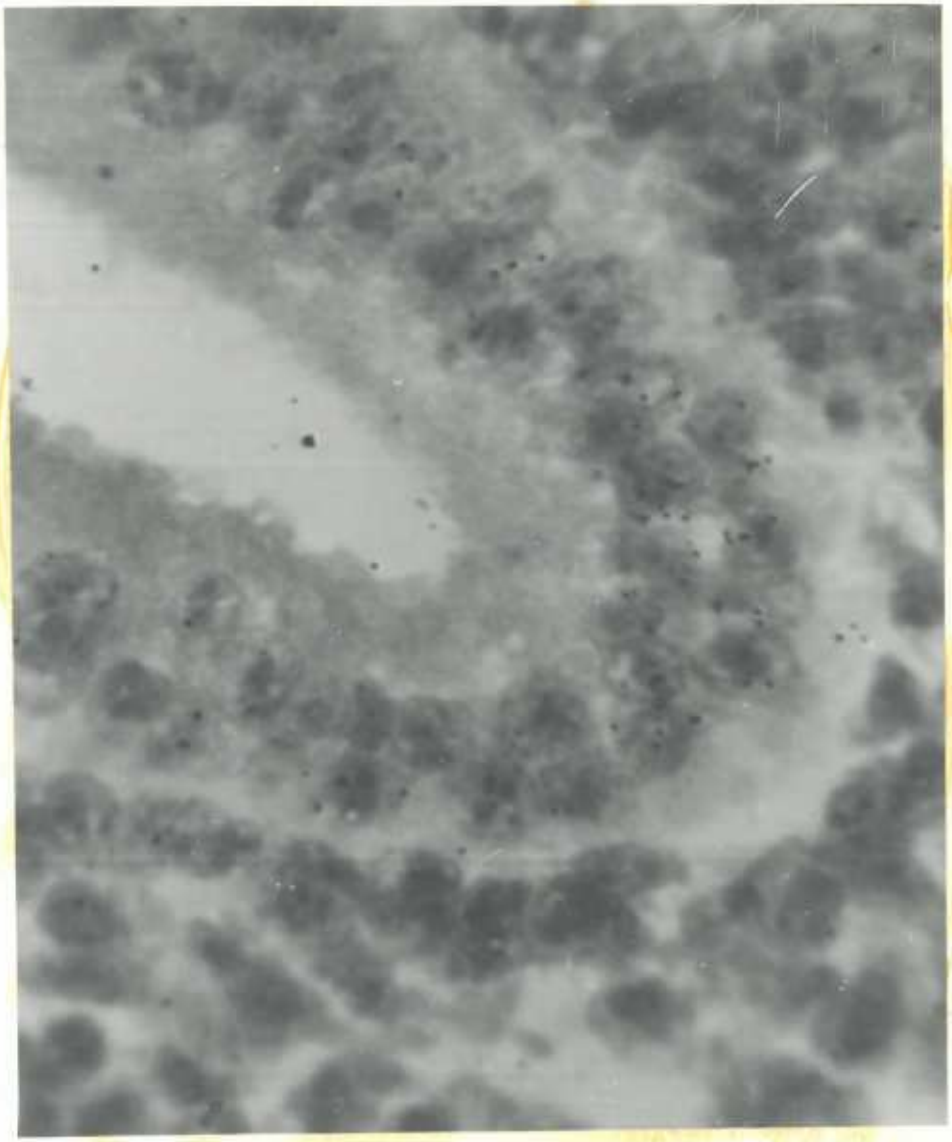

Illustration 21

Folded gonadal ridge and mesentary of embryo 8-9-D for localization of cells in illustrations 22 and 23. High-dry. (2-A-11, right gonad).

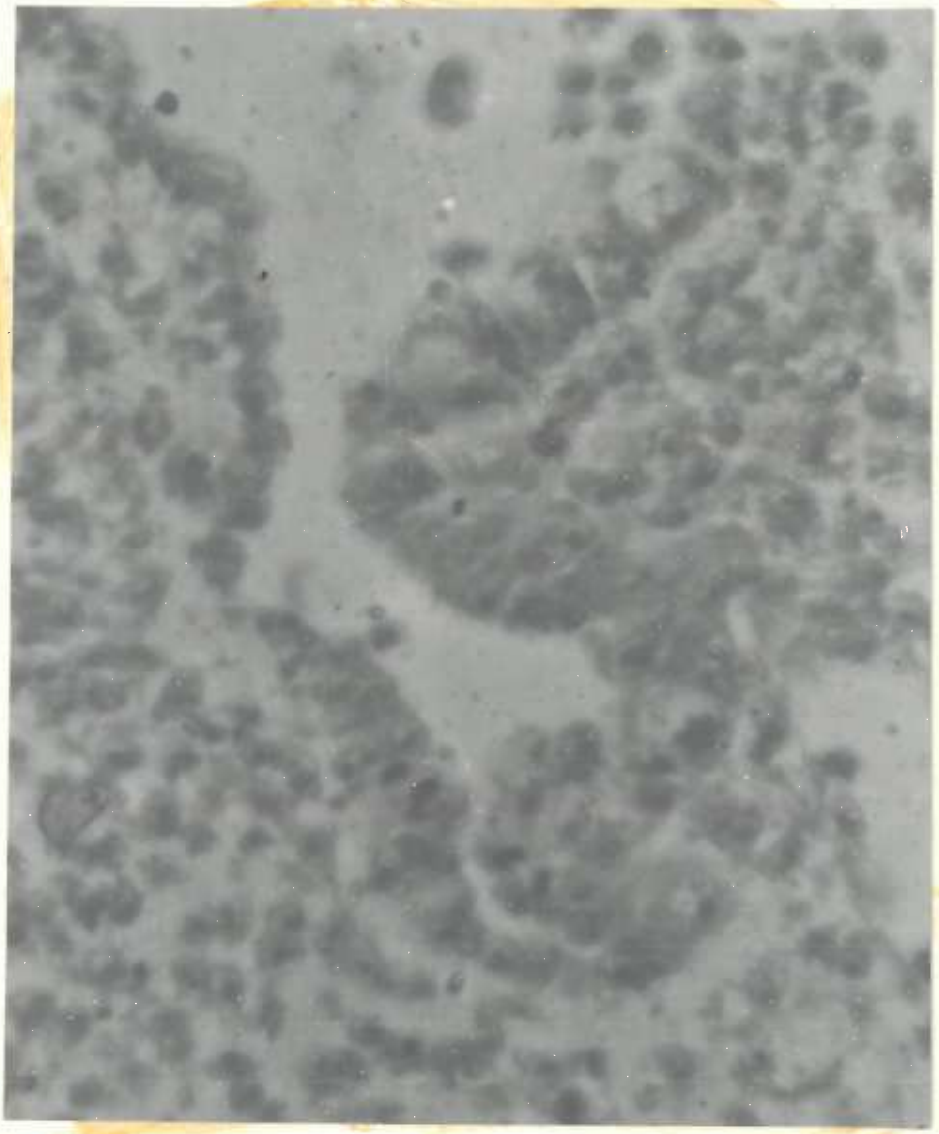

Illustration 22

Radioautograph with tagged primordial germ-cell in gonadal ridge of embryo 8-9-D. See illustrations 21 and 23. Oil-immersion. (2-A-11, right gonad).

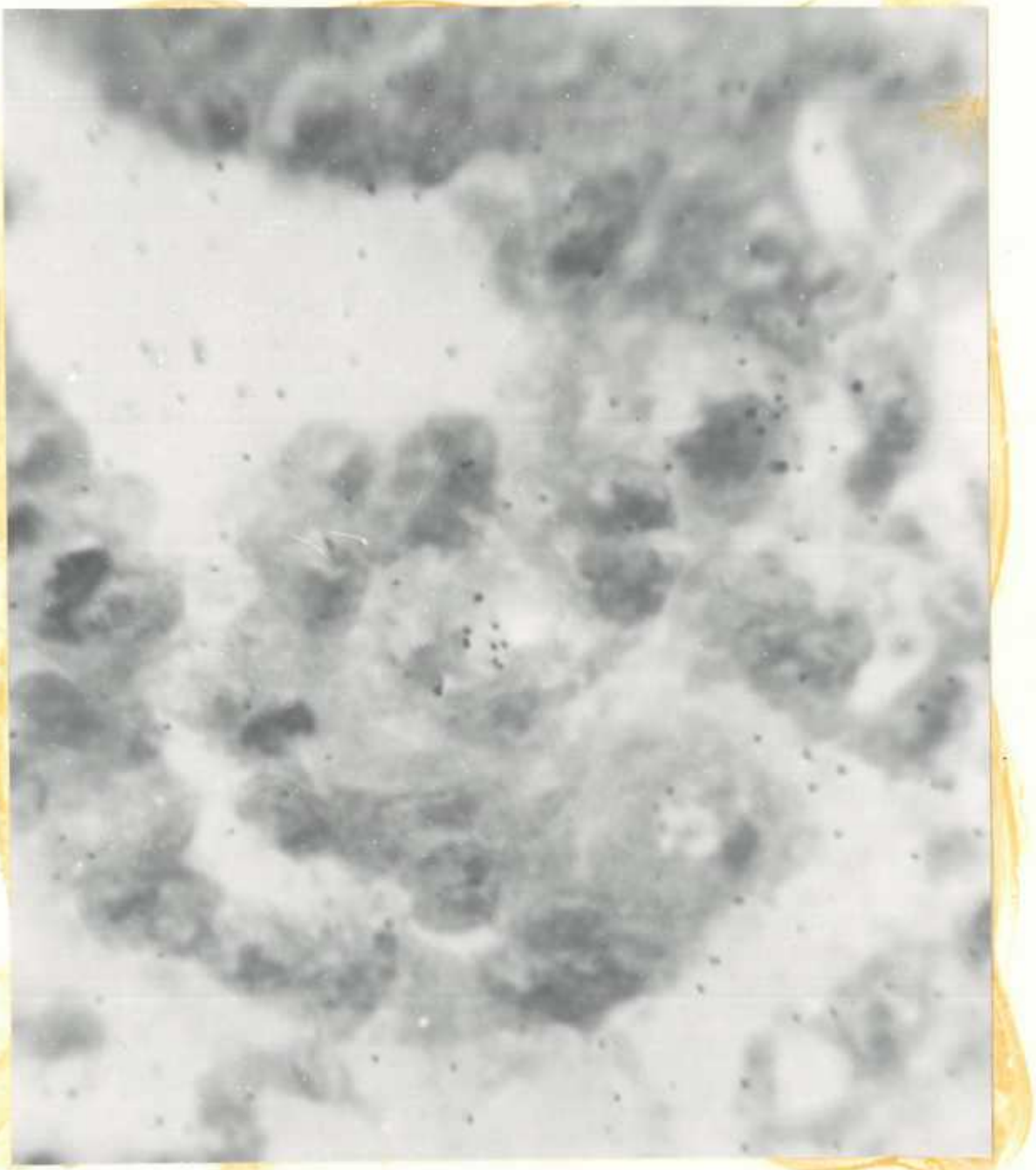

Illustration 23

Radioautograph with probable artifactual tagging over questionable cell in gonadal ridge of embryo 8-9-D. See illustrations 21 and 22. Oil-immersion. (2-A-11, right gonad).

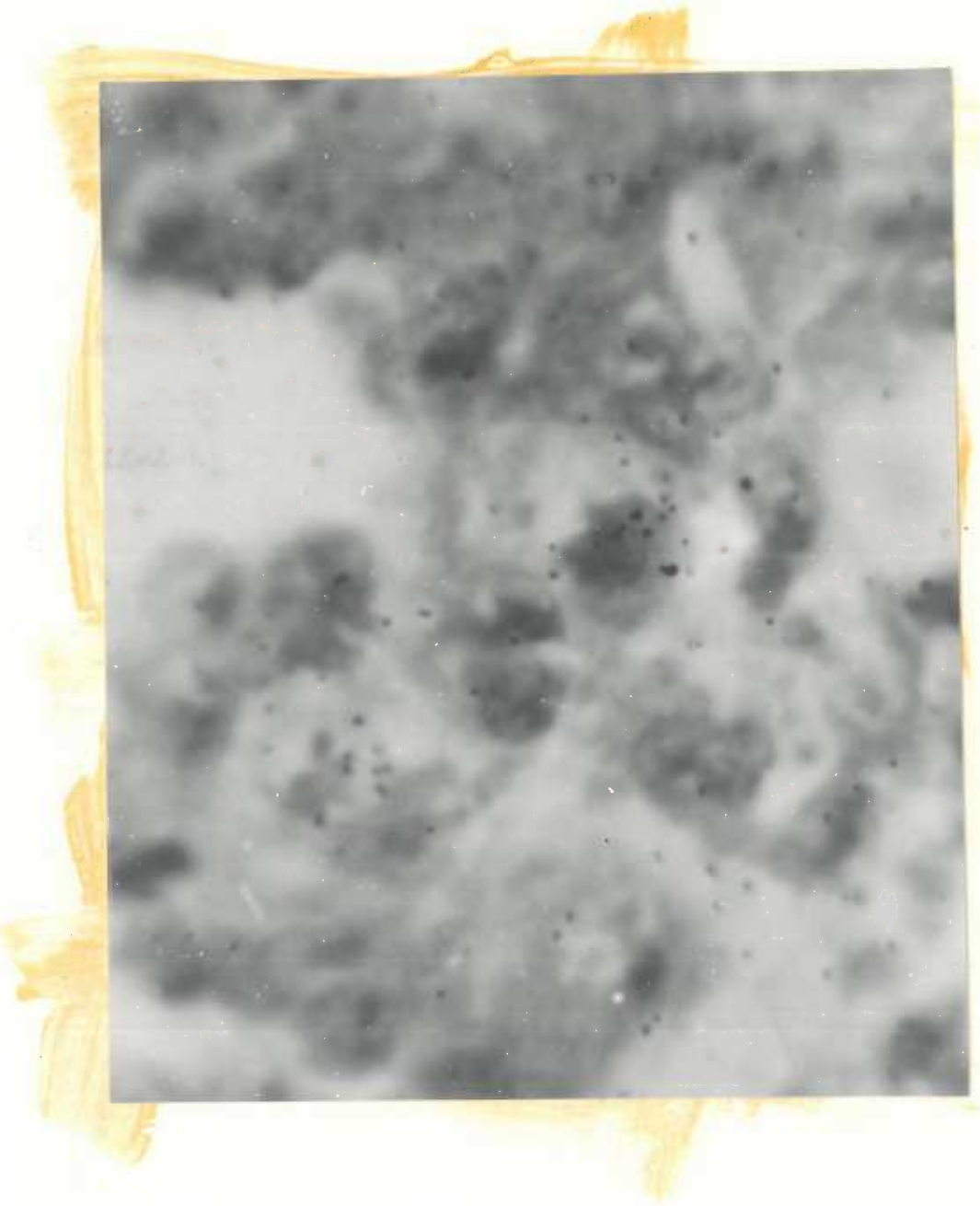

Illustration 24

Radioautograph with tagged gut mucosa of embryo 8-9-D. Oil-immersion. (2-A-11).

Illustration 25

Radioautograph with tagged gut mucosa of embryo 8-9-D. Oil-immersion. (2-C-4).

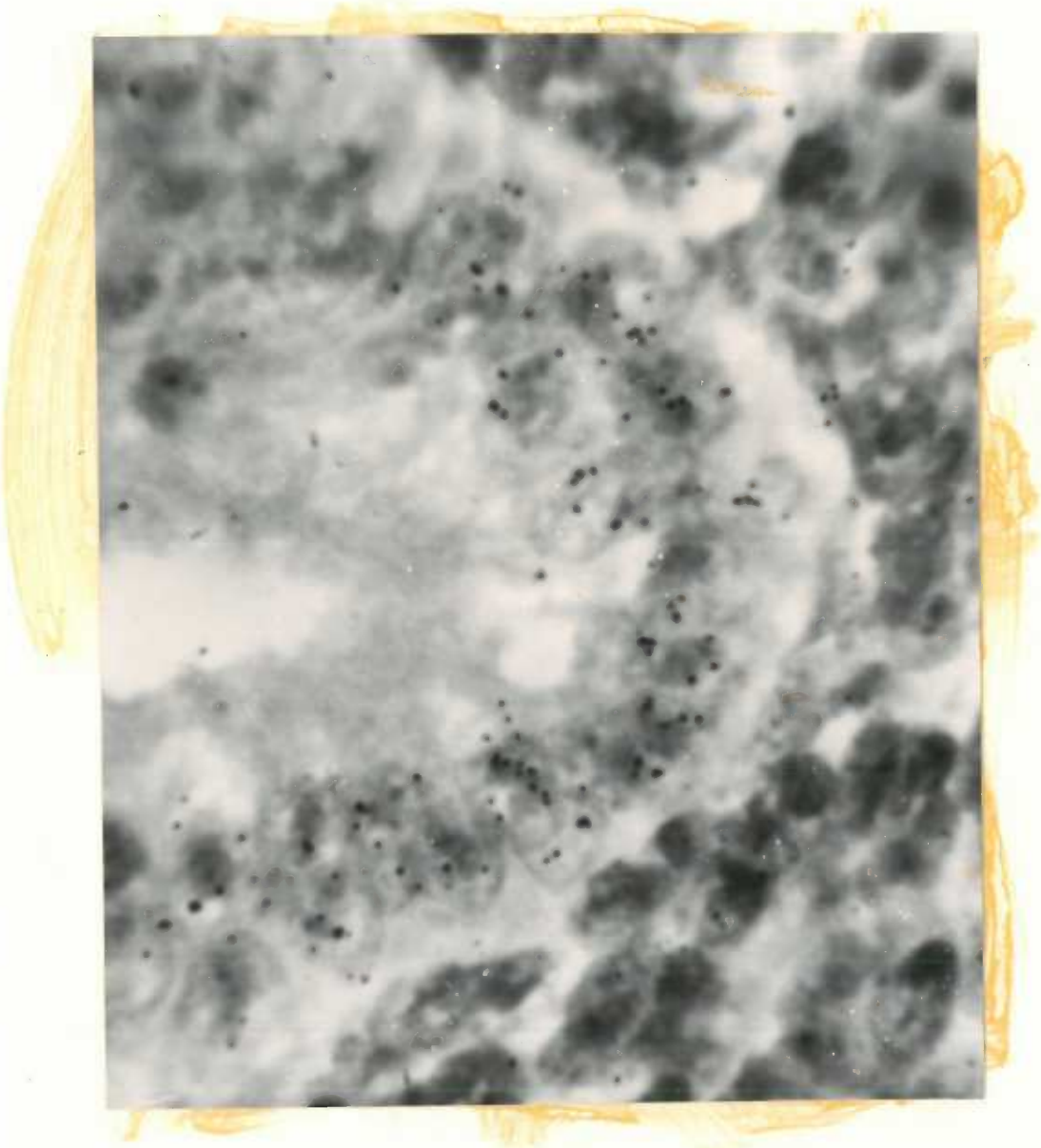

Illustration 26

Radioautograph with tagged gut mucosa of embryo 8-9-D. Oil-immersion. (4-B-11).

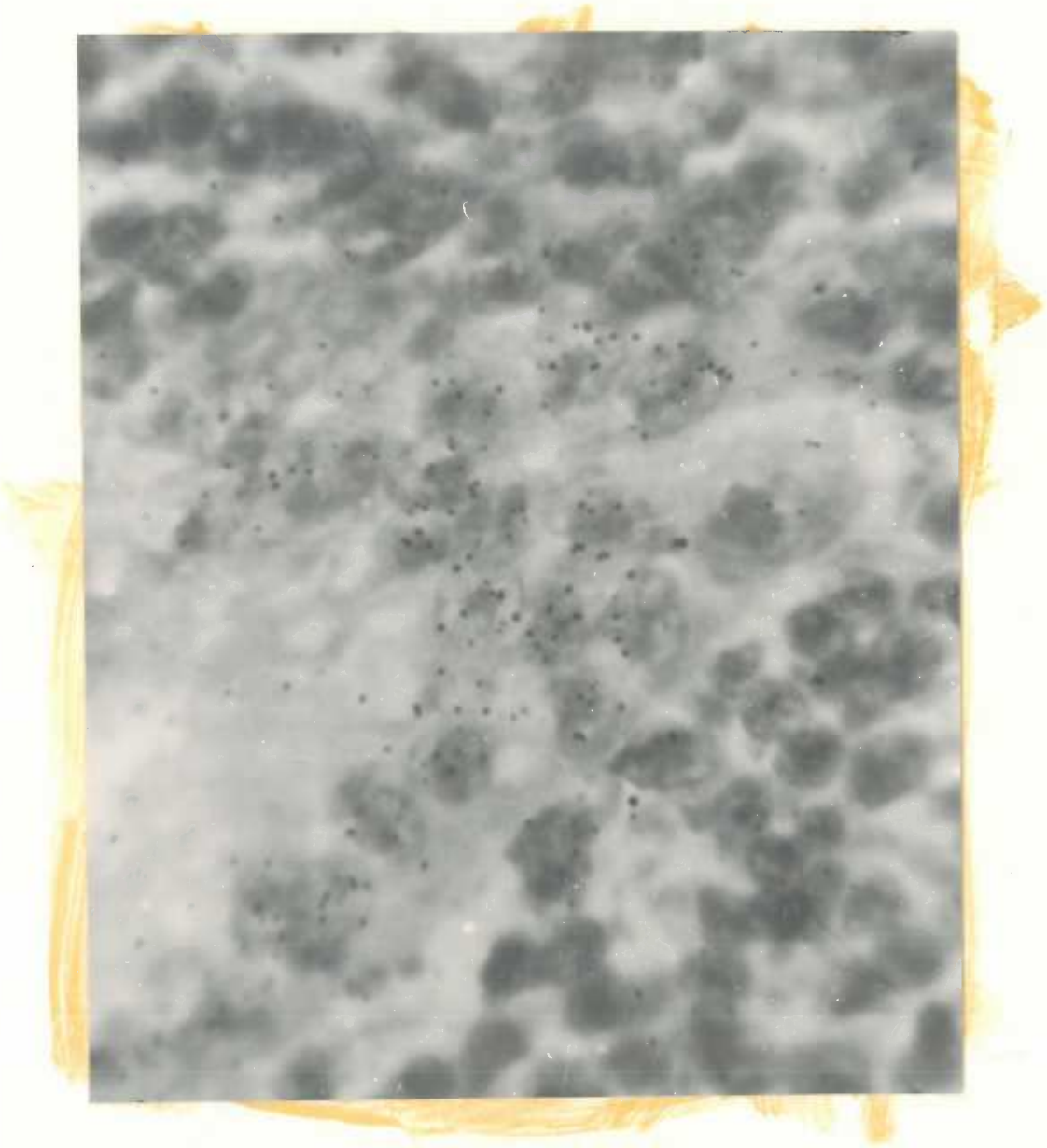

Illustration 27

Radioautograph with tagged primordial germ-cell in base of mesentary of embryo 8-9-8. Oil-immersion. (1-C-7, base of mesentary).

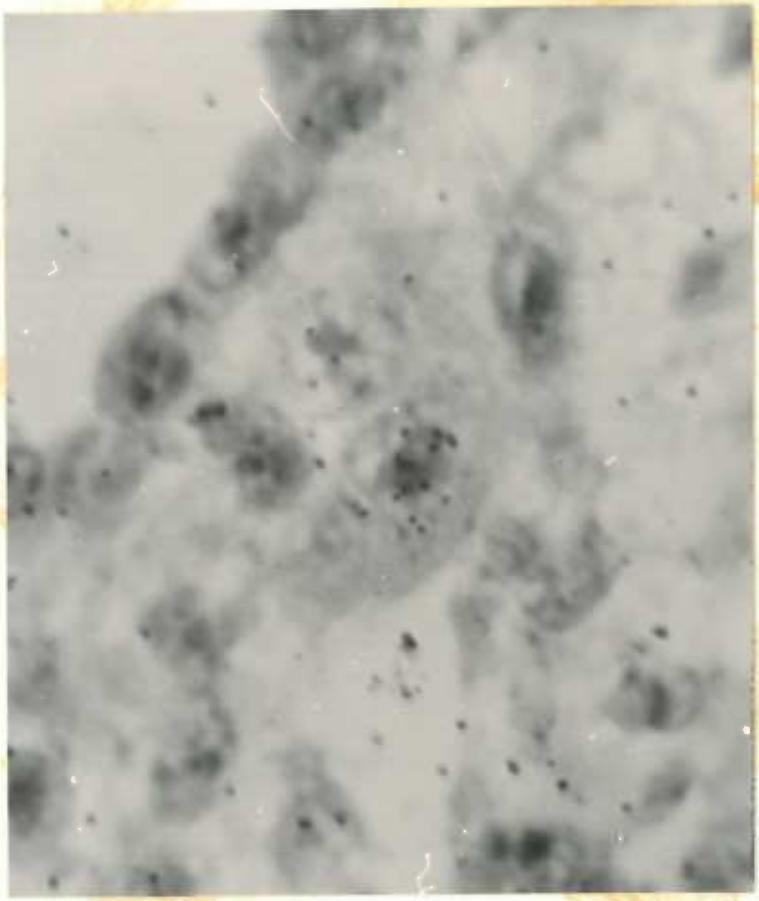

Illustration 28

Section of embryo 8-9-0 for localization of primordial germ-cell in illustration 29. High-power. (3-A-3, left gonad).

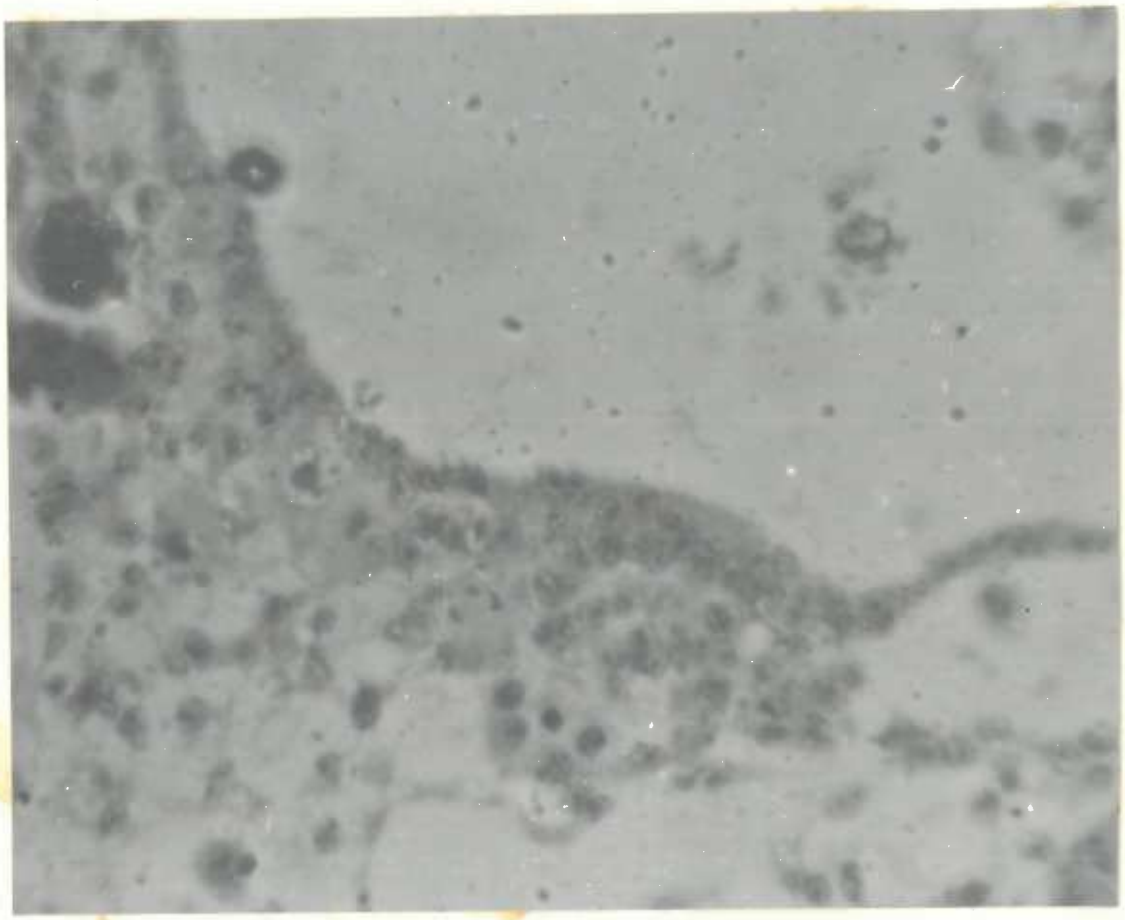

Illustration 29

Radioautograph with tagged primordial germ-cell in gonadal ridge of embryo 8-9-0. See illustration 28. Oil-immersion. (3-A-3, left gonad).

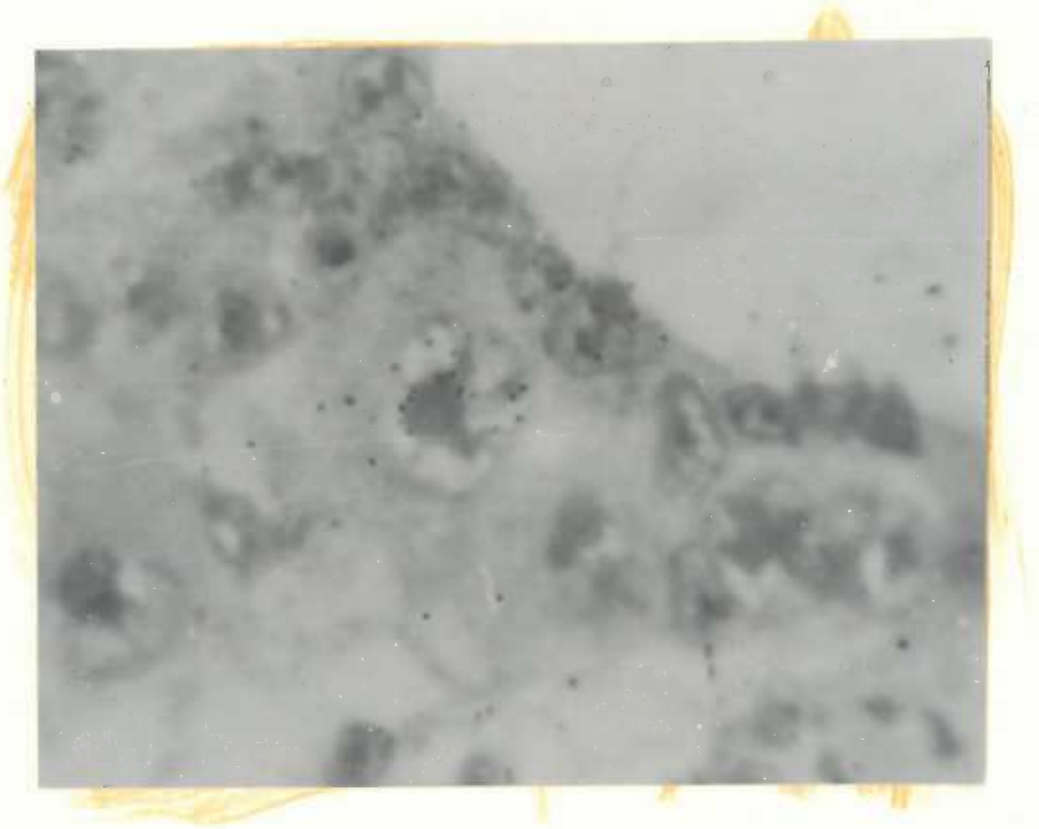

Illustration 30

Radioautograph with tagged gut mucosa of embryo 8-9-G. Oil-immersion. (4-A-11).

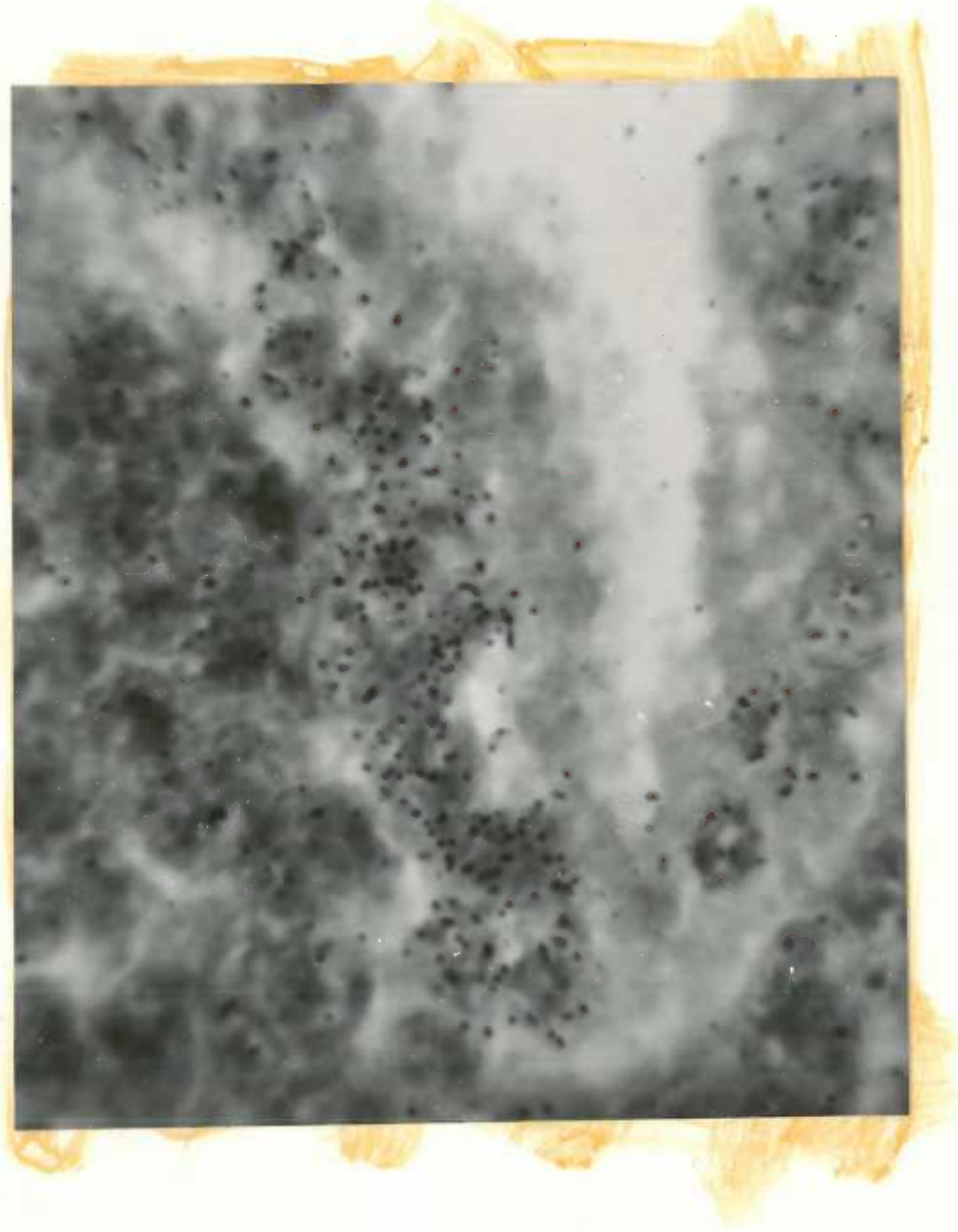

Illustration 31

Radioautograph with tagged gut mucosa of embryo 8-9-0.
Oil-immersion. (5-C-11).

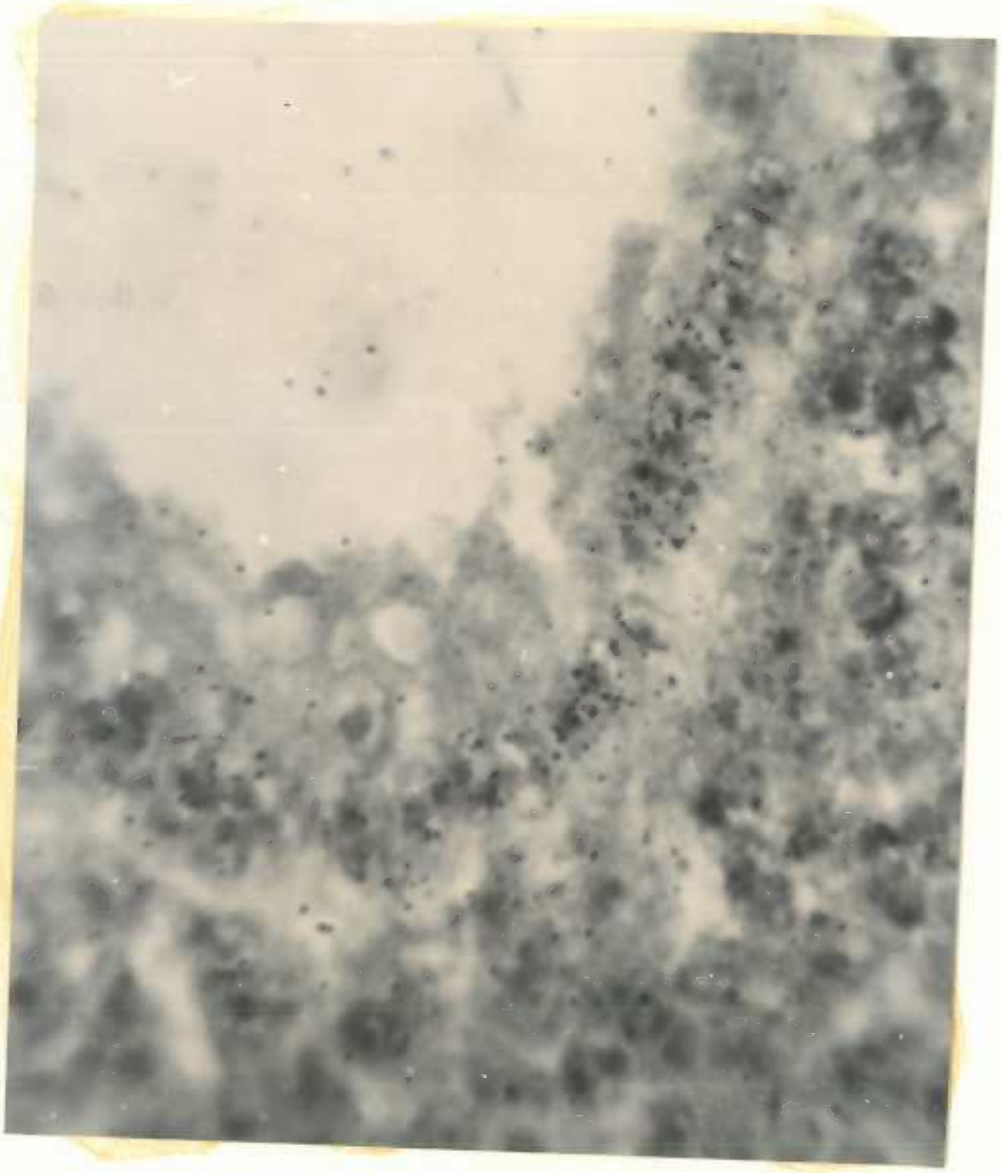

Illustration 32

Radioautograph with tagged gut mucosa of embryo 8-9-G. Oil-immersion. (3-A-1).

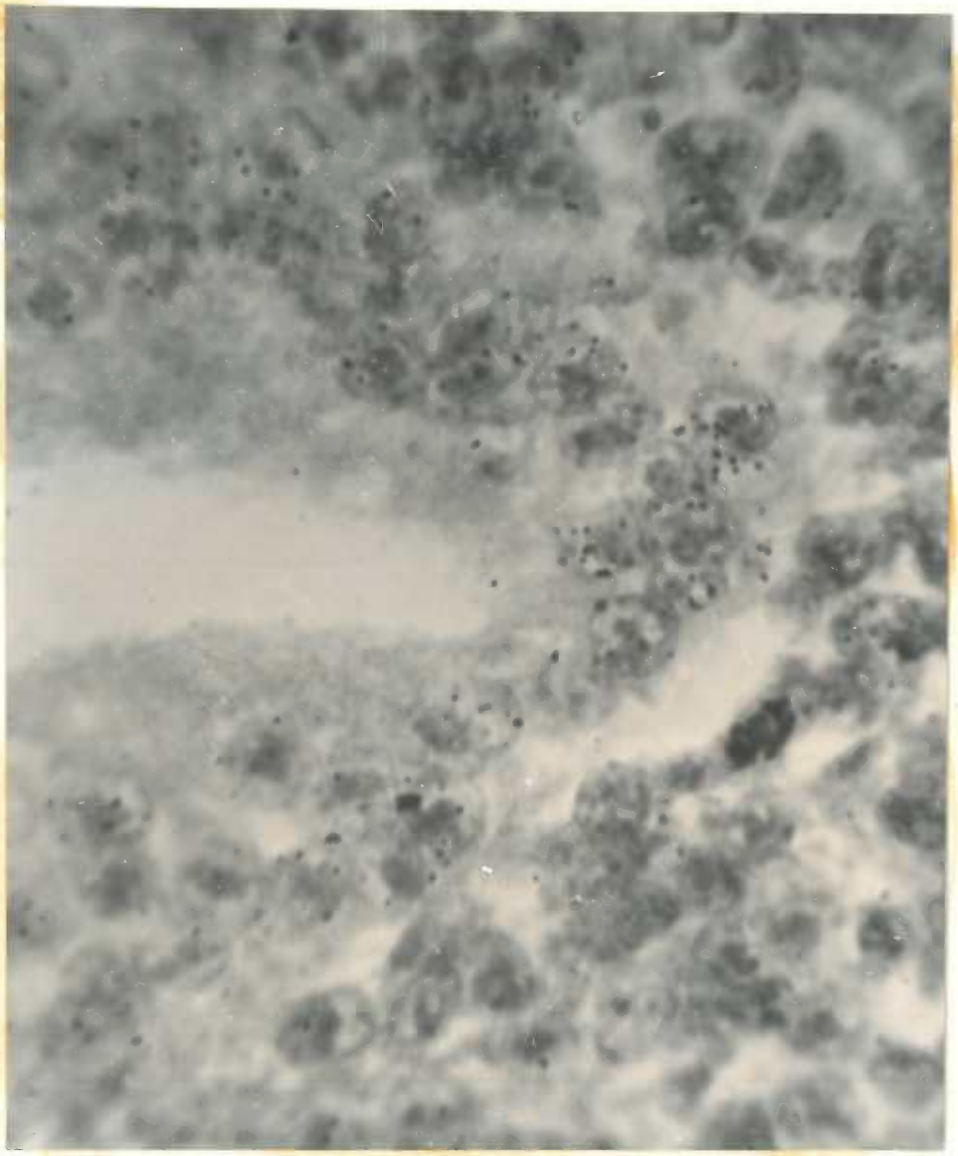

Illustration 33

Radioautograph with tagged gut mucosa of embryo 8-9-0. Oil-immersion. (2-A-1).

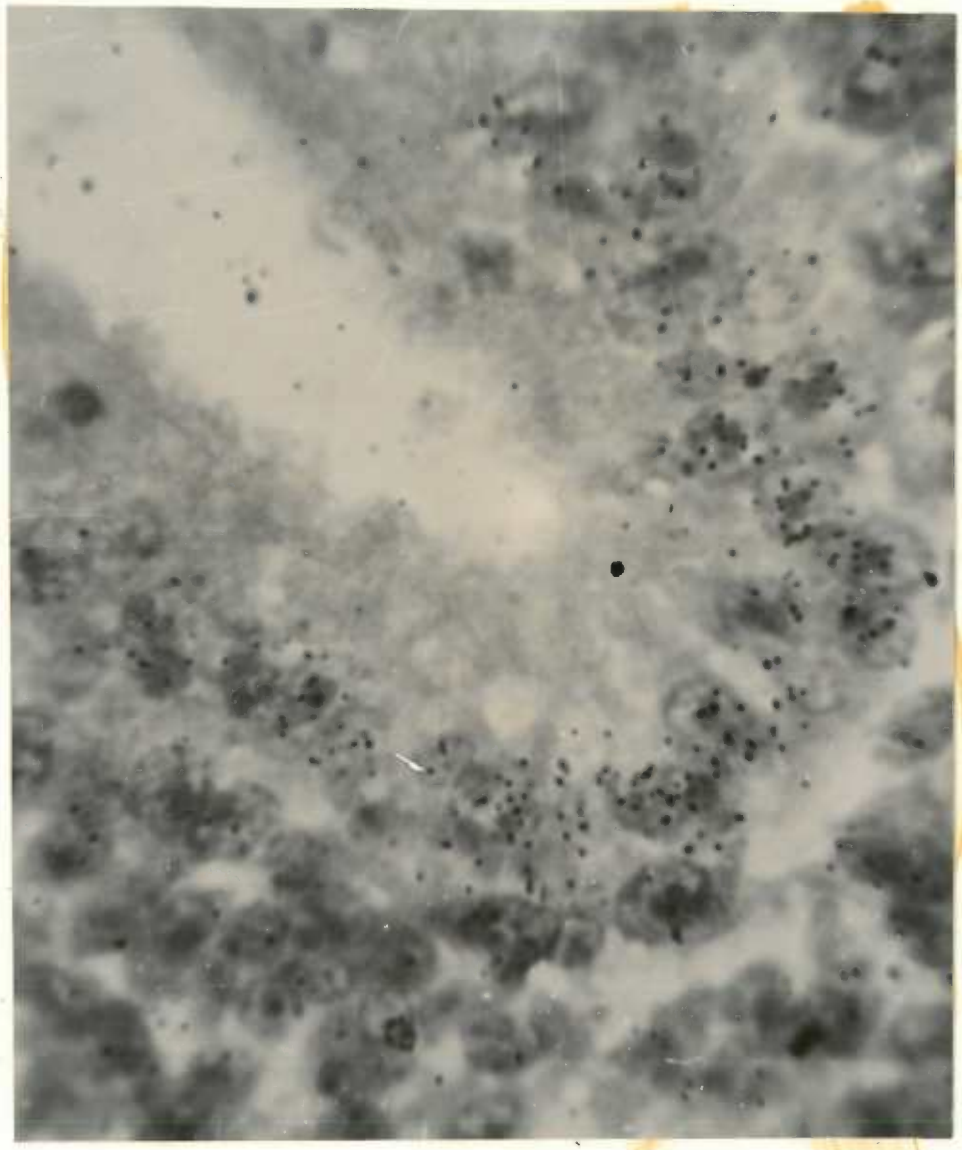

Illustration 34

Radioautograph with tagged gut mucosa of embryo 8-9-0.
Oil-immersion. (1-C-7).

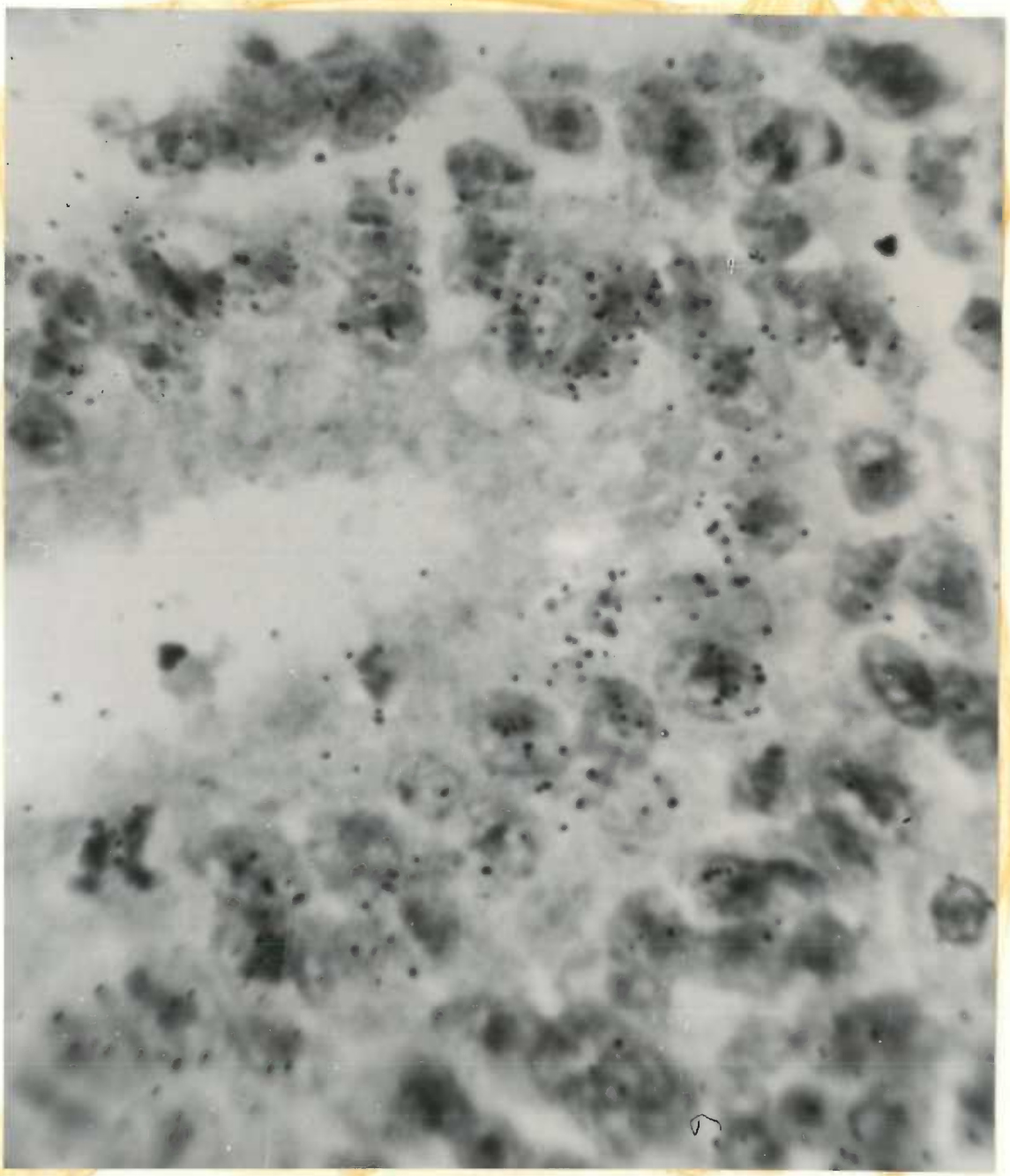

Illustration 35

Radioautograph with tagged aorta endothelial cell (cells?) in series with illustration 36. Oil-immersion. (2-B-8).

Illustration 36

Radioautograph with tagged aorta endothelial cell (cells?) in series with illustration 35. Oil-immersion. (2-B-7).

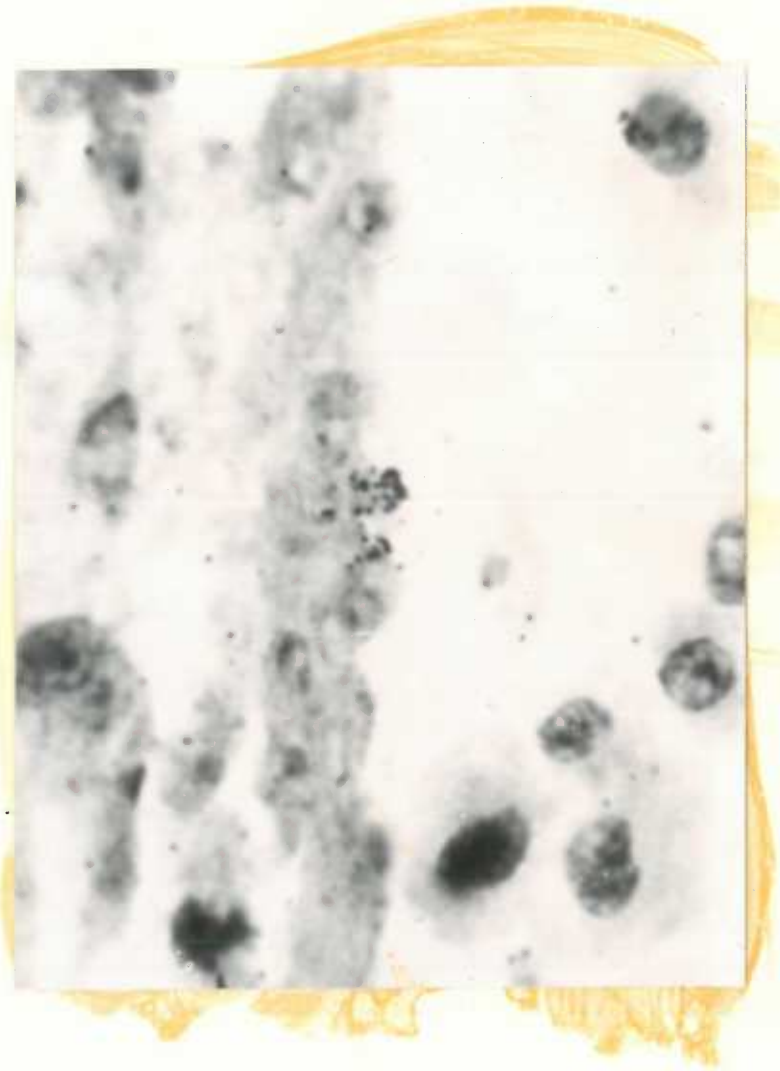

Illustration 37

Radioautograph with tagged cell in peri-spinal cord membranes of embryo 8-9-H. Oil-immersion. (5-A-14, to left of spinal cord).

Illustration 38

Radioautograph with artifact over germinal epithelial cell of embryo 8-9-H. Oil-immersion. (1-C-2, right gonad).

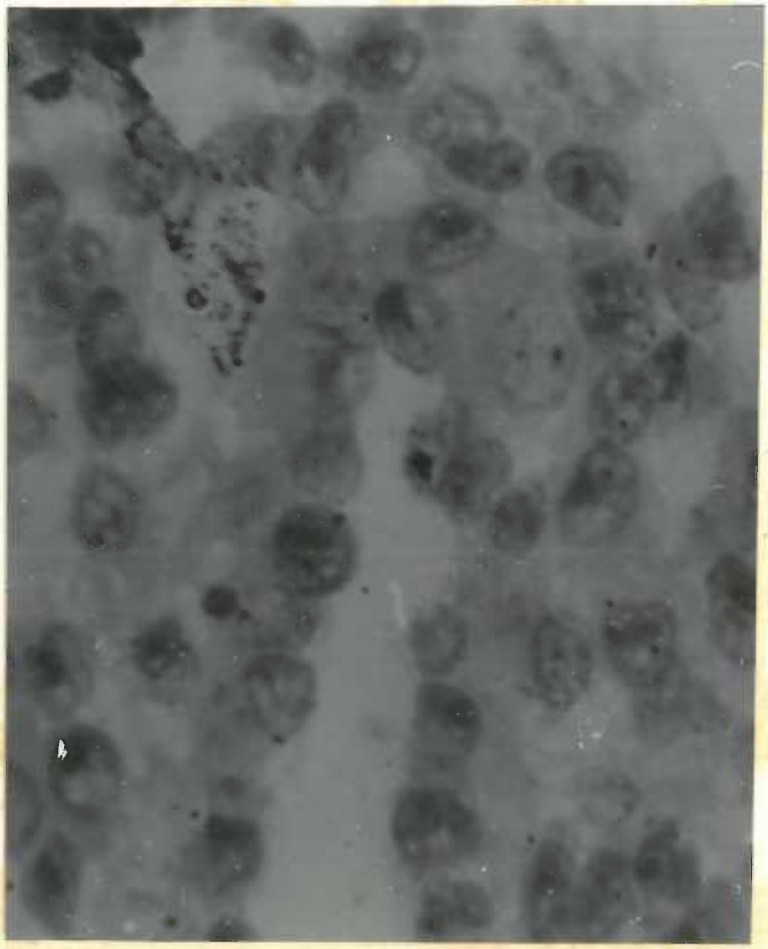

Illustration 39

Radioautograph with artifact over germinal epithelial cell of embryo 8-9-H. Oil-immersion. (1-C-12, left gonad).

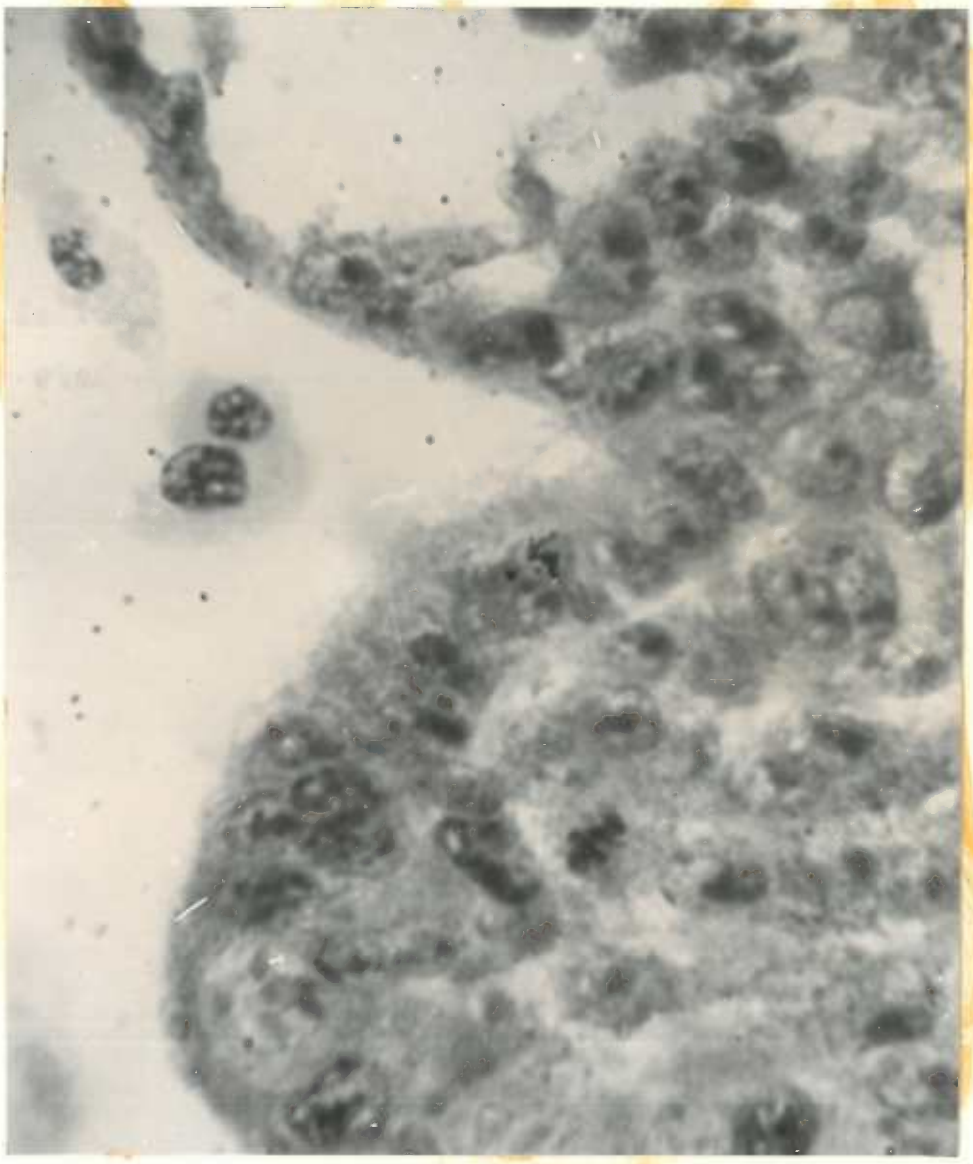

Illustration 40

Radioautograph with tagged ventral plate of spinal cord of embryo 8-9-H. Oil-immersion. (3-C-1).

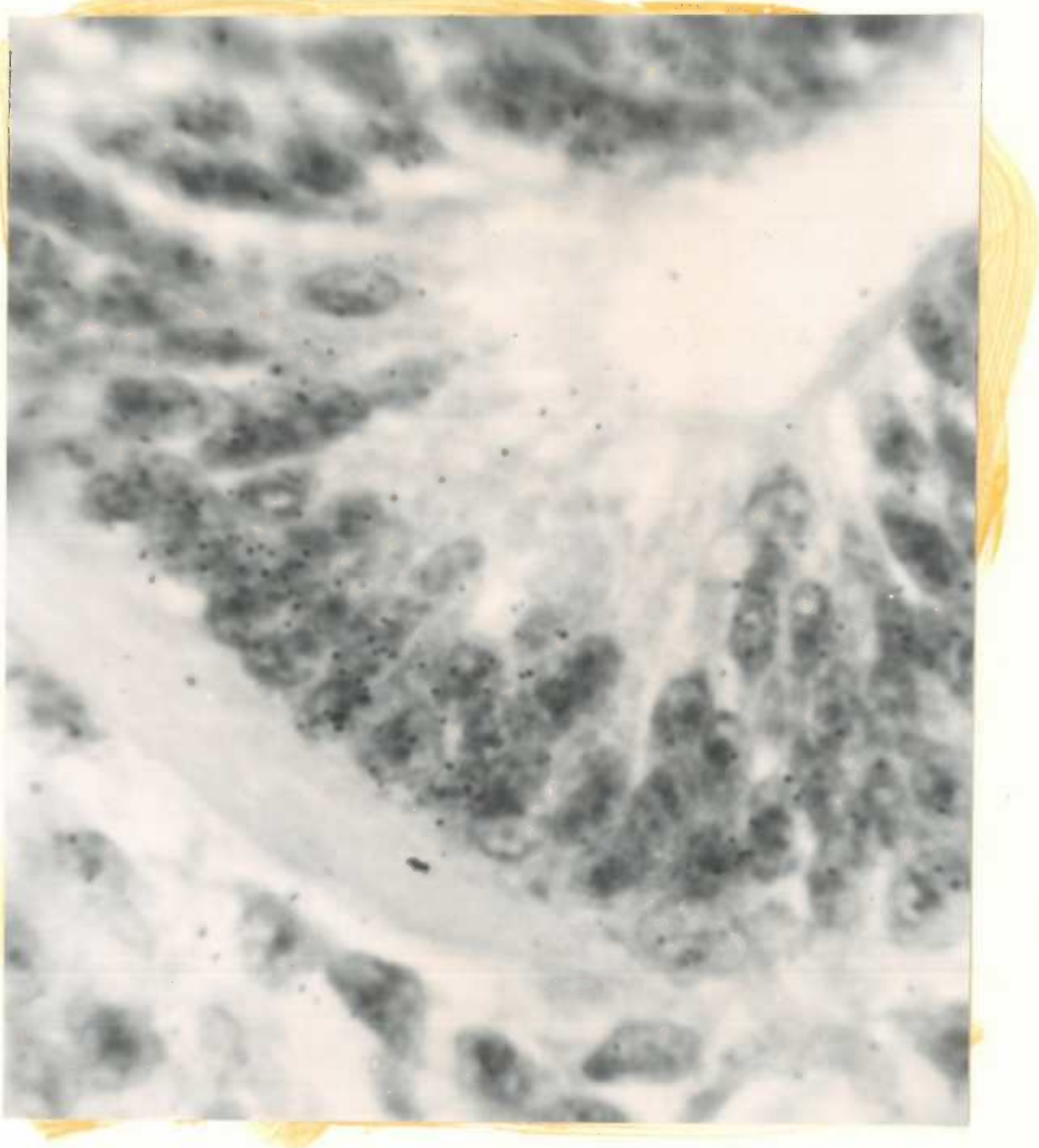

Illustration 41

Radioautograph with tagged ventral plate of spinal cord of embryo 8-9-H. Oil-immersion. (4-B-9).

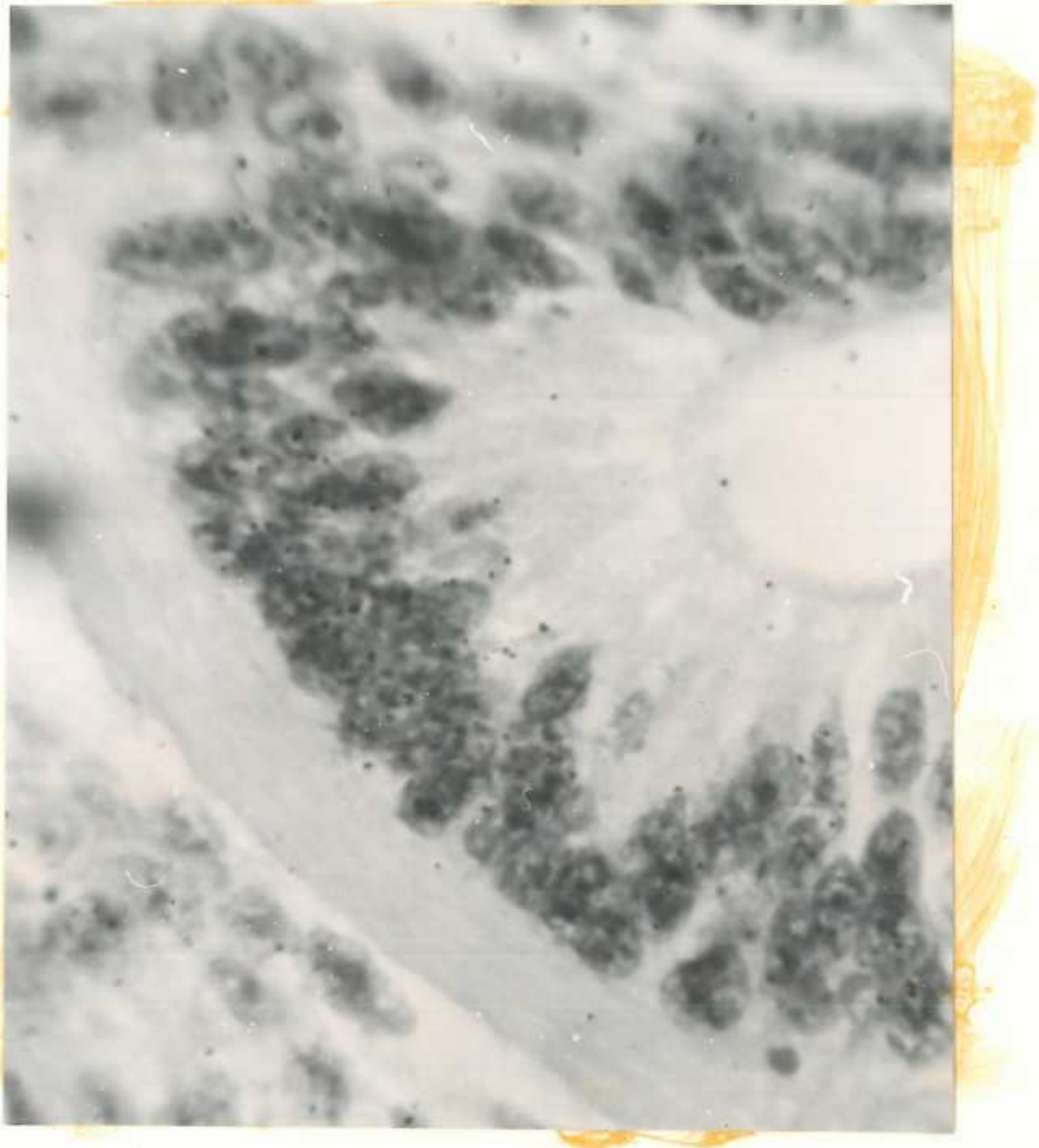

Illustration 42

Radioautograph with tagged ventral plate of spinal cord of embryo 8-9-H. Oil-immersion. (5-C-6).

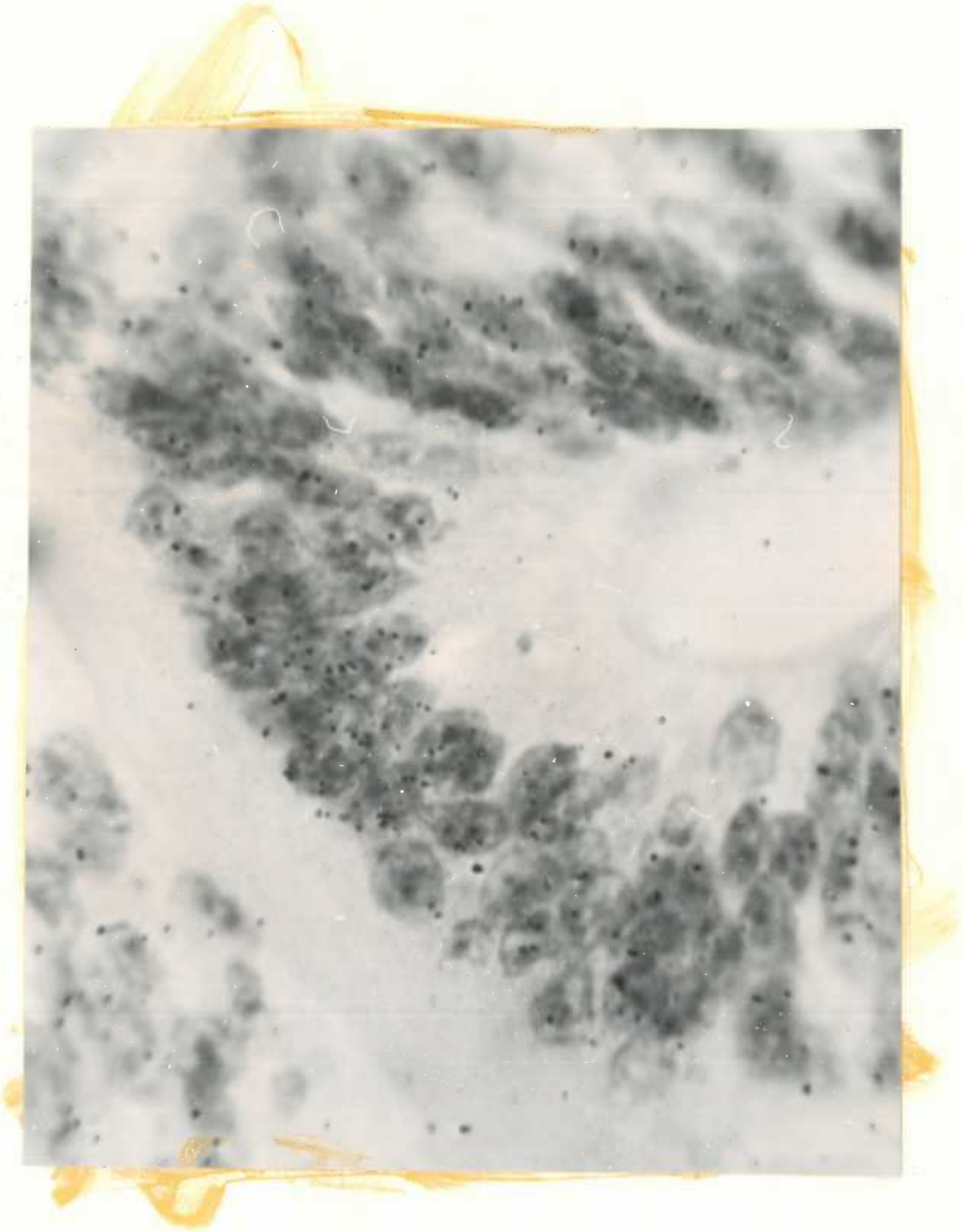

Illustration 43

Radioautograph with tagged notochord cells of embryo 8-9-H. Oil-immersion. (1-A-12).

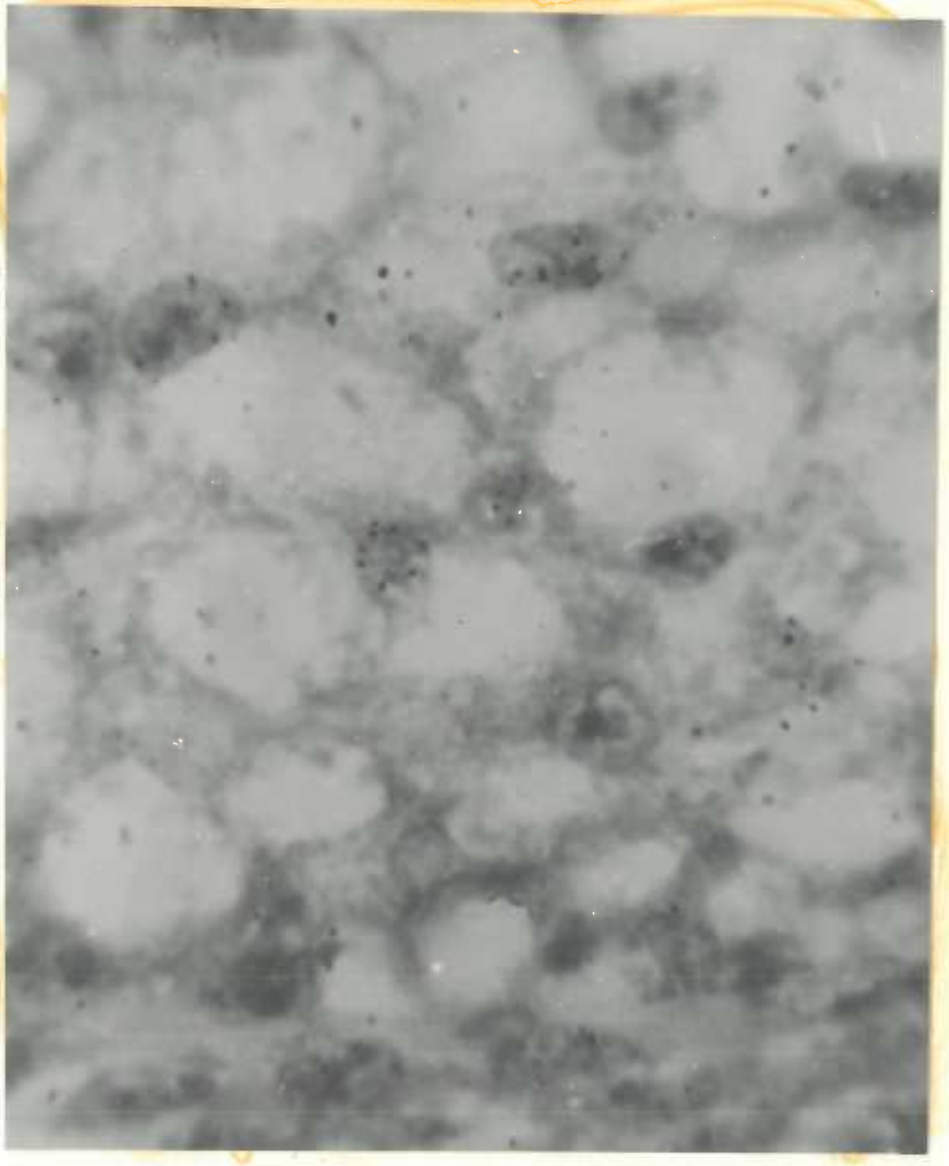

Illustration 44

Radioautograph with tagged notochord cells of embryo 8-9-H. Oil-immersion. (2-A-7).

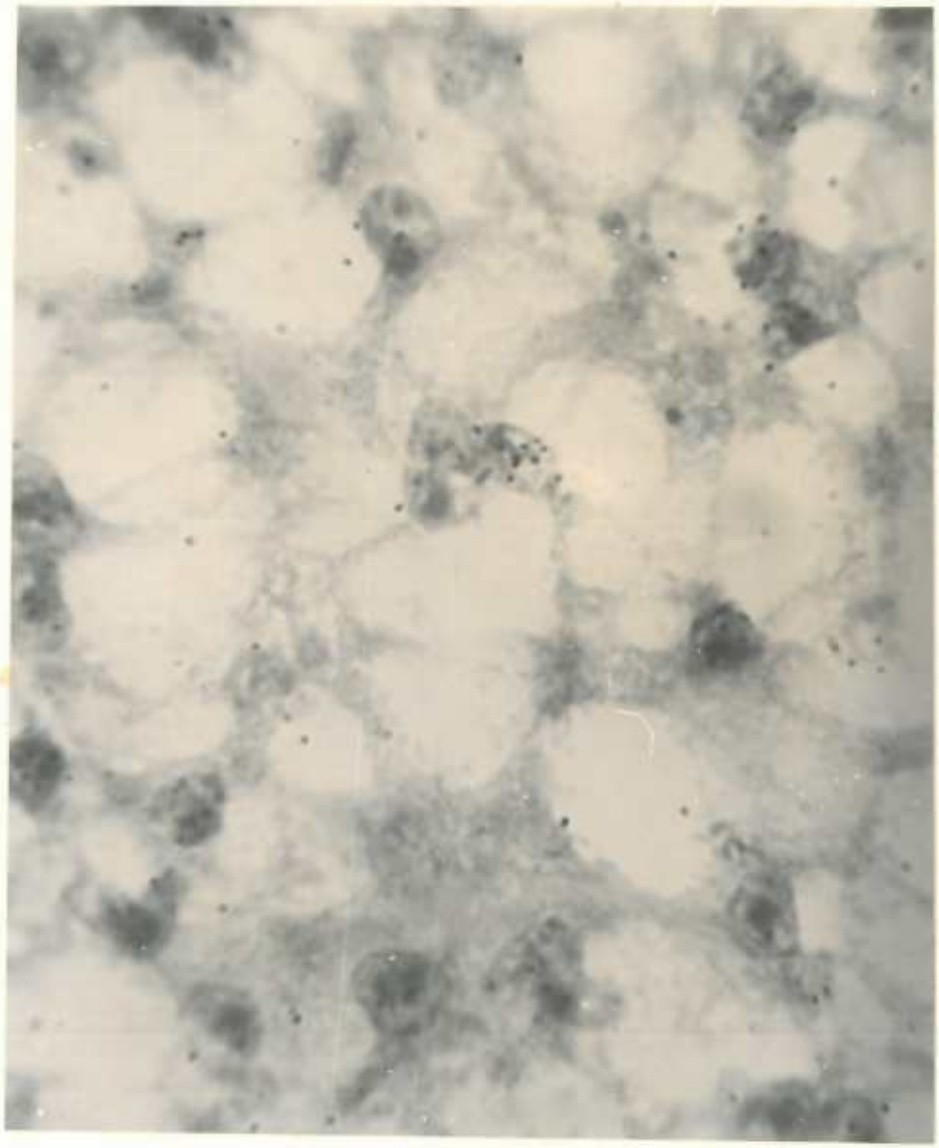

Illustration 45

Radioautograph with tagged notochord cells of embryo 8-9-H. Oil-immersion. (4-A-11).

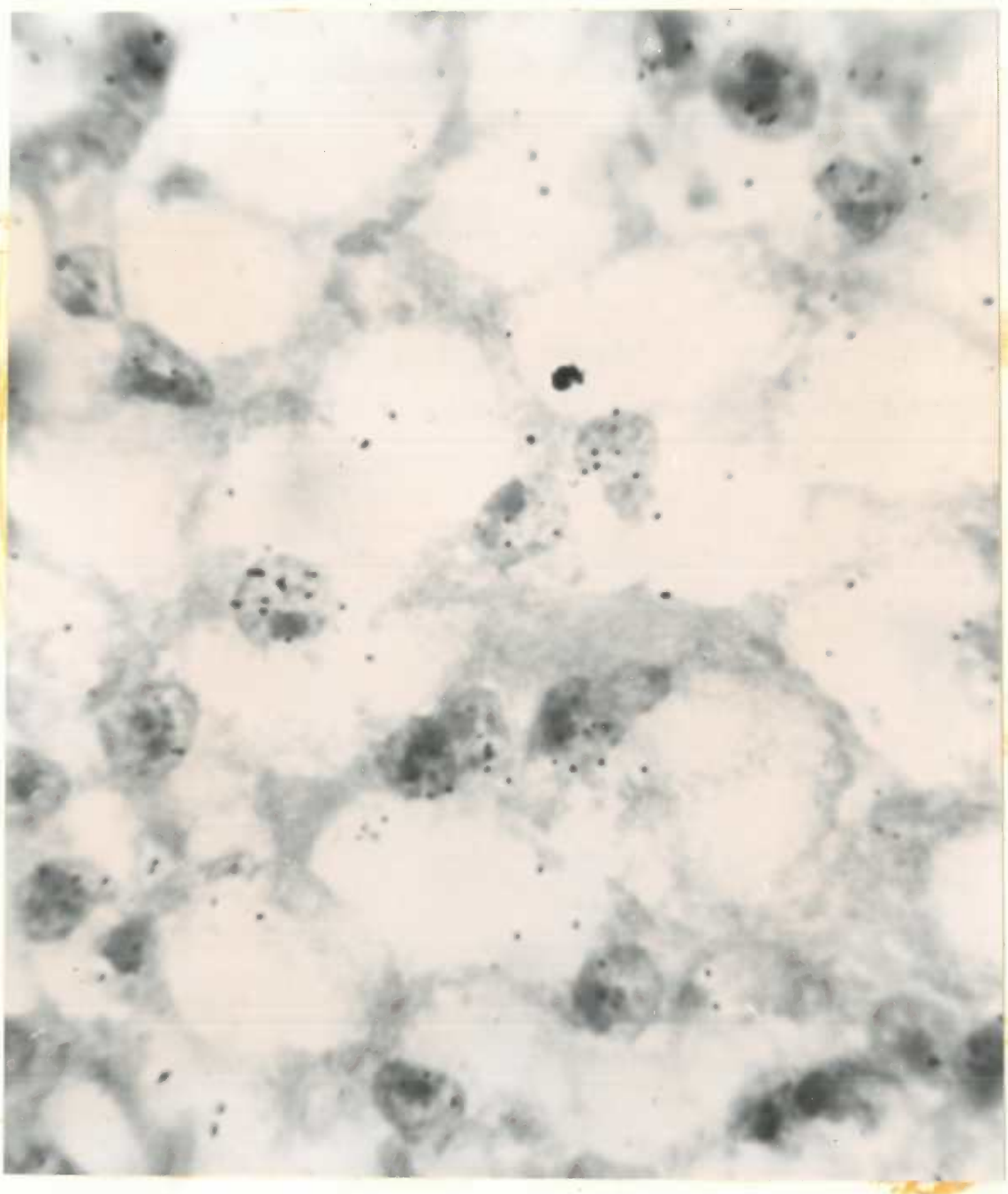

Illustration 46

Radioautograph with tagged notochord cells of embryo 8-9-H. Oil-immersion. (5-C-6).

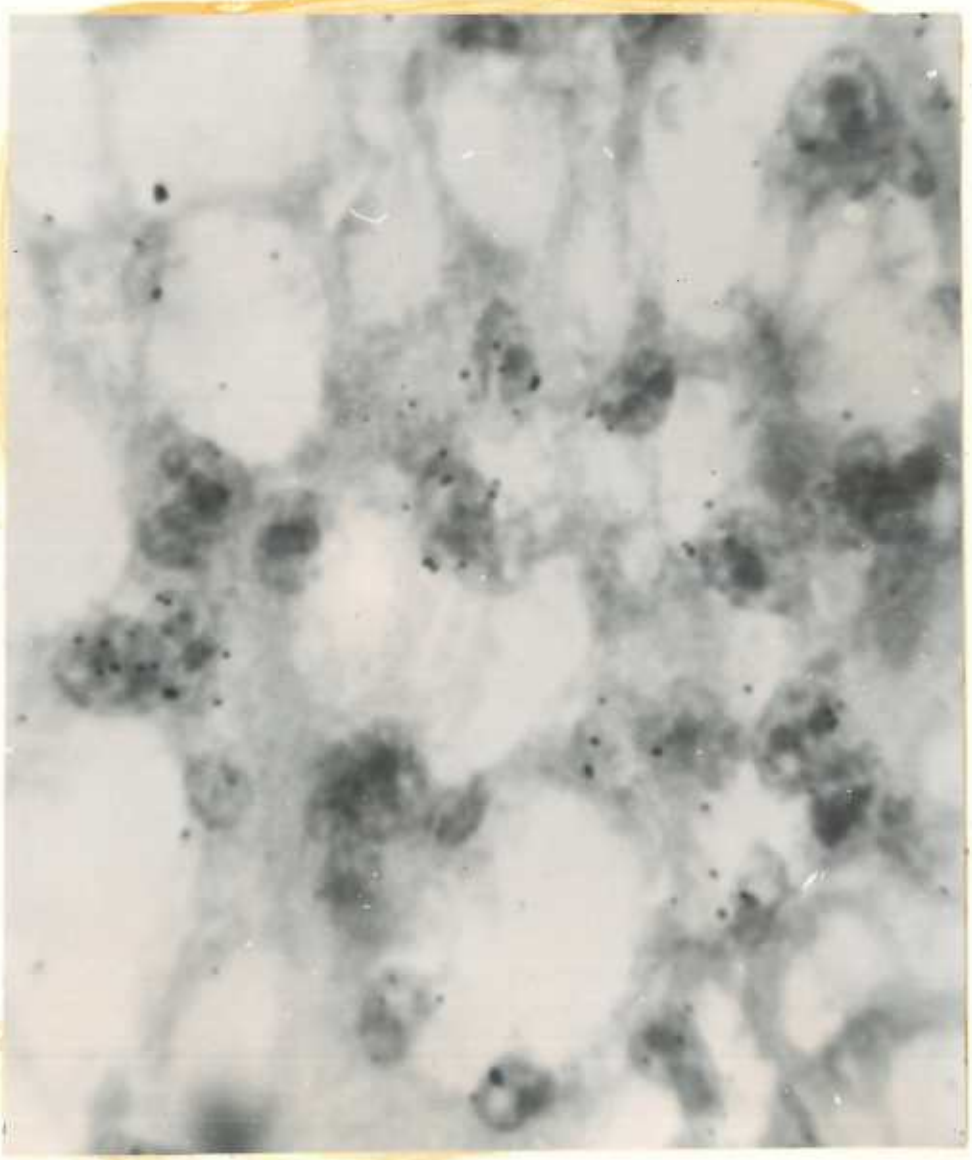

Illustration 47

Radioautograph with tagged yolk sac cells of embryo 8-9-H. Oil-immersion. (1-A-4).

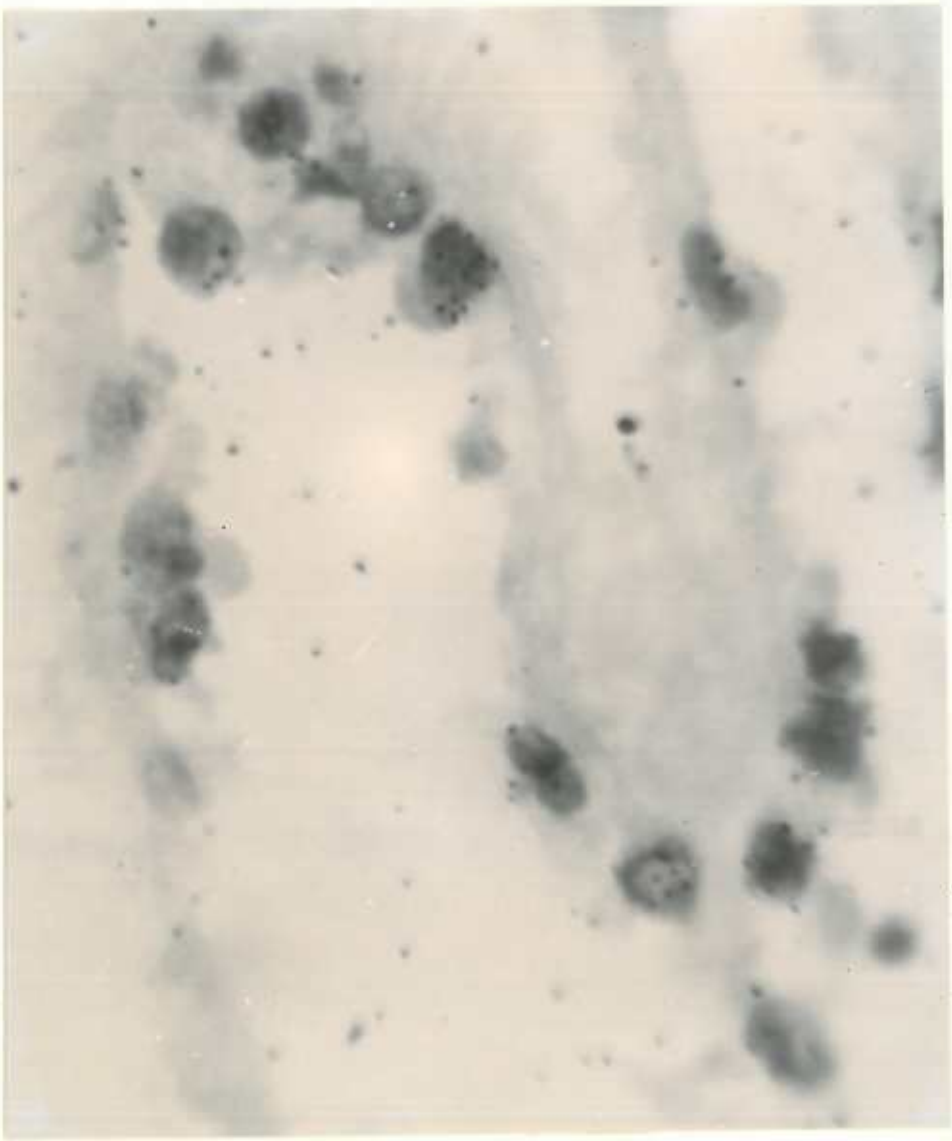

Illustration 48

Radioautograph with tagged yolk sac cells of embryo 8-9-H. Oil-immersion. (1-A-8).

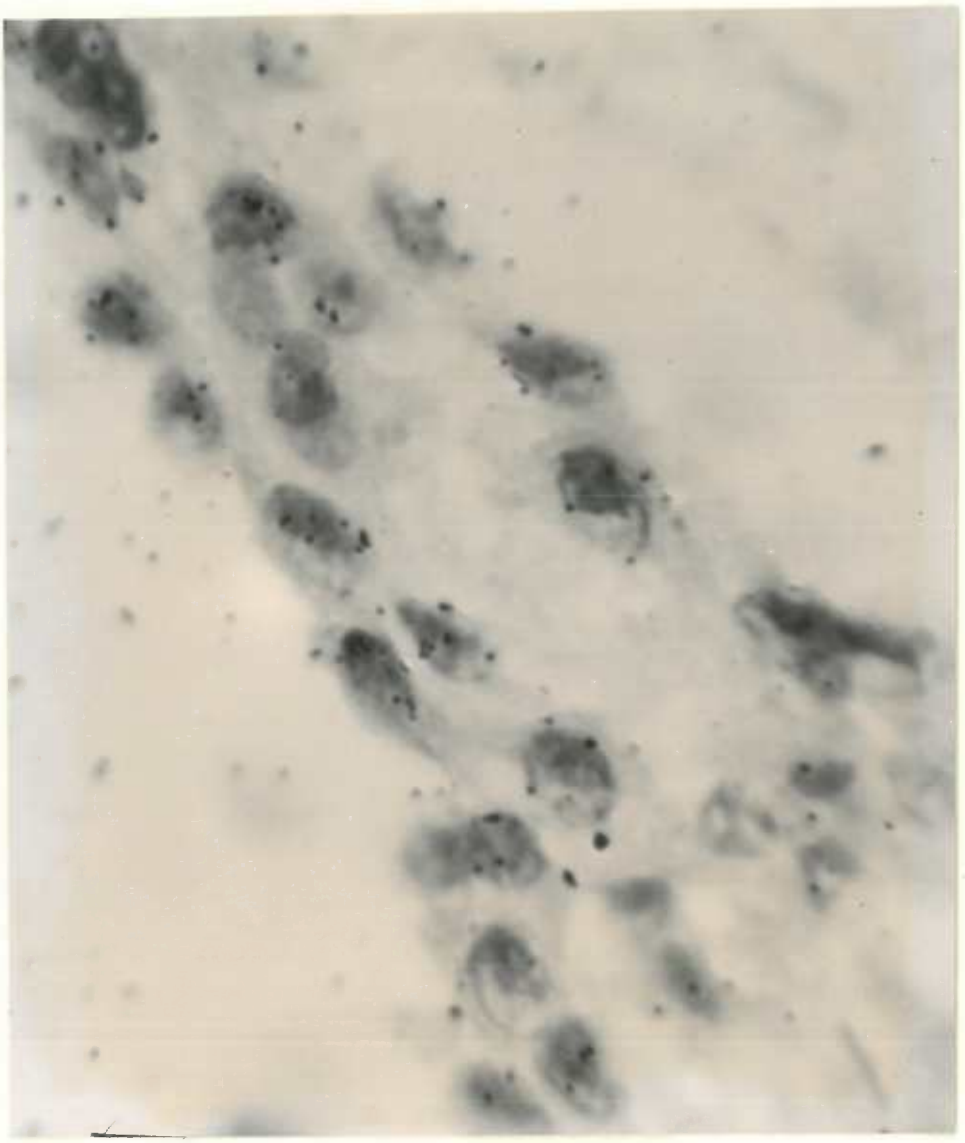

Illustration 49

Radioautograph with tagged yolk sac cells of embryo 8-9-H. Oil-immersion. (2-A-6).

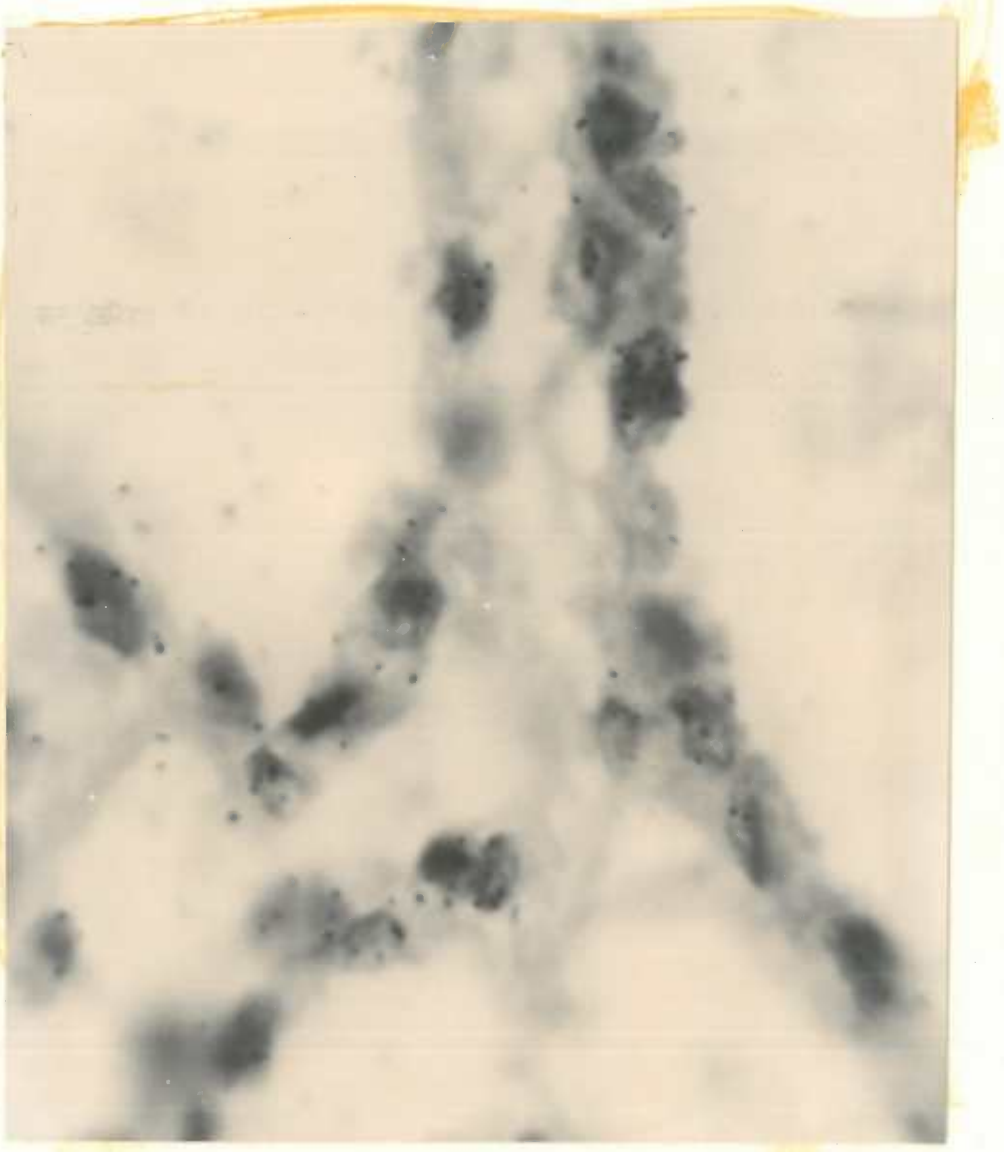

Illustration 50

Radioautograph with tagged gut mucosa of embryo 8-9-H. Oil-immersion. (1-A-7).

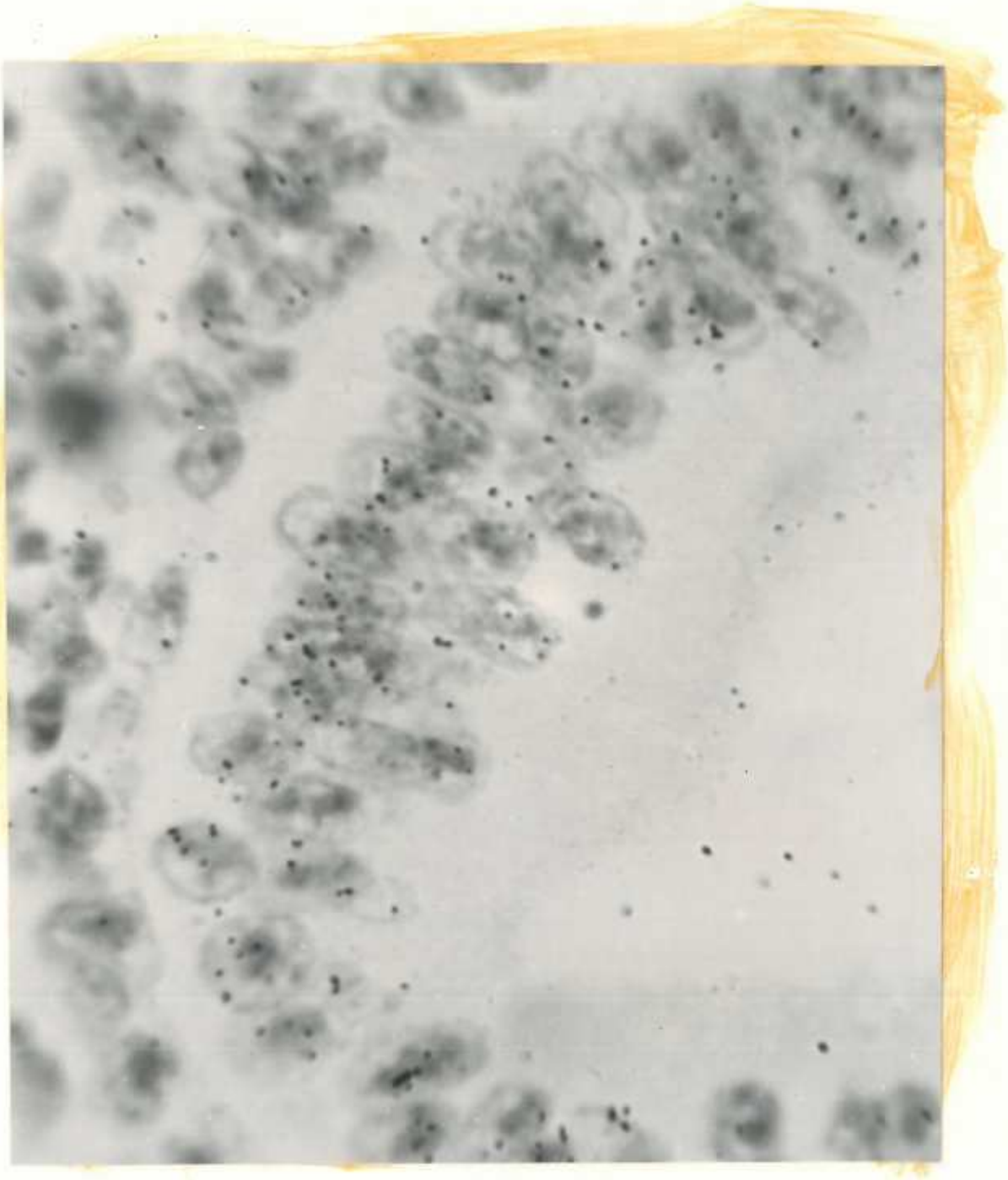

Illustration 51

Radioautograph with tagged gut mucosa of embryo 8-9-H. Oil-immersion. (2-B-7).

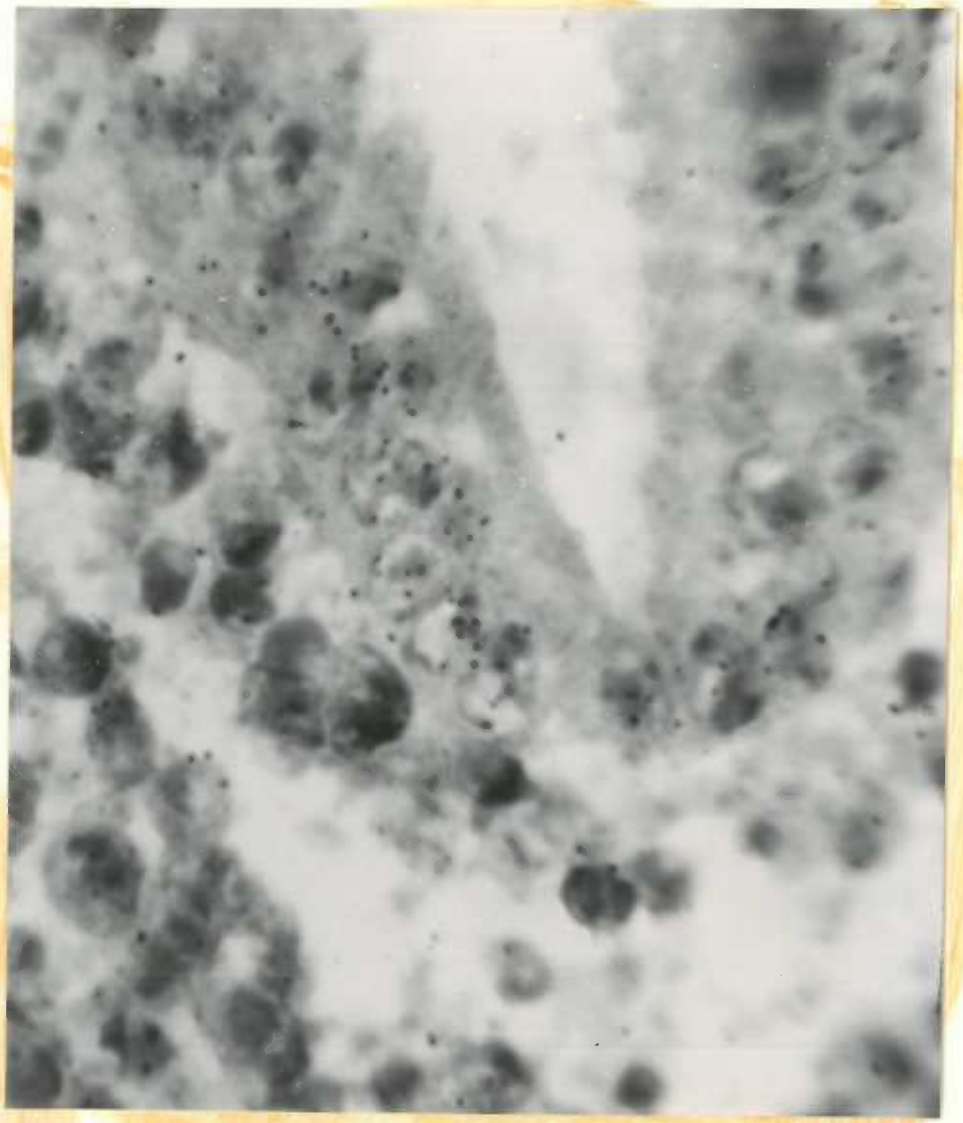

Illustration 52

Radioautograph with tagged blood cell in aorta of embryo 8-9-J. Oil-immersion. (1-C-1).

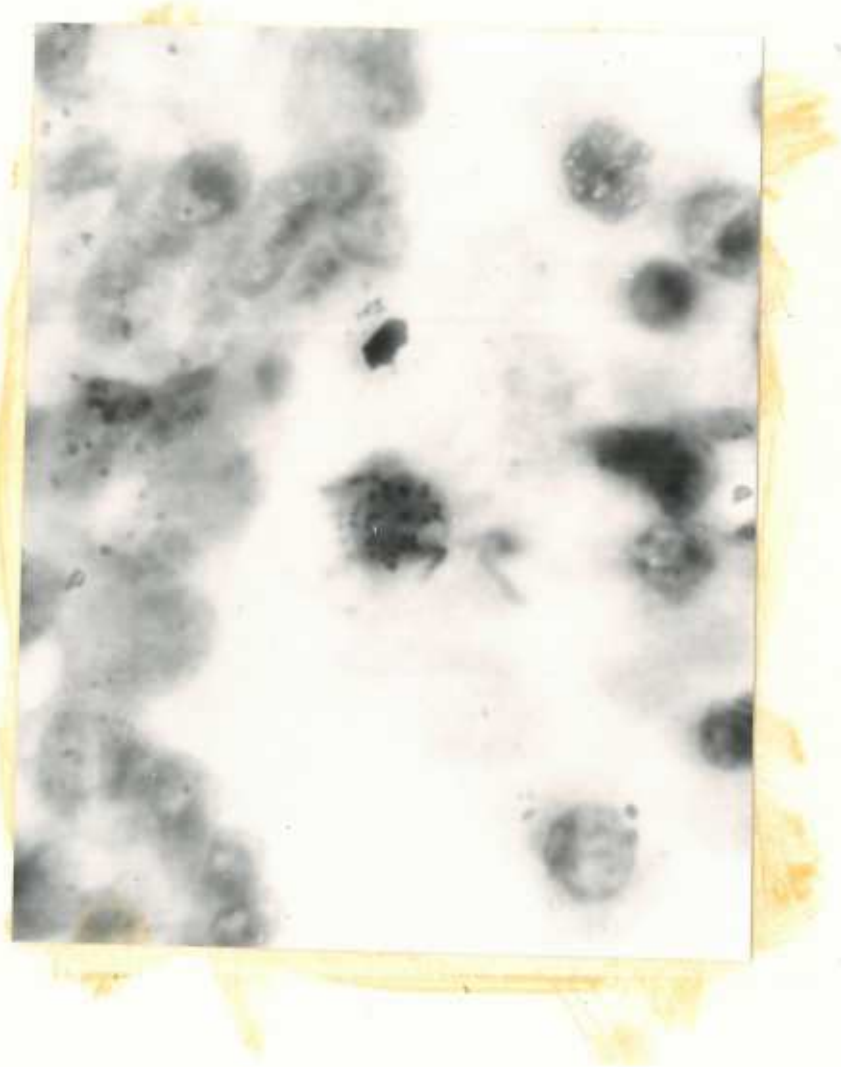

Illustration 53

Radioautograph with tagged blood cell in coelomic cavity of embryo 8-9-J. Oil-immersion. (4-A-2, by right gonad).

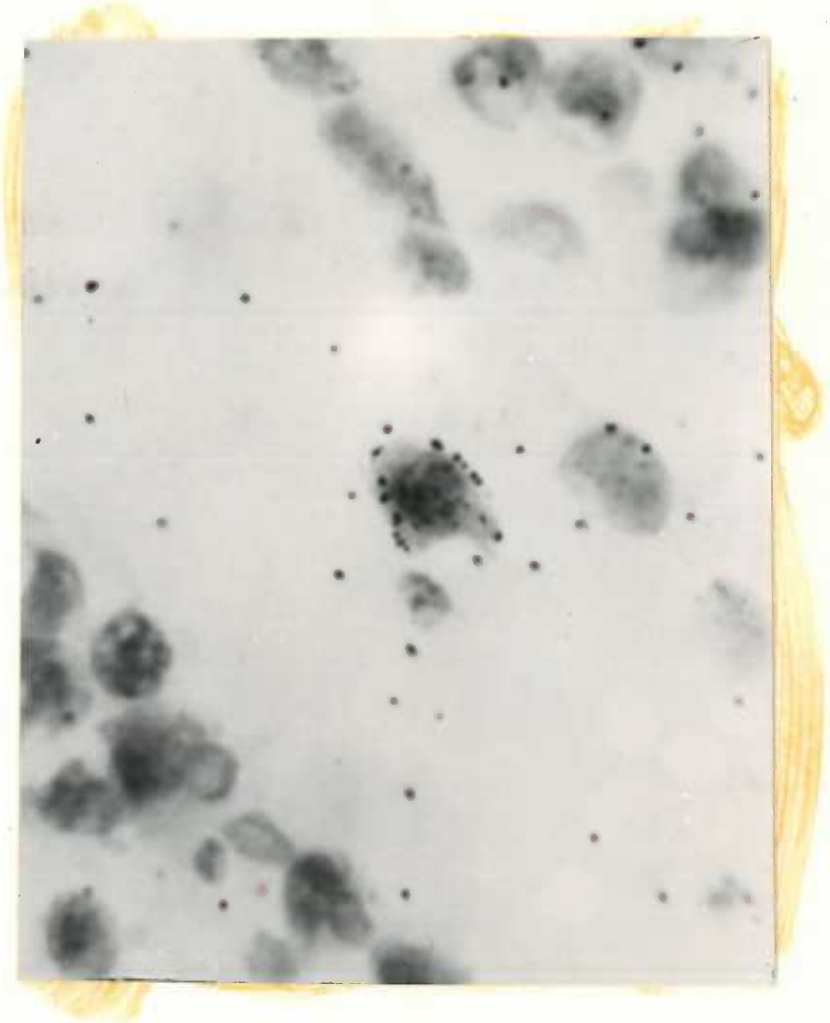

Illustration 54

Radioautograph with possible tagged aorta endothelial cell of embryo 8-9-J. Oil-immersion. (2-A-7).

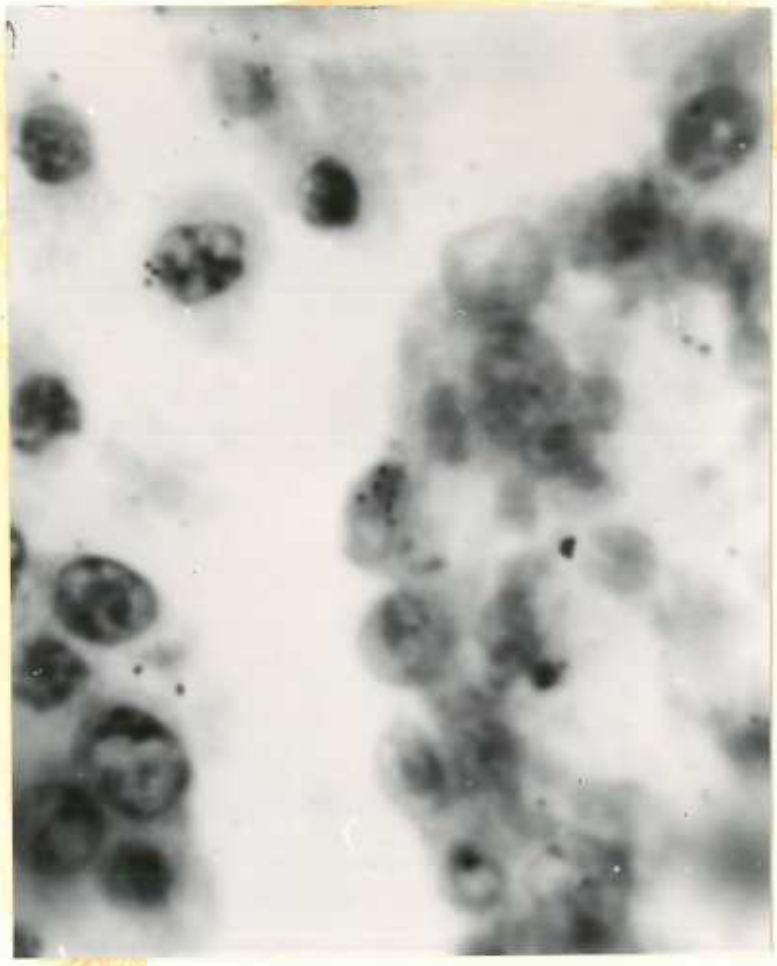

Illustration 55

Radioautograph with tagged ventral plate of spinal cord of embryo 8-9-J. Oil-immersion. (5-B-6).

Illustration 56

Radioautograph with tagged notochord cells of embryo 8-9-J. Oil-immersion. (2-B-12).

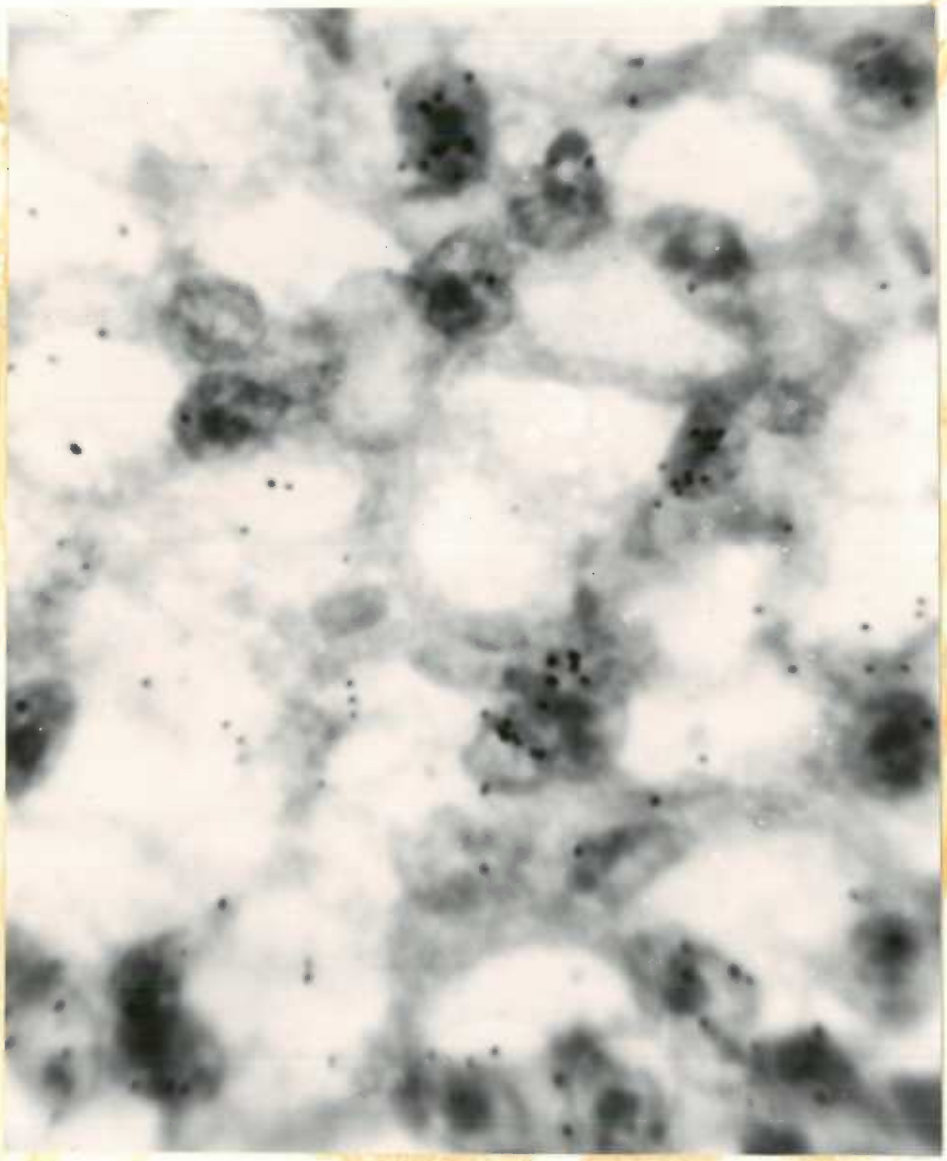

Illustration 57

Radioautograph with tagged notochord cells of embryo 8-9-J. Oil-immersion. (4-B-4).

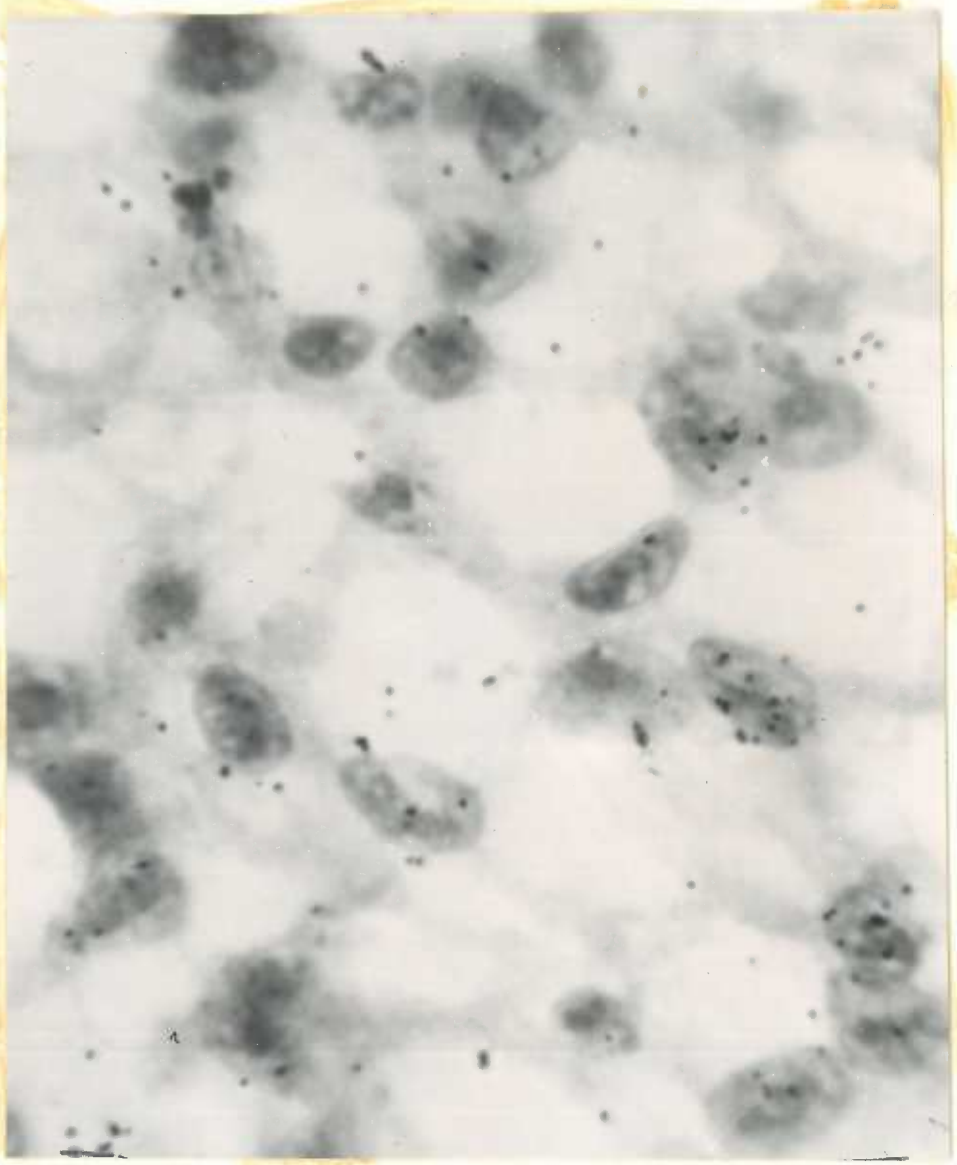

Illustration 58

Section of embryo 8-9-J showing, together with illustration 59, the opening of the avulsed gut into the yolk sac. Oil-immersion. (5-A-7).

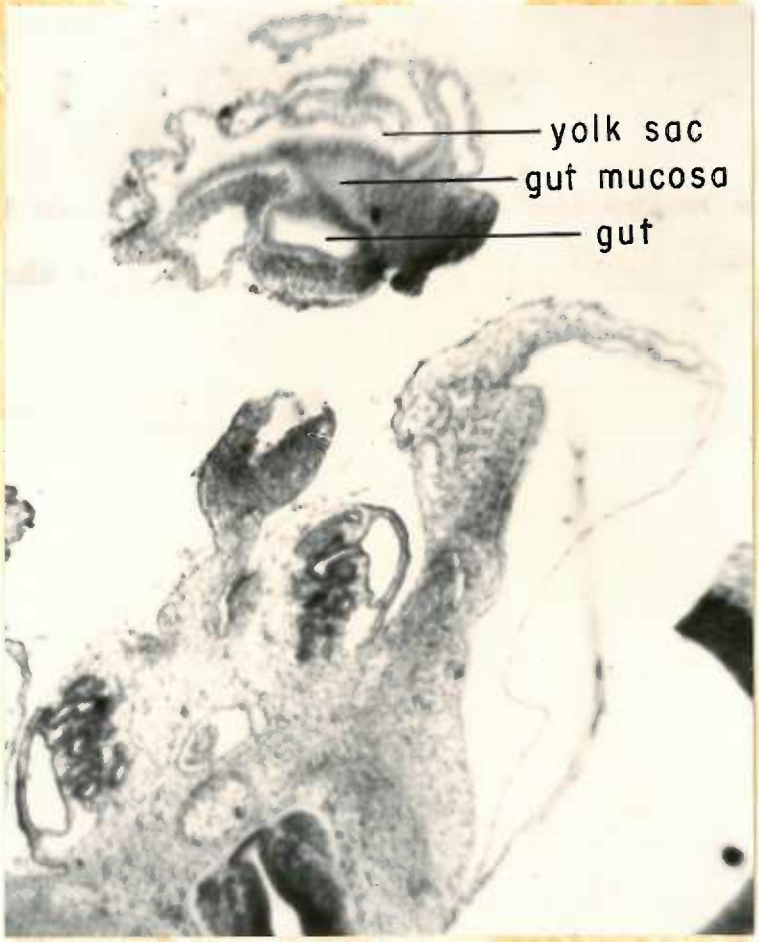
yolk sac
gut mucosa
gut

Illustration 59

Section of embryo 8-9-J showing, together with illustration 58, the avulsed gut opened into the yolk sac. Oil-immersion. (4-C-2).

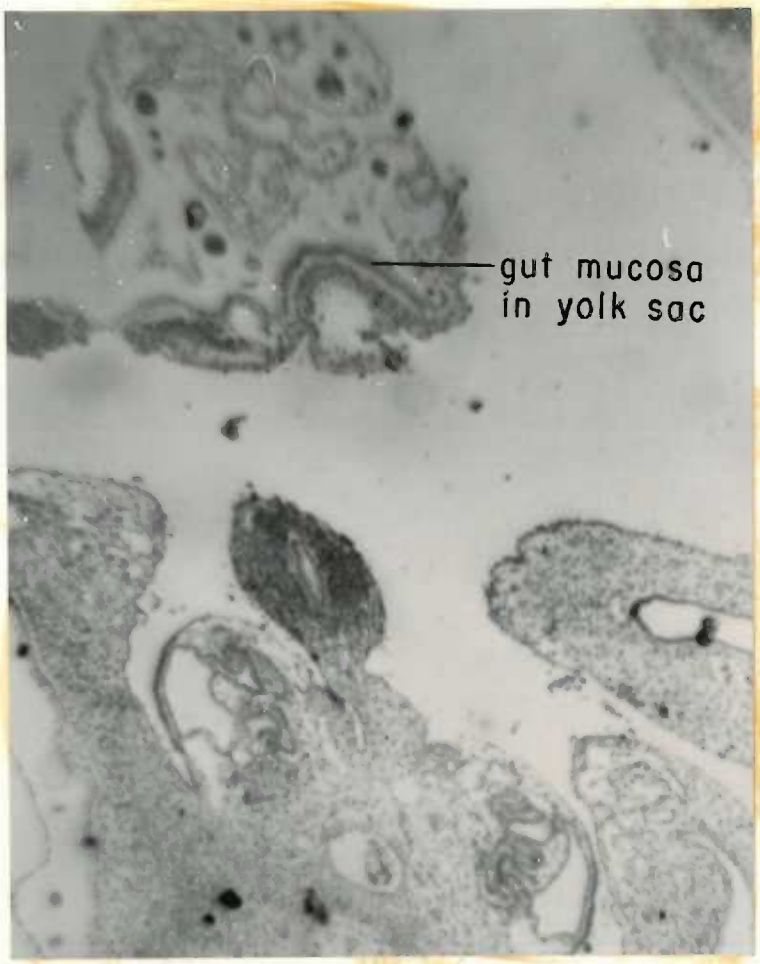
gut mucosa
in yolk sac

Illustration 60

Radioautograph with tagged yolk sac cells of embryo 8-9-J. Oil-immersion. (2-B-6).

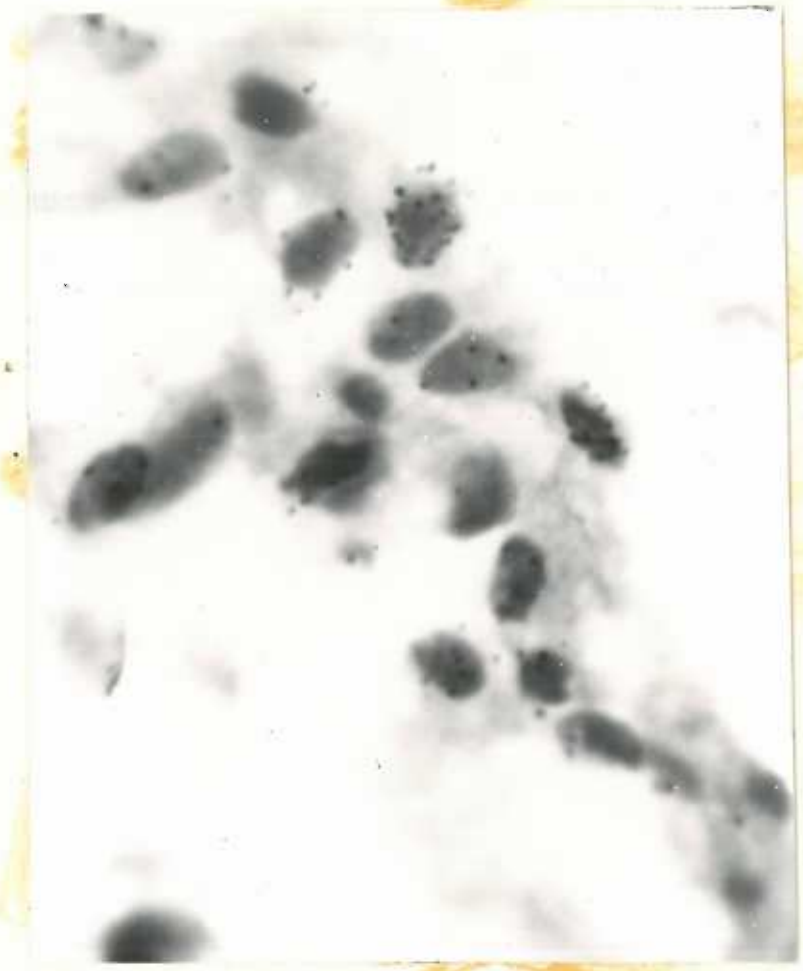

Illustration 61

Radioautograph with tagged gut mucosa of embryo 8-9-J. Oil-immersion. (1-A-8).

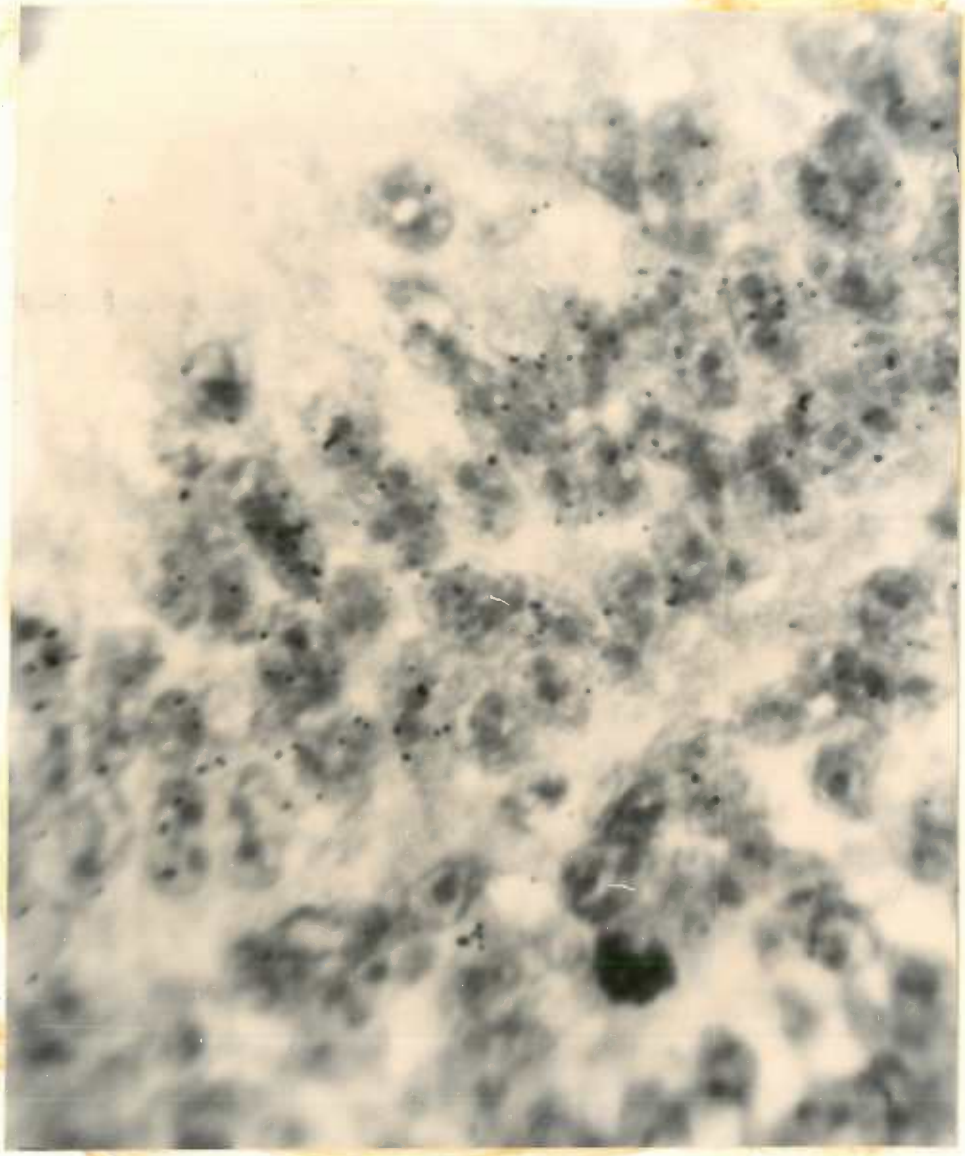

Illustration 62

Radioautograph with tagged gut mucosa of embryo 8-9-J. Oil-immersion. (2-B-10).

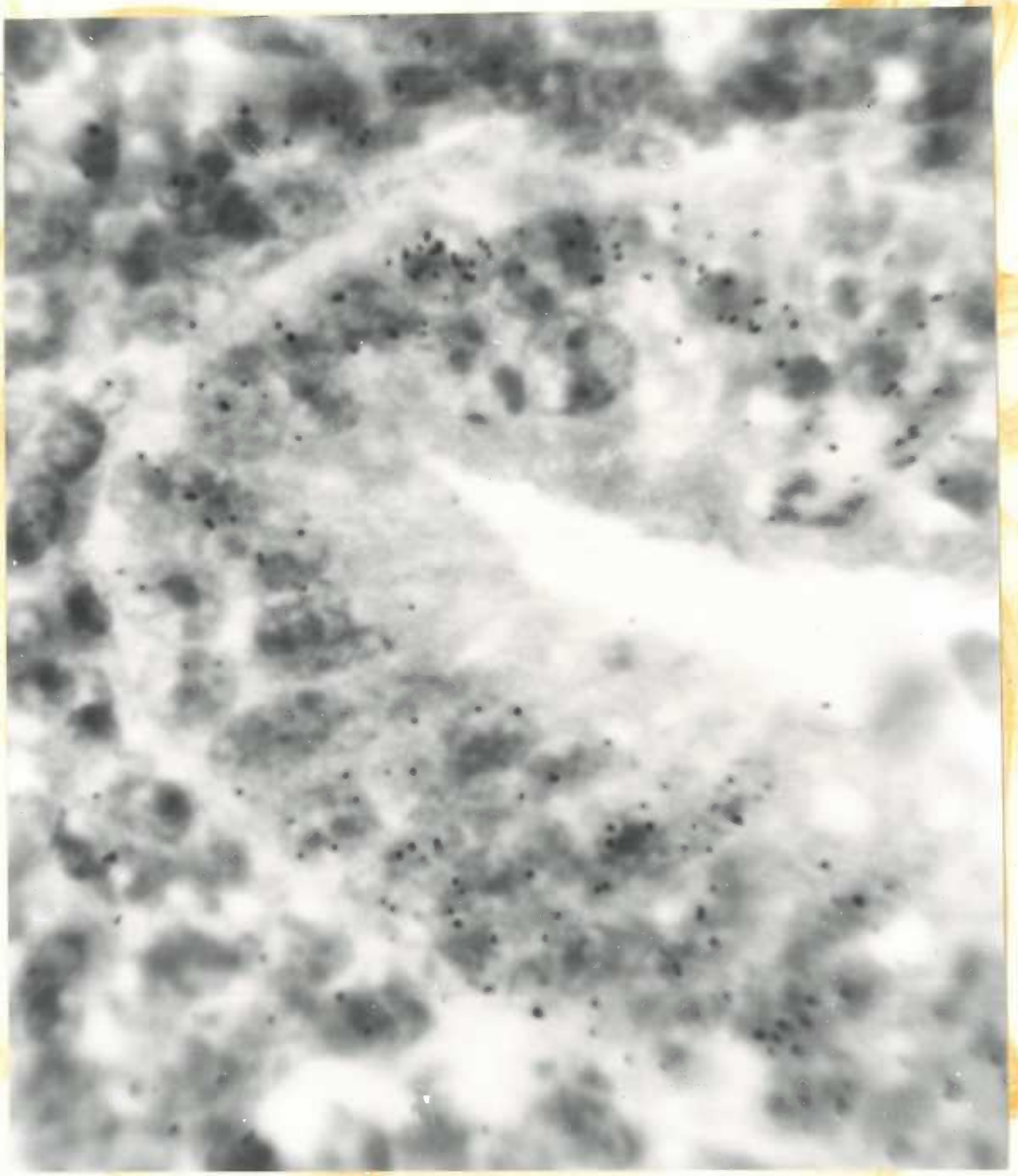

Illustration 63

Radioautograph with tagged gut mucosa of embryo 8-9-J.
Oil-immersion. (4-B-8).

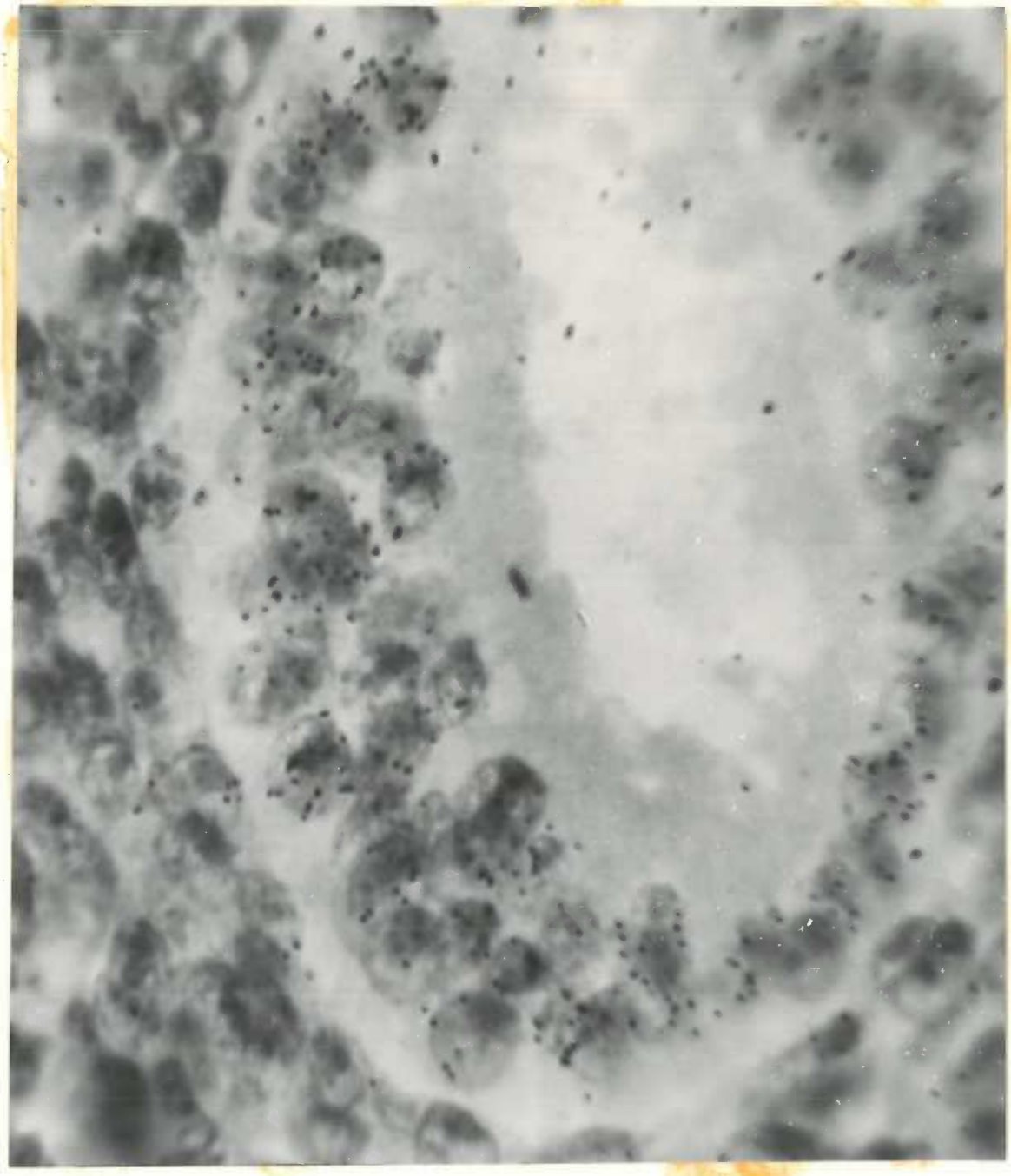

Illustration 64

Radioautograph with tagged gut mucosa of embryo 8-9-J. Oil-immersion. (5-B-7).

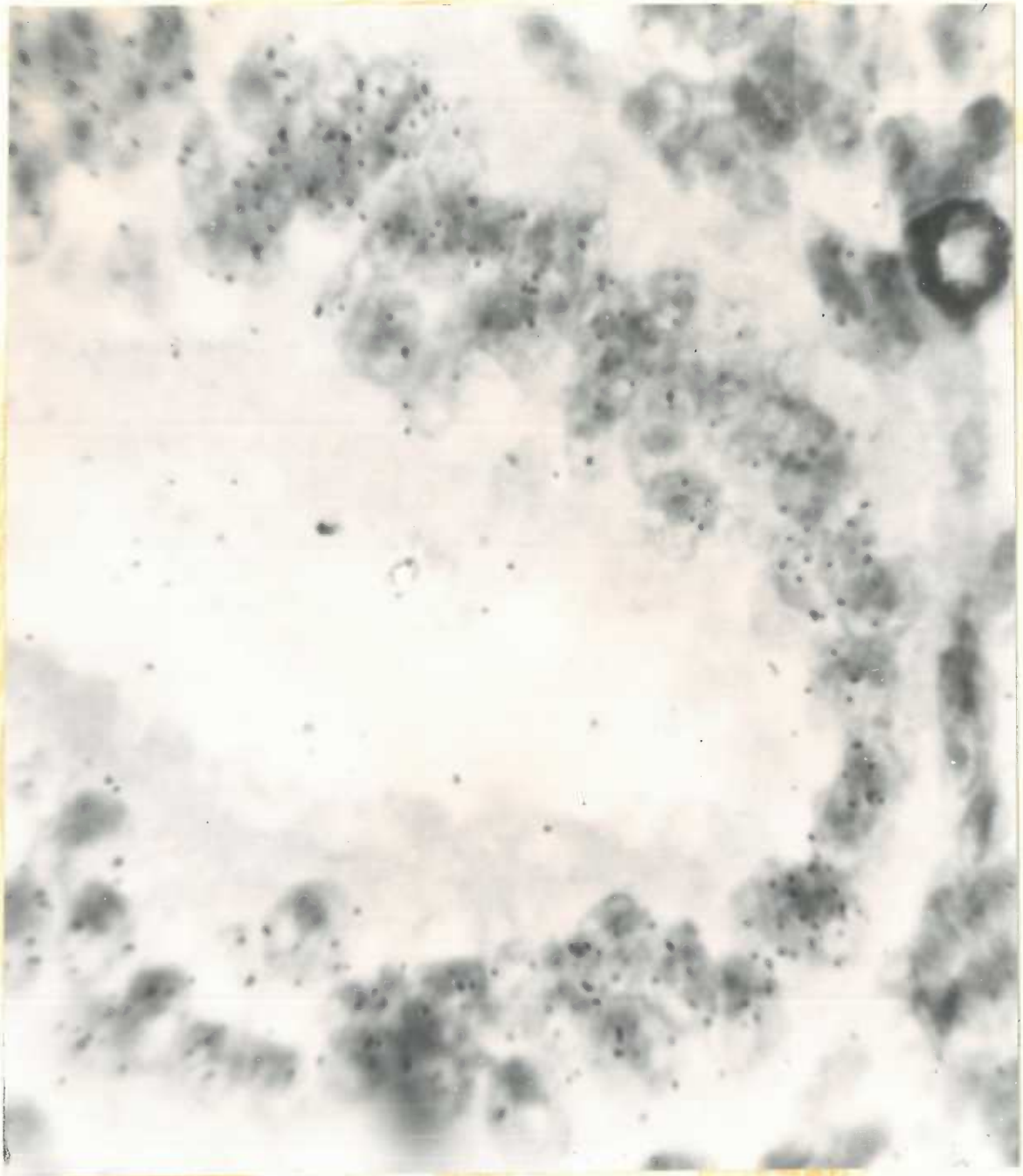

Illustration 65

Radioautograph with tangential plane of tagged gut mucosa and non-tagged stroma of embryo 8-9-J. Oil-immersion. (5-A-2).

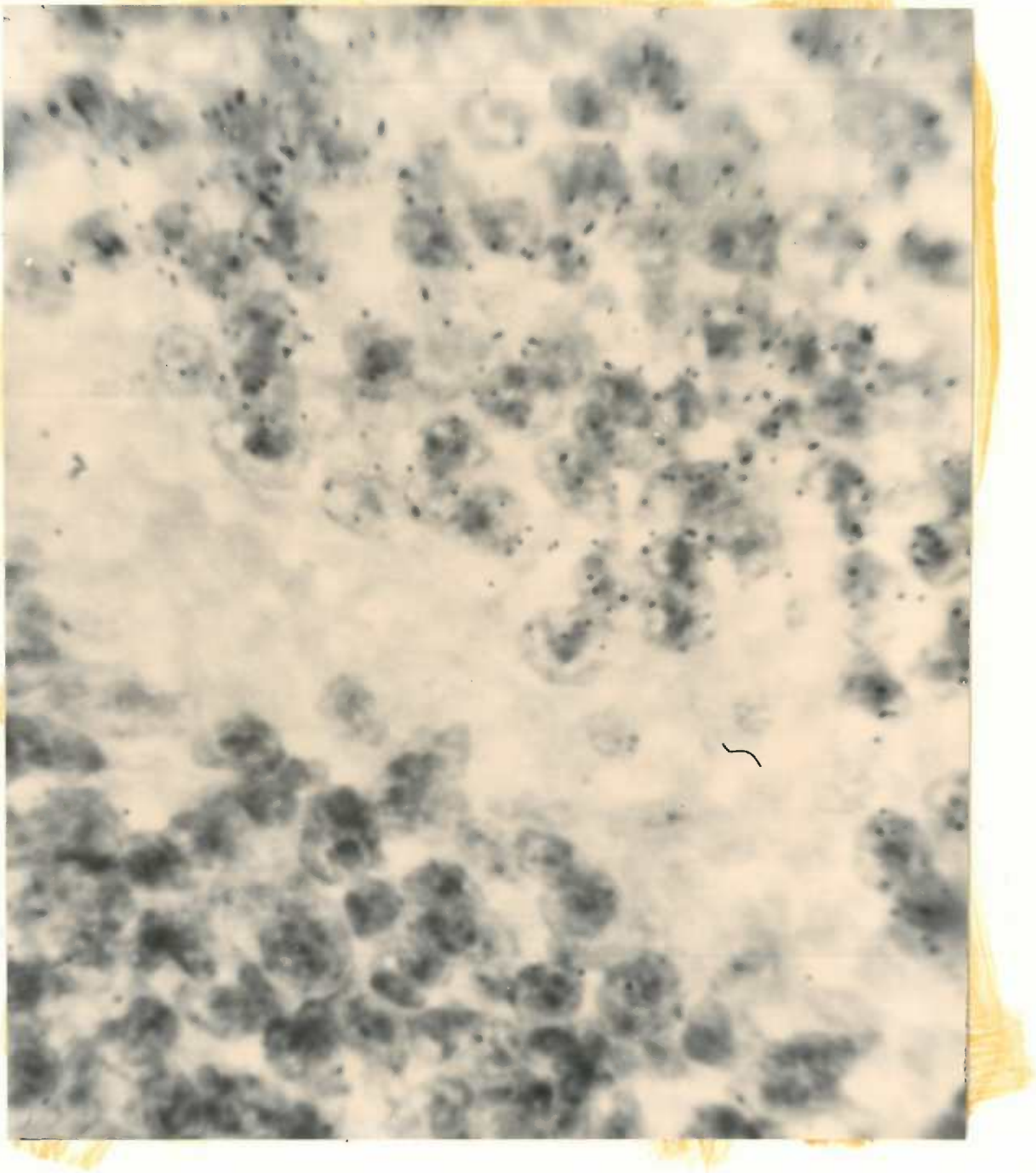

Illustration 66

Radioautograph with tagged gut mucosa of embryo 8-9-J. Oil-immersion. (6-A-3).

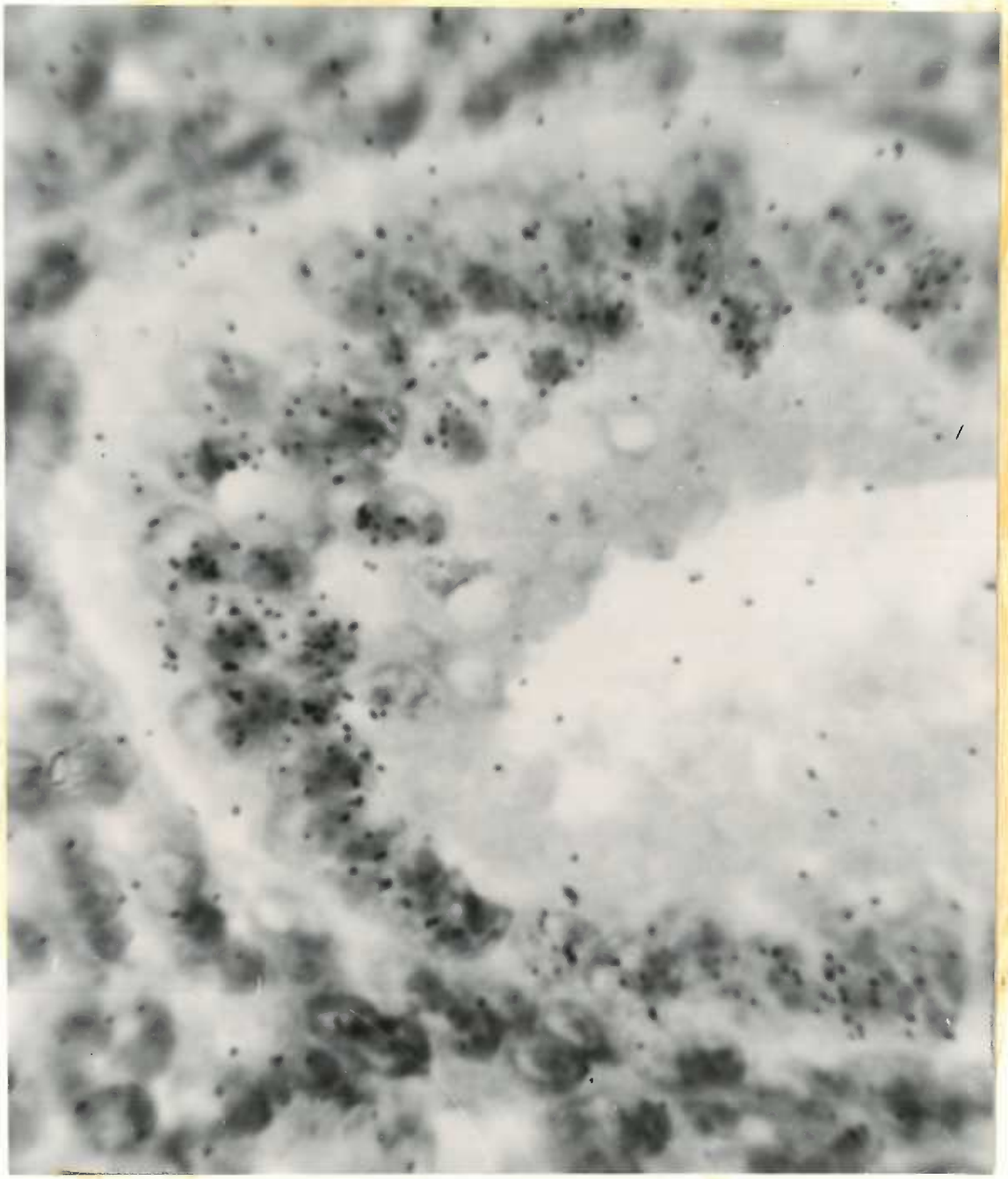

Illustration 67

Radioautograph with possible tagged vascular endothelial cell of embryo 8-9-L. Oil-immersion. (2-C-1, right gonad).

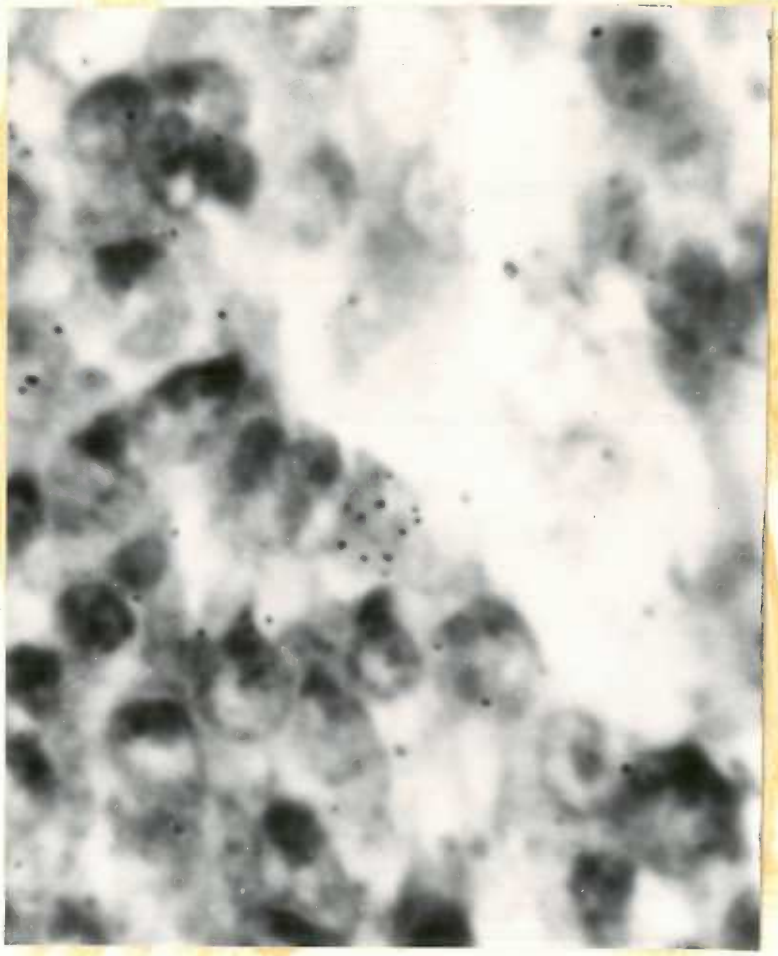

Illustration 68

Radioautograph with probable artifactual tagging over cell (cells?) in coelomic epithelium of embryo 9-6-K. Oil-immersion. (4-C-10, left coelomic epithelium).

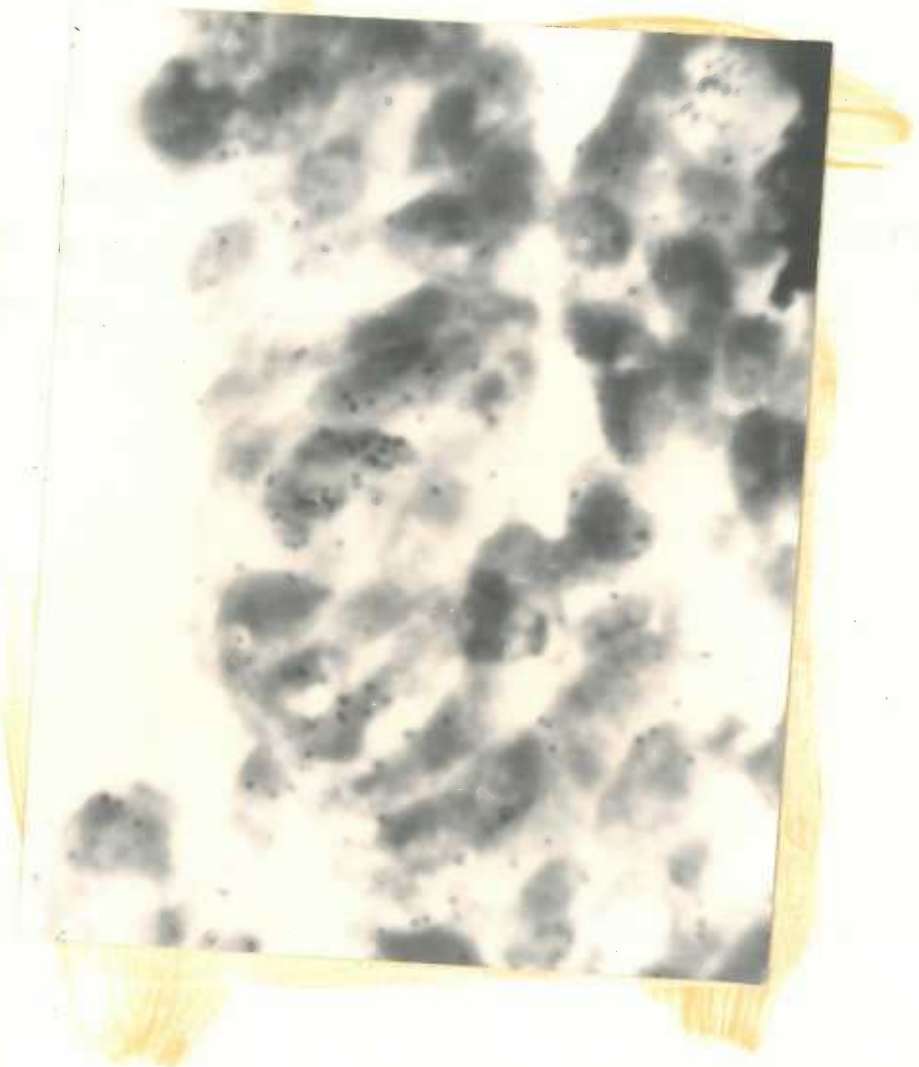

Illustration 69

Radioautograph with star track. Oil-immersion. (8-9-H, 2-C-4, over mesentary by aorta).

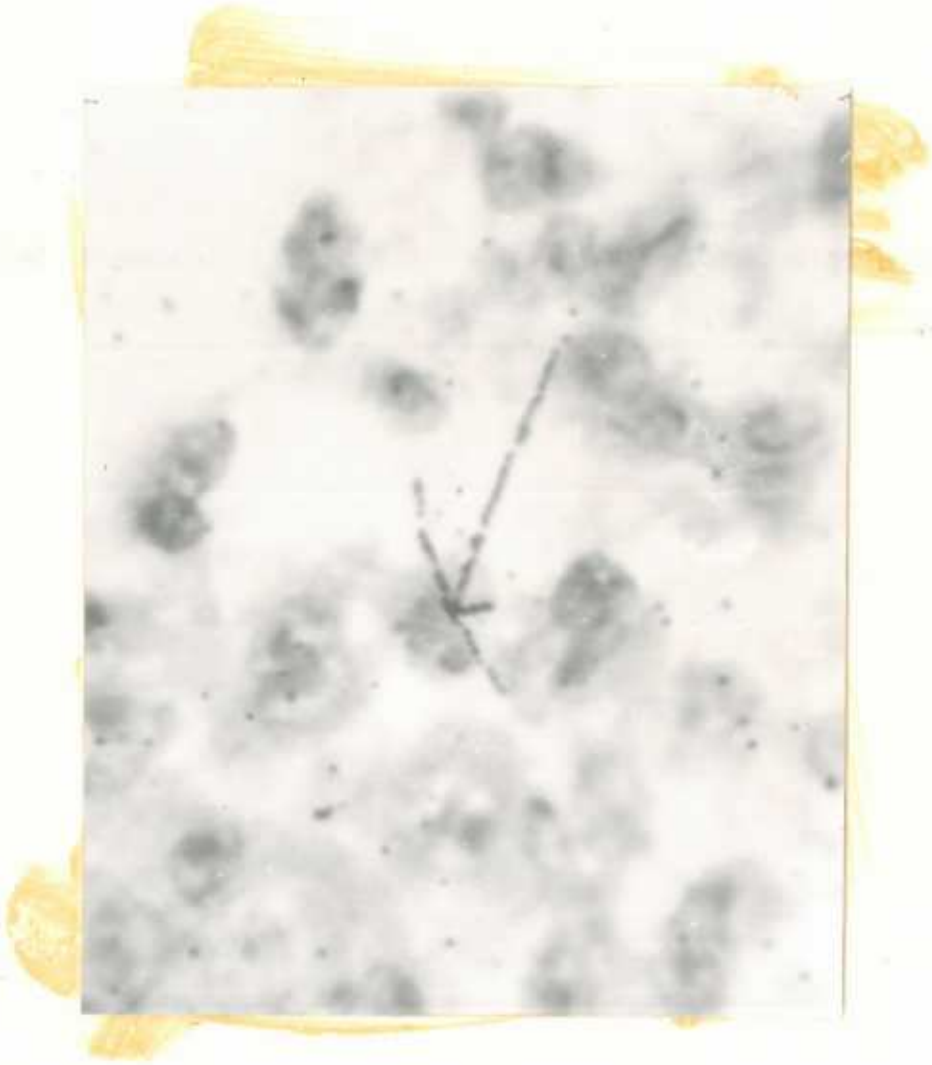

Illustration 70

Mitosing primordial germ-cell (?) in germinal epithelium of embryo 8-9-I. Oil-immersion. (1-A-5, left germinal epithelium).

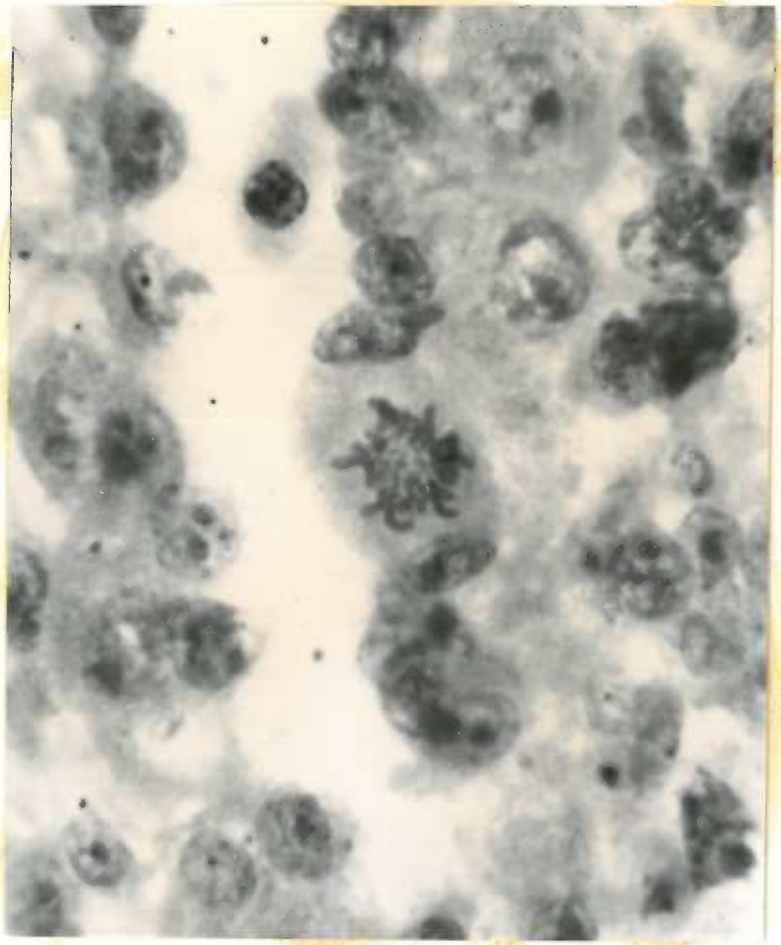

Illustration 71

Four mitosing cells including a primordial germ-cell (?) in gonadal ridge of embryo 6-6-P. High-power. (14-12, right gonad).

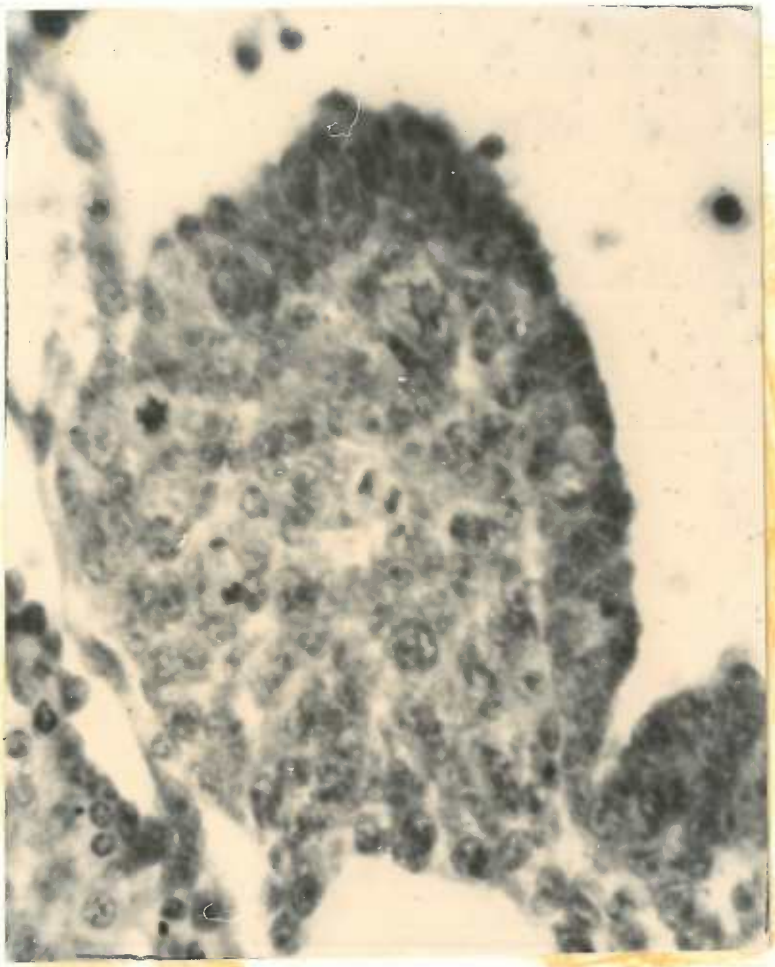

Illustration 72

Mitosing primordial germ-cell in gonadal ridge of embryo 8-9-F. Oil-immersion. (1-A-15, right gonad).

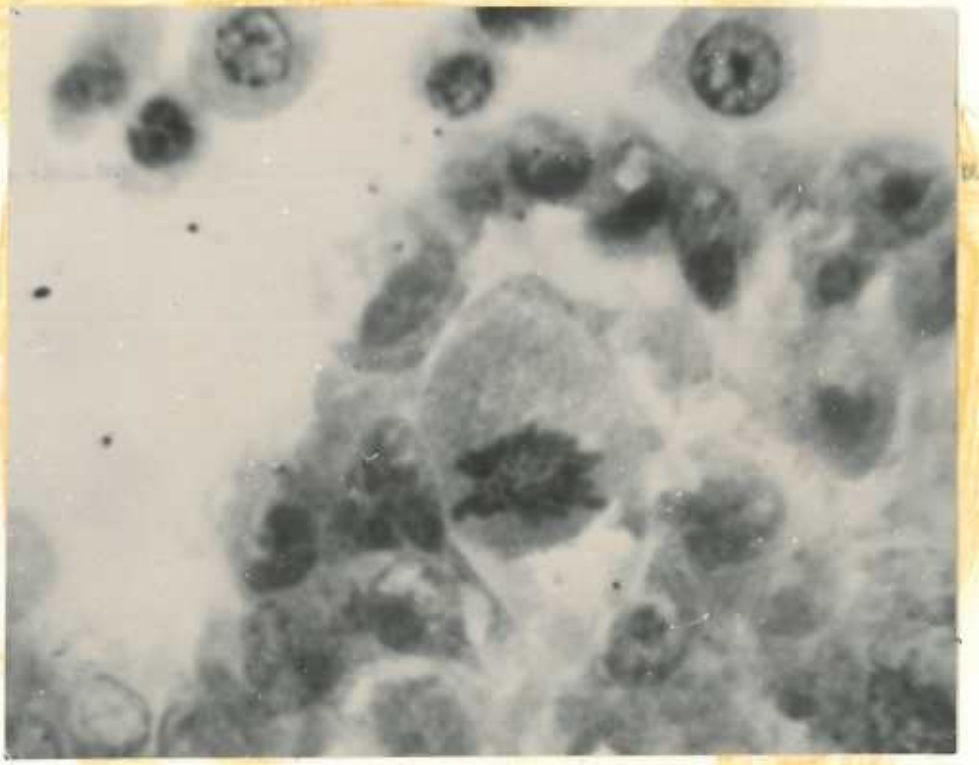

TABLE 1

COMPILED DATA FROM ALL EXPERIMENTS

Group	A				B				C				Totals
Period	6-23-60 to 9-12-60				11-2-60 to 11-24-60				6-6-61 to 9-6-61				
Relation to area pellucida	above		below	?	above		below	?	above		below	?	
Survive less than 4 days	29		10	6	2		0	6	25		5	4	87
		45				8				34			
Survive greater than 3 days	36		6	4	2		0	18	30		21	3	120
		46				20				54			
Totals	65		16	10	4		0	24	55		26	7	
			91				28				88		207

TABLE 2

EFFECT OF PERFORATION OF THE AREA PELLUCIDA ON GROUP A

Chi-square test (non-paremetric).

	survive less than 4 days	survive greater than 3 days	
above area pellucida	29	36	65
below area pellucida	10	6	16
	39	42	81

$$\text{chi-square} = \frac{81\,(29\times6 - 10\times36)^2}{65 \times 16 \times 39 \times 42} = 0.755$$

Chi-square is not significant, lying between 60 and 70%.

TABLE 3

EFFECT OF PERFORATION OF THE AREA PELLUCIDA AND TRITIATED THYMIDINE ON ALL GROUPS

Chi-square test (non-paremetric).

	survive less than 4 days	survive greater than 3 days	
above area pellucida	56	68	124
below area pellucida	15	27	42
	71	95	166

$$\text{chi-square} = \frac{166\,(56\times27 - 15\times68)^2}{124 \times 42 \times 71 \times 95} = 1.040$$

Chi-square is not significant, lying between 60 and 70%.

TABLE 4

EFFECT OF EXPERIENCE ON GROUPS A AND B

Chi-square test (non-paremetric).

	survive less than 4 days	survive greater than 3 days	
Group A	45	46	91
Group B	8	20	28
	53	66	119

$$\text{chi-square} = \frac{119\,(45\times20 - 8\times46)^2}{91 \times 28 \times 53 \times 66} = 3.790$$

Chi-square is not significant, lying between 90 and 95%.

TABLE 5

EFFECT OF EXPERIENCE AND TRITIATED THYMIDINE

ON GROUPS A AND C

Chi-square test (non-paremetric).

	survive less than 4 days	survive greater than 3 days	
Group A	45	46	91
Group C	34	54	88
	79	100	179

$$\text{chi-square} = \frac{179\,(45\times54 - 34\times46)^2}{91 \times 88 \times 79 \times 100} = 2.14$$

Chi-square is not significant, lying between 80 and 90%.

TABLE 6

DATA ON SIGNIFICANTLY TAGGED EMBRYOS

Embryo number	Transplant age: Host 0-3 somites	Transplant age: Host 4-20 somites	Transplant age: Donor 0-3 somites	Transplant age: Donor 4-20 somites	Transplant placement: above area pellucida	Transplant placement: below area pellucida	Transplant placement: unknown	age at fixing	days of exposure	Significant tagging: primordial germ-cells	single cells	gut mucosa	yolk sac	spinal cord	notochord	illustration numbers
6-28-E		x		x		x		6⅓	22		xx?					14-16
7-26-J		x		x			x	5⅔	47	?						17
8-9-A	x		x			x		5⅔	94		?	x				18-20
8-9-D	x		x		x			5⅔	94	x	?	x				21-26
8-9-G	x		x			x		5⅔	94	xx		x				27-34
8-9-H	x		x		x			5⅔	94		xx	x	x	x	x	35-51
8-9-J	x		x			x		5⅔	94		xxx	x	x	x	x	52-56
8-9-L		x		x	x			5⅔	94		?					67
9-6-K		x	x		x			3⅔	112		?					68
9	5	4	6	3	4	4	1	Totals for significantly tagged embryos								
17	3	14	7	10	10	4	3	Totals for negative embryos								

TABLE 7

EFFECT OF EXPERIENCE AND TRITIATED THYMIDINE

UPON PERFORATION OF THE AREA PELLUCIDA

Chi-square test (non-paremetric).

	survive less than 4 days	survive greater than 3 days	
Group A	10	6	16
Group C	5	21	26
	15	27	42

$$\text{chi-square} = \frac{42\,(10\times21 - 5\times6)^2}{16 \times 26 \times 15 \times 27} = 8.080$$

Chi-square is significant, lying between 99.5 and 99.9%.

TABLE 8

EFFECT OF EXPERIENCE AND TRITIATED THYMIDINE

WITHOUT PERFORATION OF THE AREA PELLUCIDA

Chi-square test (non-paremetric).

	survive less than 4 days	survive greater than 3 days	
Group A	29	36	65
Group B	25	30	55
	54	66	120

$$\text{chi-square} = \frac{120\,(29\times30 - 25\times36)^2}{65 \times 55 \times 54 \times 66} = 0.010$$

Chi-square is not significant, lying between 5 and 10%.

TABLE 9

EFFECT OF EXPERIENCE UPON PERFORATION

OF THE AREA PELLUCIDA

F-test for $\frac{\text{chi-square of Table 7}}{\text{chi-square of Table 8}} = \frac{8.080}{0.010} = 808$

degrees of freedom = $\frac{42}{120}$

F-test value equivalent to 2.21 is significant, greater than 99.95%.

APPENDIX

This is a laboratory guide for the autoradiographic stripping technique developed for this experiment. Together with the suggestions contained in the text it should prove a generally satisfactory technique comparable to, if not superior to, other stripping techniques as almost precise resolution can be obtained.

I - Stripping

Kodak autoradiographic stripping plates AR-10 are used. These have a sensitive emulsion layer 5 microns thick on a 10 micron gelatin backing mounted on a 5 x 7 inch glass plate with the sensitive surface up.

1) Float strips of film, sensitive surface down, on clean water at 28° to 30° C. These strips are cut from the plate in sizes approximately equal to the slides to be covered.
2) After the film has been maximally expanded by hydration (about 2 minutes) slip the slide with the specimen up under the film and lift it from the water, covering the entire slide except the clear end, and allowing the film to drape around the other three edges.
3) Allow the slide to stand and drain dry in a forced draft at room temperature.

4) Place the dry slide in a light tight slide box containing a sack or compartment of desecated $CaSO_4$.

5) Store in a dark container in a cool location for the required exposure time, in this instance 15 to 112 days.

II - Developing

Kodak D-19 developer and Kodak Acid Fixer are used with satisfactory results, while a weak (less than 1%) acetic acid stop bath is used. It is most satisfactory to use a slide carrier and standard staining dishes for development and staining of large numbers of slides. All solutions for developing are at 18 degrees Centigrade.

6) Place the dry exposed slides in a slide carrier and develop for 8 minutes.

7) Rinse in stop bath.

8) Fix for 8 to 10 minutes.

9) Wash in cold running water for 5 minutes, go directly to staining.

III - Staining

Harris hematoxilin was prepared by the following method, from Gurr, "Methods of Analytical Histology and Histo-Chemistry", page 307:

Haemotoxylin 10% in absolute alcohol 5 ml

Mercuric oxide	0.25 gm
Potash alum 10% aqueous	100 ml
Glacial acetic acid	4 ml

"Mix the haemotoxylin and alum solution; heat to boiling point; then add the mercuric oxide and when the solution turns deep purple turn off the heat; then cool and add the acetic acid." (30)

10) Place the wet slides in the Harris' Haemotoxylin for 10 minutes (time must be titrated for most effective stain).

11) Rinse in 50% ethanol.

12) Rinse in running cold water until dye no longer colors water.

13) Carefully wipe soft excess film from backs of slides.

14) Allow to stand-dry.

15) Cover slips are not necessary but may be added with a suitable media, either after drying or after dehydrating to xylene.

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