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Interspecific cell markers and cell lineage in birds

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[Plates 1–6]

A cell marking technique based on the structural differences existing between the interphase nucleus in two closely related species of birds, the chick and the Japanese quail, is described. In all embryonic and adult cell types of the quail, a large mass of heterochromatin is associated with the nucleolus making quail and chick cells easy to identify at the single cell level after application of any DNA-specific staining procedure and also at the electron microscope level. This method has been largely used to construct chimeras *in ovo* and to study dynamic processes such as cell migrations or cell lineage segregation during ontogeny. Recently monoclonal antibodies specific for either quail or chick antigenic determinants (for example, class II MHC antigens) have been prepared, increasing the interest of the quail–chick chimera system as an experimental model.

Introduction

The avian embryo has for long been the choice model system for studying problems related to development in higher vertebrates. Unlike the mammalian embryo and foetus, which, until recently, were totally inaccessible to experimentation, the developing bird is available in the egg from the blastula stage onward. Precise techniques of microsurgery involving extirpation and grafting of embryonic territories have been devised and, in combination with *in vitro* explantation of cells, tissues and organs, have allowed the discovery and documentation of a number of morphogenetic processes. Limb, kidney, skin, liver and skeleton morphogenesis (see, for reviews, Strudel 1953, 1955; Le Douarin 1964; Sengel 1976; Saunders 1977; Wessels 1977; Gumpel-Pinot 1984) are developmental systems to our knowledge of which avian embryology has contributed significantly.

The techniques mentioned above, however, are practically of no use in the study of cellular migrations or for cell lineage analysis in ontogeny. Such problems call for cell marking techniques that must be precise, stable and easy to use. Radioisotopic labelling of the nucleus by tritiated thymidine, introduced by Weston (1963) to follow neural crest cell migration, fulfilled some of these requirements and yielded interesting results. However, its use is limited by the dilution resulting from active proliferation of embryonic cells.

The quail–chick marker system devised in 1969 (Le Douarin 1969) represents a significant improvement compared with the labelling techniques previously applied for similar purposes. In the present article, after a brief reminder of the principle of the method, novel approaches using monoclonal antibody technology in conjunction with the construction of quail–chick chimeric tissues and embryos will be reported. Finally, examples of cell lineage analyses based on the quail–chick system will be described.

1. The quail–chick marker system

1.1. *The nuclear marker*

Avian chimeras were developed following the original observation that the structure of the interphase nucleus differs strikingly in two closely related species of birds, the chick (*Gallus gallus*) and the Japanese quail (*Coturnix coturnix japonica*) (Le Douarin 1969, 1971*a*,*b*, 1973*a*,*b*). The difference resides in the distribution of heterochromatin, which, in virtually all types of embryonic and adult cells of the quail, is concentrated in one (or a few) large mass (or masses) closely associated with the nucleolus. In chick cell nuclei, as in most other animal species, the heterochromatin is evenly distributed throughout the nucleoplasm in small chromocentres. Quail and chick cells associated in organ culture or grafted into an embryo *in ovo* can be recognized, irrespective of the duration of the coculture or of the graft, through various staining procedures. The most widely used is the Feulgen–Rossenbeck technique for DNA staining, which affords easy distinction of all types of quail and chick cells, even when a single quail cell is isolated within a chick tissue (figure 1, plate 1). Although some variations occur in the nucleolar size and in the respective distributions of RNA and DNA in the nucleolus of different cellular categories in quail and chick (see Le Douarin 1973*a*), the Feulgen–Rossenbeck reaction allows, in all instances, homologous cell types of the two species to be distinguished.

Electron microscopy also reveals the differences between quail and chick nucleoli and has the advantage of disclosing cell-type-specific cytoplasmic features: relevant information in studies on cellular differentiation (figure 2*a*,*b*). A technique described by Bernhard (1968), using EDTA treatment associated with uranyl acetate staining, shows even more clearly the unique feature of quail cells, that is, heterochromatin condensation in the nucleolus. With this technique, RNA stains preferentially, while DNA and most of the proteins remain uncontrasted. The nucleolar heterochromatin of the quail then appears as a large unstained area in the nucleus, juxtaposing the nucleoli proper (figure 3, plate 2).

Other cytological methods currently used to stain quail and chick nuclei differently are based on the affinity of certain fluorescent dyes for DNA molecules. Staining with acridine-orange (Fontaine-Pérus *et al.* 1985*a*) or with the Hoechst reagent (bisbenzamide 33258, Calbiochem) according to a procedure described by Franklin & Martin (1980) gives an excellent contrast in ultraviolet light microscopy between cells of the two species and allows immunocytochemical techniques to be used on the same preparations (figures 4 and 5, plate 3).

Among the cytological techniques used to follow the differentiation of quail or chick cells in chimeric tissues, formaldehyde-induced fluorescence, for the detection of biogenic amines (Falck 1962), has been particularly useful. This method, described in detail elsewhere (see Le

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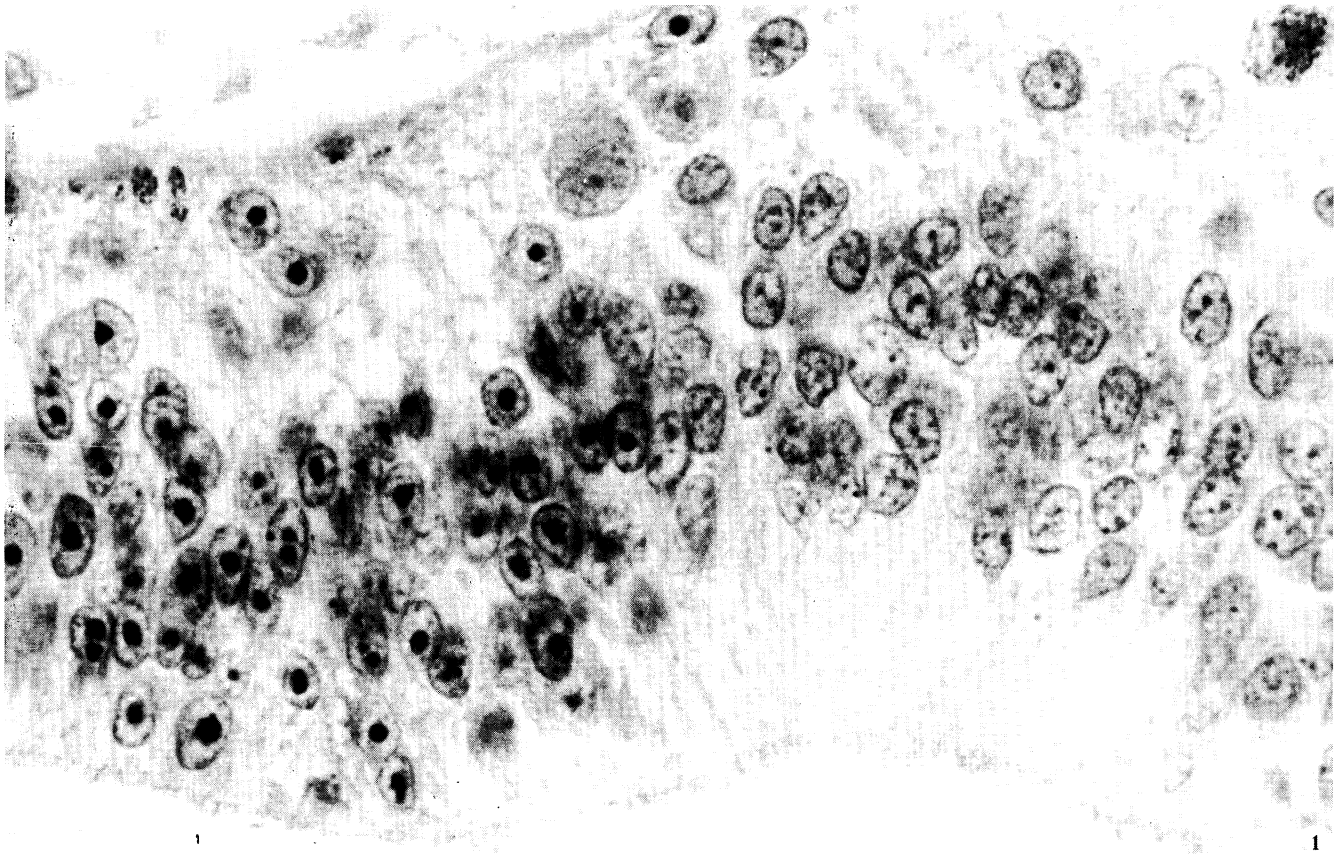
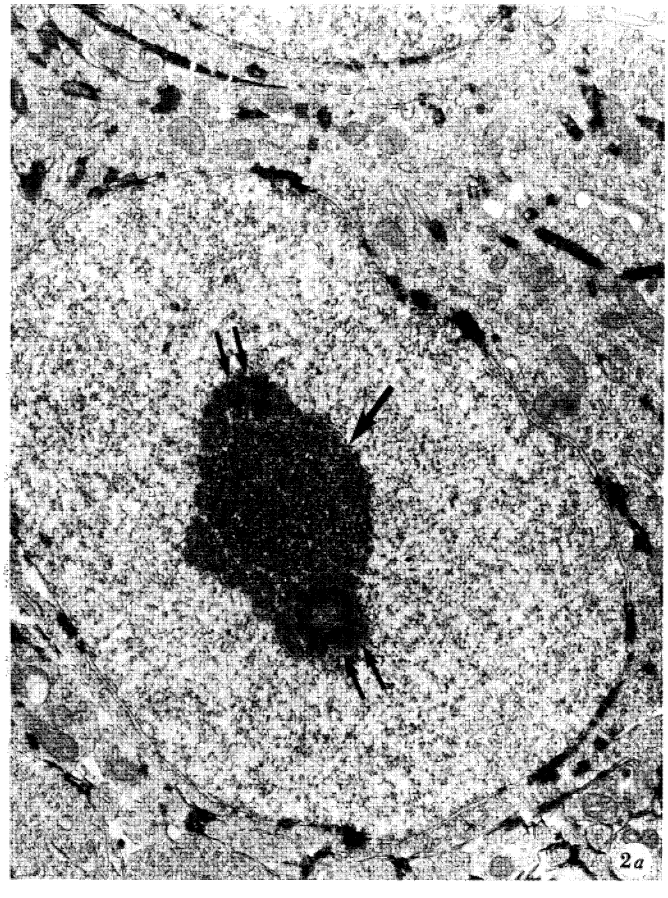
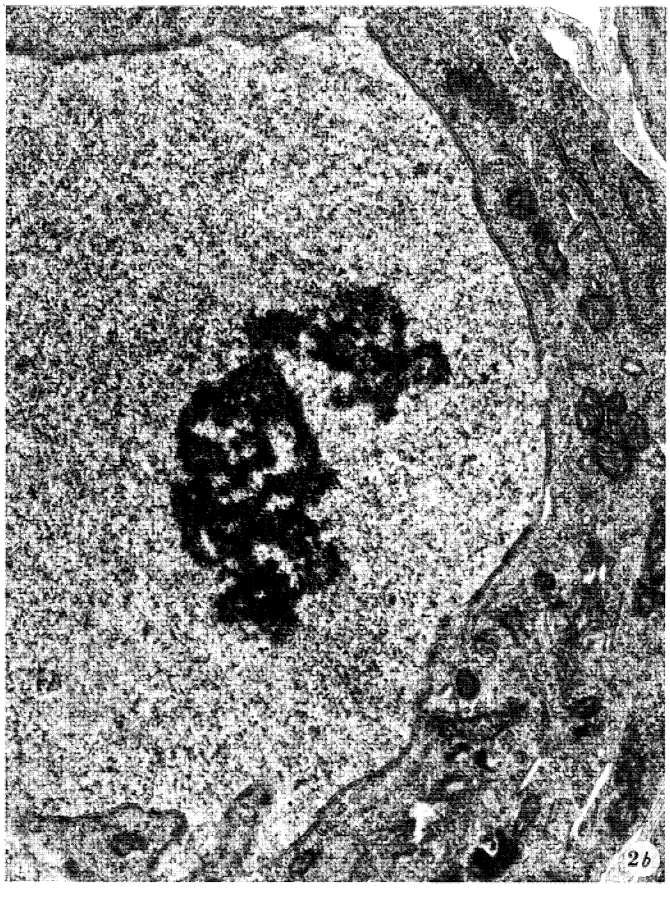
Description of plate 1

Figure 1. Neuroblasts in the mesencephalic neural epithelium of quail (left) and chick (right) embryo at E6 stained with the Feulgen–Rossenbeck's reaction. Note the large mass of heterochromatin of the quail nuclei. The chimaeric neural epithelium results from the isotopic graft of half the mesencephalon of a quail into a chick embryo at 10-somite stage. (Magn. ×1815.)

Figure 2. Comparison of the nucleolar structure in neurons of the peripheral nervous system in quail and chick cells at the electron microscope level. (Magn. ×1700.) (*a*) Quail enteric ganglion neuron in the myenteric plexus in the gizzard of a 10-day embryo. Large mass of heterochromatin (arrow) associated with the nucleoli (double arrows). In the cytoplasm acetylcholinesterase activity is revealed according to Tsuji (1974). (*b*) Chick ciliary ganglion neuron at E11. Note the reticular structure of the nucleolus in which no heterochromatin is present.







FIGURES 1 AND 2. For description see opposite.

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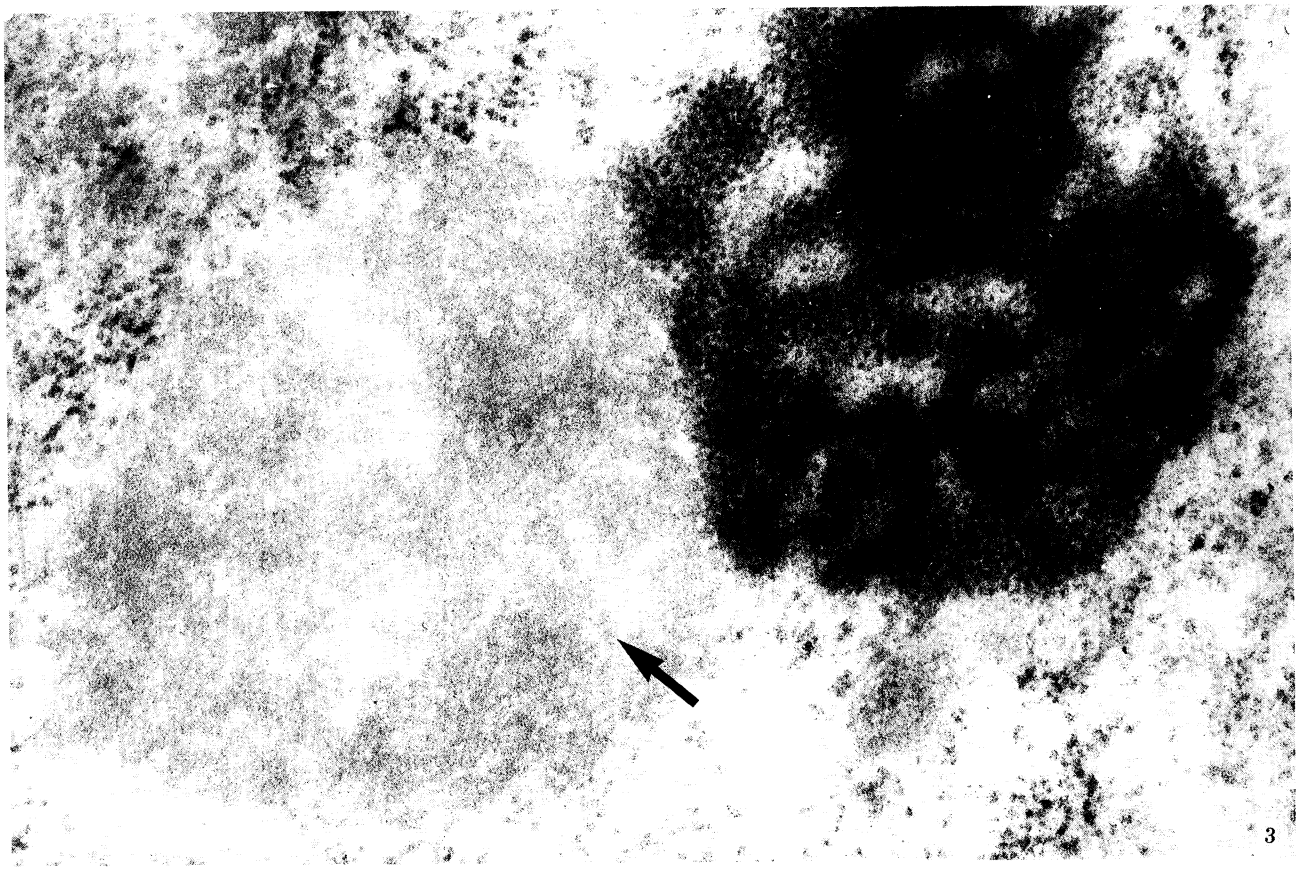
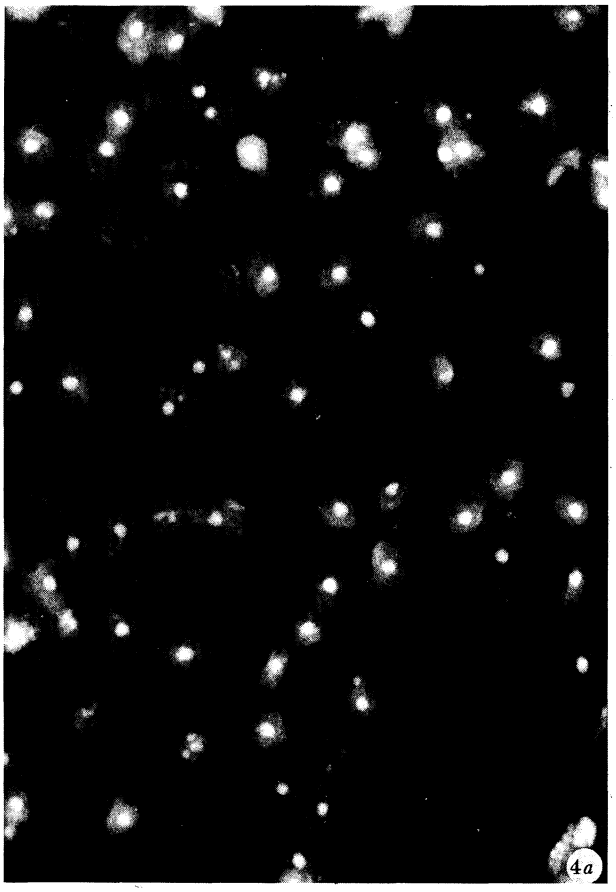


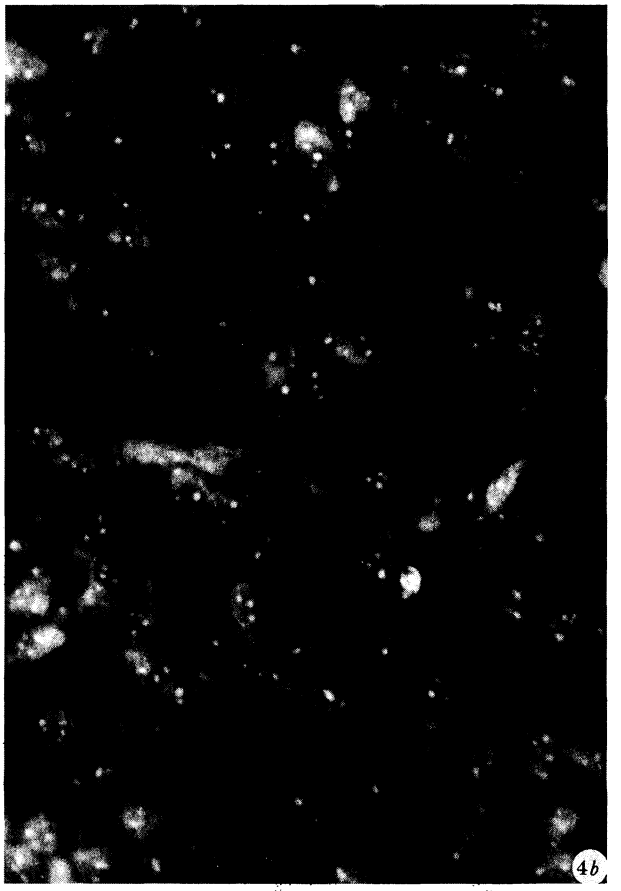
FIGURE 3. Quail adrenocortical cell nucleolus. After EDTA treatment the heterochromatin (arrow) and the intranucleolar strands of DNA are unstained while RNA fibrils and granules are contrasted. (Magn. × 72000.)

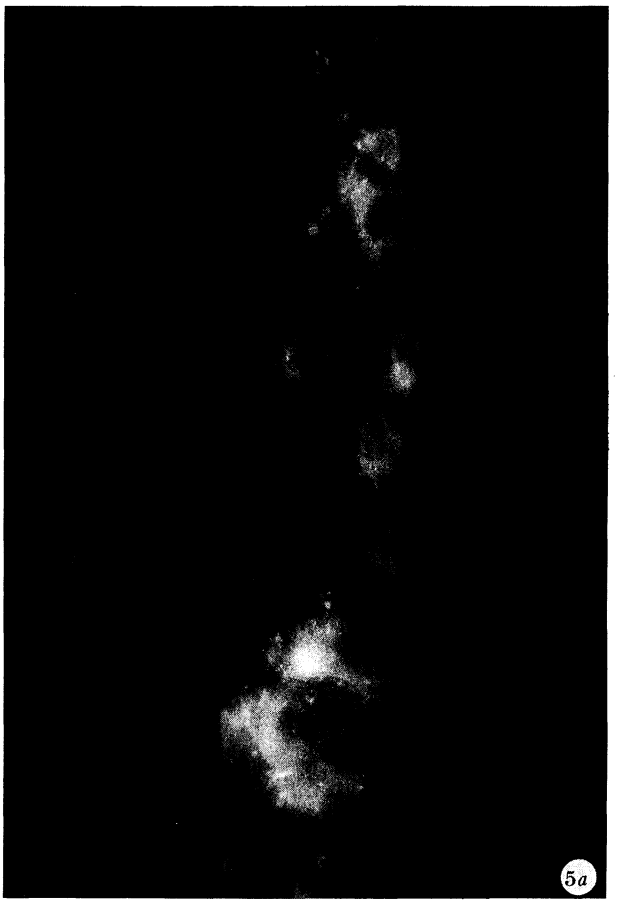
DESCRIPTION OF PLATE 3

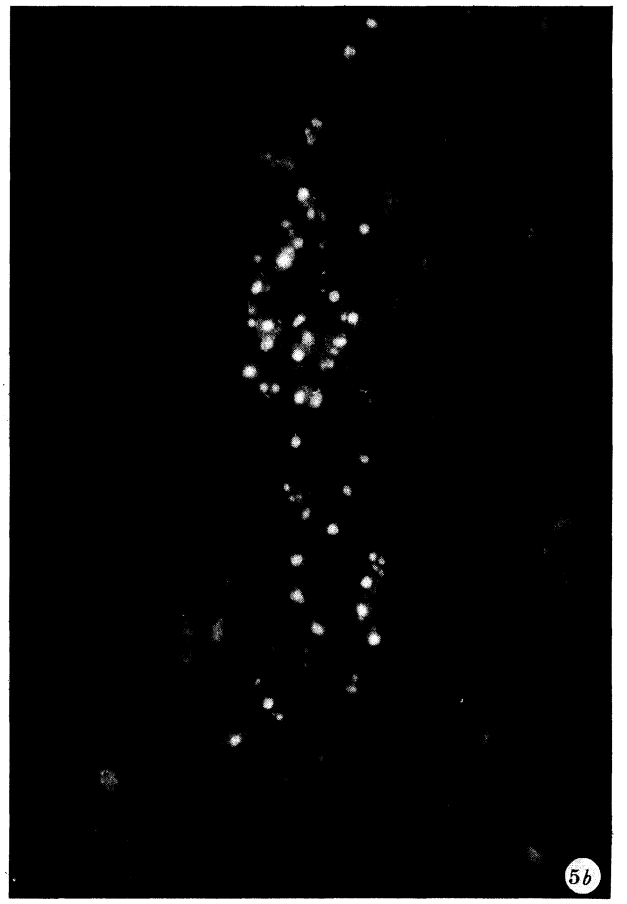
FIGURE 4. Neuroblasts of E5 quail (*a*) and chick (*b*) stained with acridine-orange. Note the large heterochromatin mass in the centre of quail nuclei and the numerous chromocentres in chick nuclei. (Magn. × 1600.)

FIGURE 5. Ileum of a chick embryo colonized by quail cells which differentiated into myenteric plexus ganglia. Vasoactive intestinal peptide is evidenced by immunofluorescence in certain neurons of the ganglion in (*a*). In (*b*), the same section is stained with acridine orange. It appears that most ganglion cells belong to the quail species while the mesenchyme is of the chick type. (Magn. × 1750.)









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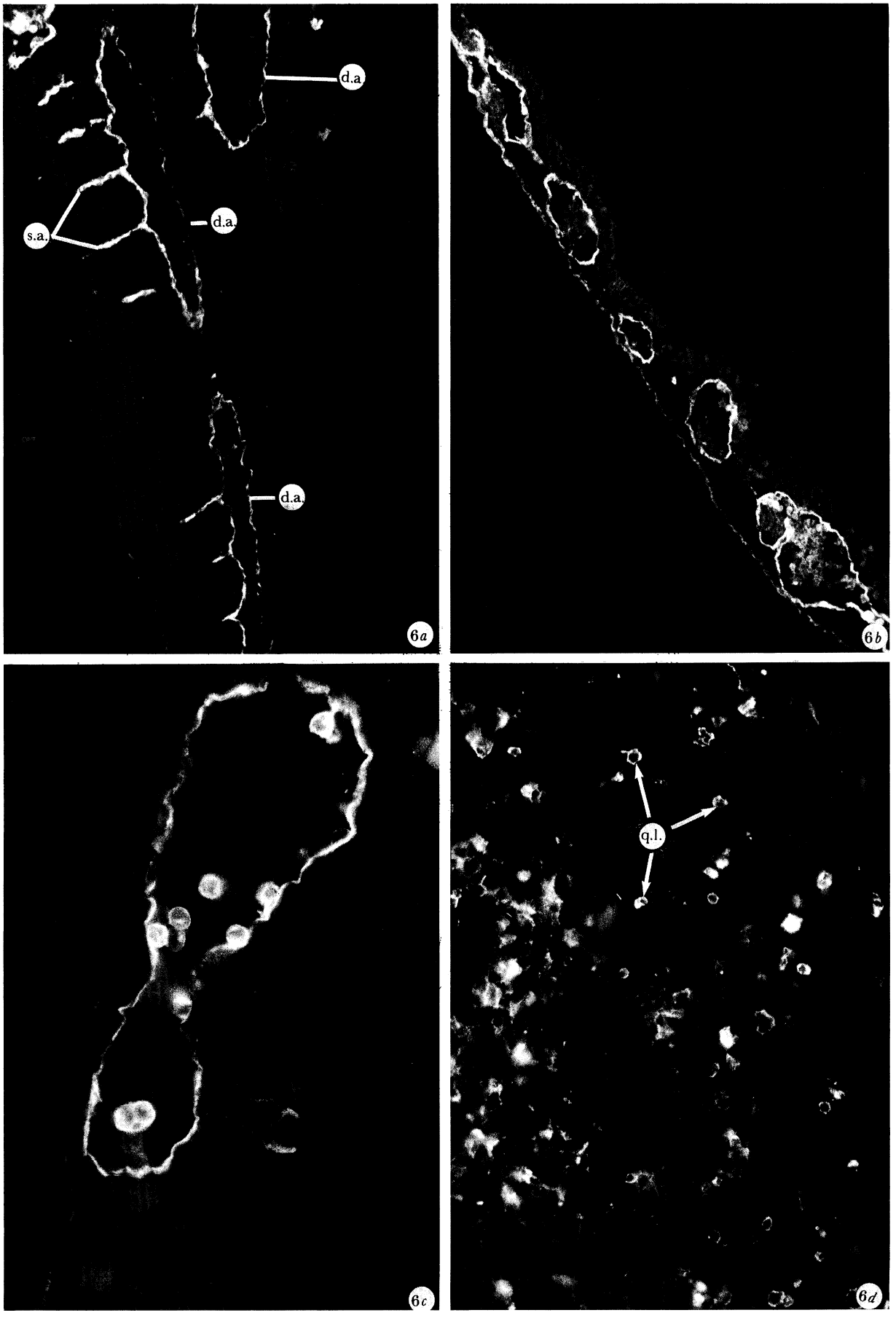
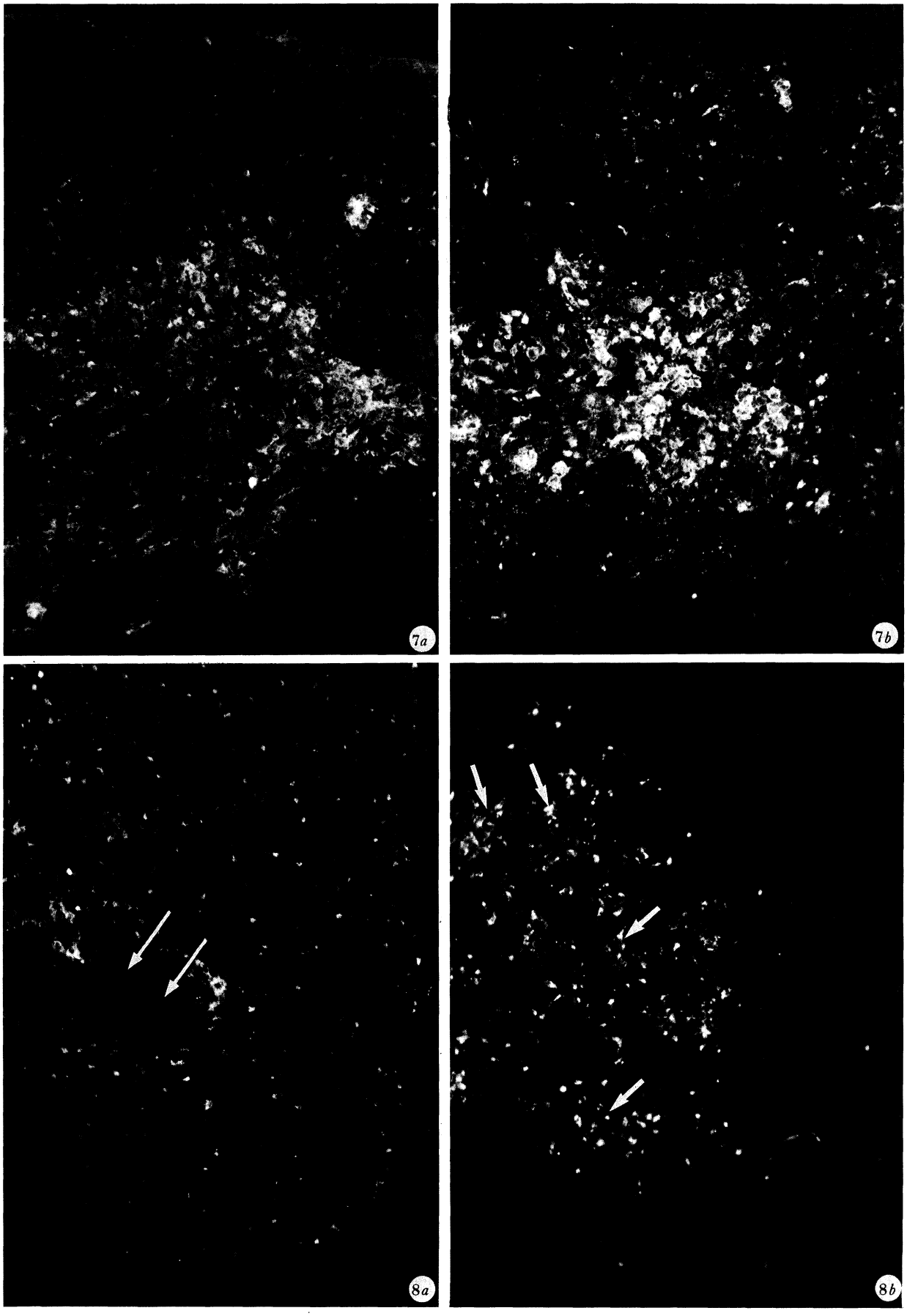


FIGURE 6. For description see page 155.



FIGURES 7 AND 8. For description see page 155.

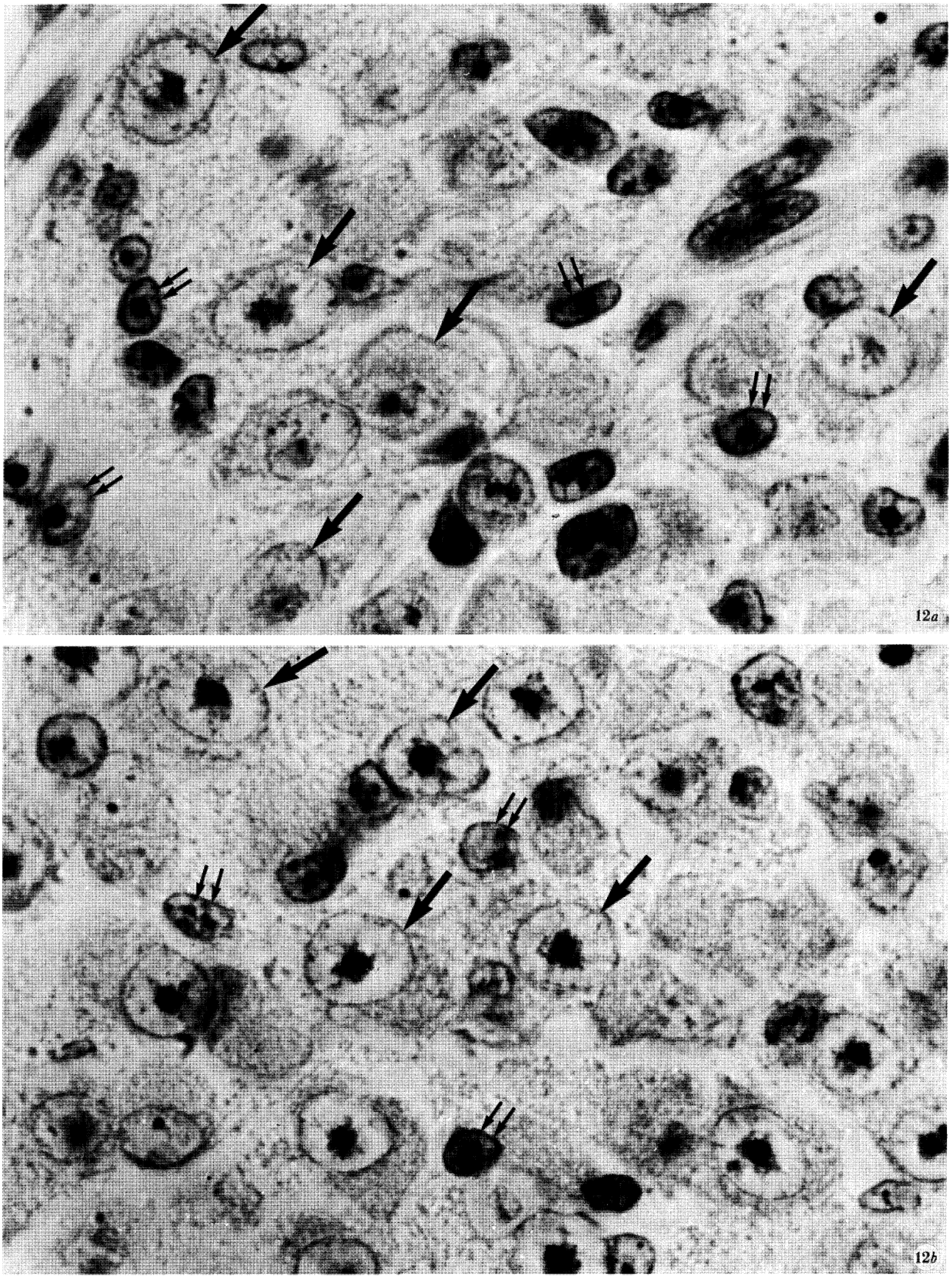


FIGURE 12. For description see opposite.

Douarin (1982) for references), reveals on the same tissue section whether a cell contains a fluorigenic monoamine (catecholamine or indolamine) and whether it belongs to the quail or to the chick species.

1.2. *Quail–chick cell distinction through species-specific monoclonal antibodies*

In recent years, the quail–chick chimera system has benefited from a new tool with which both species specificity and other particular characteristics can be simultaneously shown (that is, with a single reagent) at the single cell level.

These reagents are monoclonal antibodies (Mabs), raised either against a purified antigen or a given cell type belonging either to the quail or to the chick species.

1.3. *The anti-MB1 monoclonal antibody*

A BALB/C mouse was immunized with the isolated plasma immunoglobulin μ chain of the quail and the fusion of its spleen cells with the myeloma cell line Sp2/O yielded several clones. Some of them exhibited μ-chain specificity and therefore recognized the surface-IgM of bursal and peripheral B-lymphocytes, showing a cross-reactivity with chick-IgM molecules as well. In addition to these reagents a clone with an unexpected specificity was produced. It recognized a surface marker (called MB1) broadly distributed on all endothelial and haemopoietic cells (except mature erythrocytes) of the quail (Péault *et al.* 1983) and also on the early precursor of endothelial and blood cells, the *haemangioblast*, which first appears in the yolk-sac blood islands (Le Douarin *et al.* 1986). Moreover, it was recently found that the endodermal endothelium of the bursa of Fabricius synthesizes the MB1 antigen when it is invaded by B-cell precursors (Belo *et al.* 1985). In the chick no cell type whatsoever possesses a molecular entity recognized

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Description of plates 4, 5 and 6

Figure 6. Immunostaining of quail endothelial and blood cells by anti-MB1 monoclonal antibody. (*a*) Longitudinal section at the somitic level in a 17-somite quail embryo showing the specific staining of the dorsal aorta (d.a.) of the segmentary arteries (s.a.) and of the endocardium (end.) (Magn. × 180.) (*b*) Section in the yolk sac of the same embryo where the endothelial wall of the blood vessels is immunoreactive. (Magn. × 180.) (*c*) Higher magnification of a yolk-sac blood vessel in a 22-somite quail embryo showing immunoreactive endothelial and blood cells and non-reactive erythrocytes. (Magn. × 750.) (*d*) Quail thymus grafted into a chick embryo at E8 after being colonized by quail haemopoietic cells: 22 days after the graft, most quail lymphocytes have been replaced by chick cells in the cortical area. However, single quail lymphocytes (q.l.) are recognizable dispersed within the chick lymphoid cells by their MB1 immunoreactivity. In the medulla, many cells of the quail type are still numerous and brightly immunoreactive (arrows). (Magn. × 450.)

Figure 7. Immunolabelling of cells expressing Ia in normal and chimeric thymuses. (Magn. × 170.) (*a*) Thymus of a quail 10 days after birth stained with TaC2. Note that the epithelial cells of the cortex and the dendritic cells of the medulla are immunoreactive. (*b*) Thymus of a 19-day-old chick embryo stained with TaP1. The distribution of Ia-positive cells is the same as in the quail thymus represented in (*a*).

Figure 8. (*a*) The thymic rudiment of a 3-day-old quail embryo has been implanted for 17 days in the somatopleure of a 3.5-day chick embryo. The lymphocytes and dendritic cells are of the chick type. TaC1 (anti-quail Ia) binds only to the Ia molecules expressed by the quail thymic epithelial cells while the medulla remains unstained (arrows). (Magn. × 170.) (*b*) The same thymus as in (*a*) is stained with TaP1 (anti-chick Ia). Only the medullary dendritic cells are immunostained (arrows) while the cortical epithelium remains unstained. (Magn. × 170.)

Figure 12. Section in a chimeric nodose ganglion at E8 in which the non-neuronal cells (double arrows) are of the quail type and the neurons (arrows), devoid of the heterochromatin marker, belong to the chick host. This results from the graft of a quail rhombencephalon into a chick embryo (that is, quail → chick chimera). Bottom: chick → quail chimeric nodose ganglion with quail neurons (arrows) and chick non-neuronal cells (double arrows). Feulgen–Rossenbeck staining. (Magn. × 2400.)

by anti-MB1 Mab. As a consequence, a quail haemopoietic or endothelial cell present within a quail–chick chimeric tissue can easily be detected on the basis of its immunoreactivity with anti-MB1 (figure 6*a–d*, plate 4).

Another Mab with comparable specificity, QH_1, has been obtained by immunizing mice with 13-day embryonic quail bone marrow cells. QH_1 also recognizes endothelial and haemopoietic cells of the quail (Dieterlen-Lièvre 1984*a*). Its affinity range is somewhat broader than that of α-MB_1, extending to submature erythrocytes and to primordial germ cells. Like α-MB1, it is restricted to the quail species. It is currently being used to investigate the origin of intraembryonic blood stem cells (Dieterlen-Lièvre 1984*a*, *b*).

1.4. *Species-specific monoclonal antibodies to class II MHC antigens*

The avian MHC, or B locus (essentially studied in the chick), is considered to be analogous to the mammalian MHC (Briles *et al.* 1982). Within the B locus, the B-L subregion is the counterpart of murine H2-I and human HLA-D (Pink *et al.* 1977; Ewert & Cooper 1982) generally designated as Ia-antigens (for immune response antigens). B-L antigens are known to be distributed on B cells and also on monocytes, macrophages, and dendritic cells, including those found in the thymus (Ewert & Cooper 1978; Peck *et al.* 1982; Oliver & Le Douarin 1984).

The immunogens used to raise the anti-Ia Mabs were dendritic cells and macrophages isolated from thymic cell suspensions by virtue of their glass adherence properties. Three reagents were selected by the following criteria: (i) they react with a surface determinant carried by thymic adherent cells and bursal lymphocytes; (ii) they can be used to immunoprecipitate, from spleen cell membrane extracts, molecular entities of an apparent molecular mass close to 55000 Da, which can be fractionated, under dissociating conditions, into monomers of apparent molecular mass 30000 Da. Among these three reagents, two are strictly species specific, that is, they recognize either chick (TaP1) or quail (TaC1) Ia determinants, whereas the third, TaC2, recognizes both chick and quail Ia molecules.

2. Examples of cell lineage studies based on the use of the quail–chick chimera system

2.1. *In the immune system: identification and embryonic origin of the cells expressing Ia-determinants in the avian thymus*

By using anti-Ia reagents on the thymus (TaC1 on quail thymus, TaP1 on chick thymus, TaC2 on either of them) the distribution of the Ia-determinants on the various thymic cell types was revealed. They are expressed in the cortex on small cells with intricate processes but not at all on lymphocytes. In the medulla, large areas of densely packed cells are brightly fluorescent (figure 7*a*, *b*, plate 5).

A series of experiments involving the construction of chimeric thymuses have allowed a distinction to be made between intrinsic and extrinsic Ia-positive cells of the avian thymus. Quail thymic primordia (that is, the third and fourth pharyngeal pouches) were removed from a 3-day quail embryo and grafted into the somatopleure of a 3.5-day chick for 17 days (total age of the thymus at the time of observation: 20 days). As demonstrated before, all the haemopoietic cells developing in these thymuses were of chick host origin (Le Douarin & Jotereau 1973, 1975; Le Douarin *et al.* 1984). The quail Ia-specific reagent (TaC1) stained the small epithelial cells with processes in the cortex but not the compactly arranged cells of

the medulla (figure 8*a*). Immunoreactivity of the latter elements was observed with TaP1, which, in contrast, did not stain the cortical epithelial cells (figure 8*b*). Owing to its double chick and quail specificity, TaC2 exhibited a positive immune reaction with both cortical and medullary Ia-bearing cells of these chimeric thymuses.

These results demonstrate that, while the Ia-positive cells of the cortex are derived from the thymic primordium itself, those of the medulla are of extrinsic haematopoietic origin.

2.2. *In the nervous system: demonstration that the ganglia of the peripheral nervous system contain undifferentiated neuronal precursors of the autonomic cell lineage*

The peripheral nervous system arises mainly from the neural crest, with an additional contribution of the ectodermal placodes to the sensory ganglia of the cranial nerves (see Le Douarin (1982) for a review). One of the most striking characteristics of the neural crest is the fact that its component cells are endowed with migratory properties and differentiate into a number of cell types after a phase of migration leading them to home into various embryonic tissues. A number of studies over the last ten years or so have shown the pluripotentiality of the neural crest cell population in the different regional areas of the neural crest and the decisive role played by the target organs, in which the neural crest derivatives develop, in selecting among these potentialities, thus defining the phenotypic expression of crest-derived cells (see Le Douarin (1984, 1985*a*, *b*) for reviews).

Two non-exclusive mechanisms may be responsible for the generation of diversity in the progeny of neural crest cells. One is that developmentally labile cells will be enticed by environmental cues to differentiate along a particular differentiation pathway. Another is that the neural crest is initially (that is, from the onset of migration) composed of cells with restricted developmental capacities, hence endowed with the capacity to respond differently to definite factors arising from the local environment.

The experiments to be described below demonstrate that, during peripheral nervous system ontogeny, certain cell lines become segregated early. This is the case for the precursors of sensory and autonomic neurons which belong to two different cell lineages that appear from the earliest stages of neural crest cell migration. It was possible to show that the developmental potentials contained in the avian peripheral ganglion cells are not all used during peripheral nervous system ontogeny. Neuronal precursors are present in the non-neuronal cell population of the developing peripheral nervous system ganglia long after all the neurons of these ganglia have withdrawn from the cell cycle. Such precursors (even those contained within spinal or cranial sensory ganglia) have their developmental potentials restricted to the autonomic cell lineage and cannot, under the experimental conditions devised for their identification, develop into sensory neurons.

The experiments consisted in the retro-transplantation of embryonic quail peripheral ganglia into the chick neural crest migration pathway at E2 (embryonic day 2), as depicted in detail in figure 9. The solid piece of ganglion loses cohesiveness soon after implantation and its histological structure becomes disrupted, while its component cells disperse in the host somitic structures. Twenty-four hours after grafting, quail cells can be seen, singly or in small groups, among the host dorsal mesenchymal cells. After 48 h, this dispersion is followed by homing of the progeny of the grafted cells into the host's neural crest-derived tissues.

Although the phase of dispersion varies little, homing of the grafted cells changes with the nature, sensory or autonomic, and the age of the grafted ganglia. Quail cells populating the

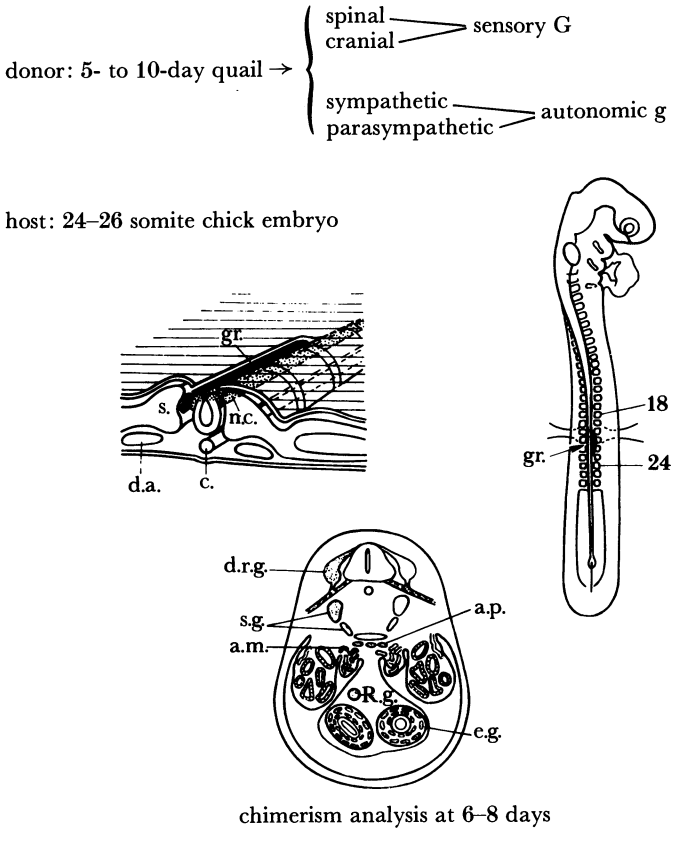

FIGURE 9. Diagram showing the experimental procedure followed in the back-transplantation of quail p.n.s. ganglia into the chick neural crest migration pathway. 1 and 2 show the positioning of the graft, 3 shows the various host crest derivatives in which quail cells are found in the 6- to 8-day chick host. a.m., Adrenal medulla; a.p., aortic plexus; c., notochord; d.a., dorsal aorta; d.r.g., dorsal root ganglion; e.g., enteric ganglia; gr., graft; n.c., neural crest; R.g., ganglion of Remak; s., somite; s.g., sympathetic ganglia.

host dorsal root ganglion and differentiating there into sensory neurons and glia were found only after grafts of dorsal root ganglia. In contrast, Schwann cells and autonomic derivatives (that is, sympathetic neurons, chromaffin cells and, in some cases, enteric ganglia) were obtained after both dorsal root ganglion and autonomic ganglion grafts. The latter (for example, ciliary, Remak, sympathetic ganglia), when transplanted under similar conditions, never gave rise to sensory neurons in the host dorsal root ganglion. Only rarely were a few glial cells of graft origin found in this position (figure 10) (Le Douarin *et al.* 1975; Le Lièvre *et al.* 1980; see also Le Douarin (1982) for a review).

Moreover, the capacity of quail dorsal root ganglion cells to populate the host sensory ganglia is developmentally restricted to the time when sensory neuroblasts are still mitotic. As shown by Schweizer *et al.* (1983), when all the sensory neurons have withdrawn from the cell cycle, dorsal root ganglion grafts give rise exclusively to ganglion cells of the autonomic type. This result suggested that postmitotic neurons did not survive when implanted in a younger host embryo, and that all the neurons arising in the host were in fact derived from the non-neuronal cell population of the ganglion. This was fully confirmed by the use of the nodose ganglion in retrotransplantation experiments.

Owing to the mixed placodal and crest origin of the distal ganglia of certain cranial sensory

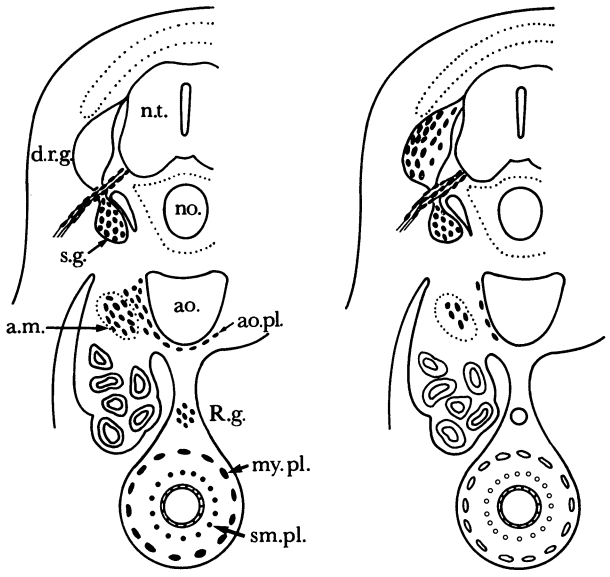

FIGURE 10. Diagram showing the localization of quail cells 4.5 days after the graft of 4- to 7-day quail autonomic and dorsal root ganglia (d.r.g.) at the adrenomedullary level of chick embryos. The black spots indicate the quail cells (that is, derived from the graft) localized in the host neural crest-derived structures. The differences between the two types of grafts are conspicuous, since only d.r.g. grafts result in the colonization of the host d.r.g. and in the differentiation of quail sensory neurons in the host. This never occurs when autonomic ganglia are grafted. In this case, the graft-derived quail cells become localized in the region of the ventral root of the rachidian nerves, the sympathetic ganglia (s.g.), and, more ventrally, in the area of the aortic plexus (ao.pl.) and adrenal medulla (a.m.) of the host. Quail cells are also present at these levels after d.r.g. grafting. If quail d.r.g. are taken from a donor older than 7 days, so sensory neurons arise from the graft and only autonomic derivatives are found. In addition, the contribution of quail cells to the ganglion of Remak (R.g.) and to the enteric plexuses, while fully apparent for ciliary and cervical ganglion grafts, has not been observed after grafting of sympathetic chain ganglia. ao., Dorsal aorta; no., notochord; n.t., neural tube; my. pl., myenteric plexus; sm. pl., submucosal plexus.

nerves (geniculate ganglion for nerve VII; petrosal for nerve IX and nodose for nerve X) (Le Douarin *et al.* 1985), it is possible to label at will either their neuronal or non-neuronal (destined to become glial) component cells (figure 11). Advantage was taken of this developmental feature to follow selectively the fate of these cell categories in the back-transplantation system (Ayer-Le Lièvre & Le Douarin 1982). Labelling of the non-neuronal cells of the nodose and petrosal ganglia is obtained when a chick embryo is chosen as a host and receives an isotopic and isochronic graft of a quail neural primordium at the rhombencephalic level. The chick epibranchial placodes then provide the ganglia with neuronal cells, while the implanted quail crest cells will form the entire non-neuronal cell population. If the host is a quail and the donor of the rhombencephalon a chick, the reverse situation will be established (figure 12, plate 6).

Back-transplantation into the chick crest migration pathways of a nodose ganglion with quail neurons and chick non-neuronal cells did not result in colonization of the host crest derivatives by graft-derived neurons or glia. Only a few fibroblasts, included in the implant, could be recognized at the graft site. In contrast, a considerable number of quail cells invaded the host when the grafted chimeric nodose ganglion contained a quail non-neuronal cell population. The invasiveness of the cells derived from the graft was considerable: numerous quail neurons and glia were found, not only in adrenal medulla, sympathetic ganglia and plexuses but also in enteric ganglia. However, no quail-labelled neurons or glial cells (with a few exceptions for the latter cell types) were found in the dorsal root ganglion of the host following the graft either

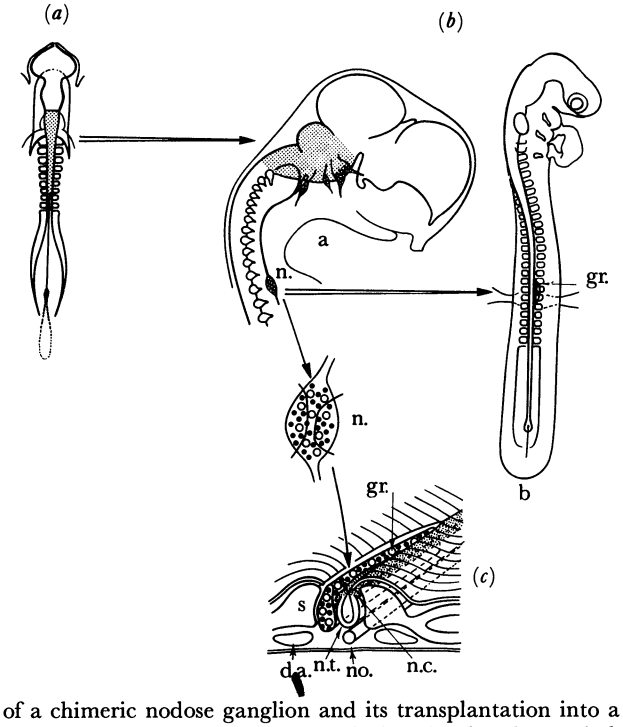

FIGURE 11. Construction of a chimeric nodose ganglion and its transplantation into a two-day chick host. (*a*) A quail→chick (or chick→quail) chimera is made by replacing the rhombencephalo-vagal neural primordium of the host by the equivalent piece of neural primordium from the donor. The graft is performed in embryos at 6- to 11-somite stages.

of a nodose or a petrosal ganglion with quail-labelled non-neuronal cells (Ayer-Le Lièvre & Le Douarin 1982; J. Fontaine-Pérus, M. Chanconie and N. M. Le Douarin, unpublished). The presence of autonomic neuronal precursors in the non-neuronal cell population of the cranial sensory ganglia, is also likely in all developing peripheral nervous system ganglia.

CONCLUDING REMARKS

Since its discovery in 1969, the quail–chick marker system has been widely used to investigate a number of problems relating to cellular interactions, cell migration and morphogenesis in the avian embryo (for references and reviews see Le Douarin & MacLaren (1984)). Implantation of single quail cells into chosen localizations of chick embryos is a tempting way to approach problems of cell lineage and determination in a privileged manner. This has been applied to neural crest cells in the author's laboratory but has not yet been successful, for technical reasons, probably related to the early embryonic cells susceptibility to *in vitro* manipulations. Further improvements of this technique have to be brought about before it becomes fruitful.

It is noticeable that the advent of the monoclonal antibody technology allowing the production of reagents with both species and cell-type specificities is an additional advantage which, in the future, will increase the interest of quail–chick chimeras as an experimental model.

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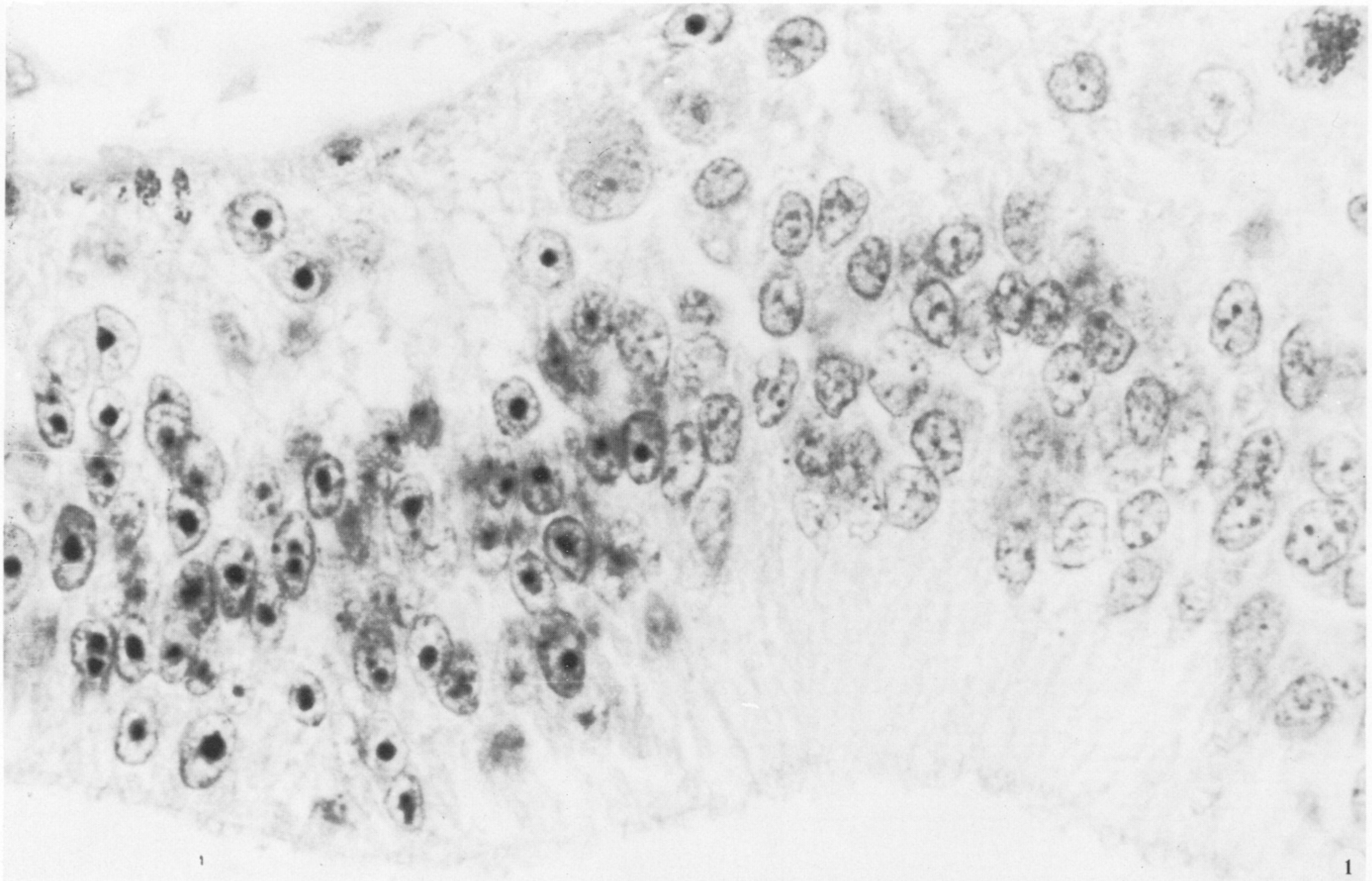

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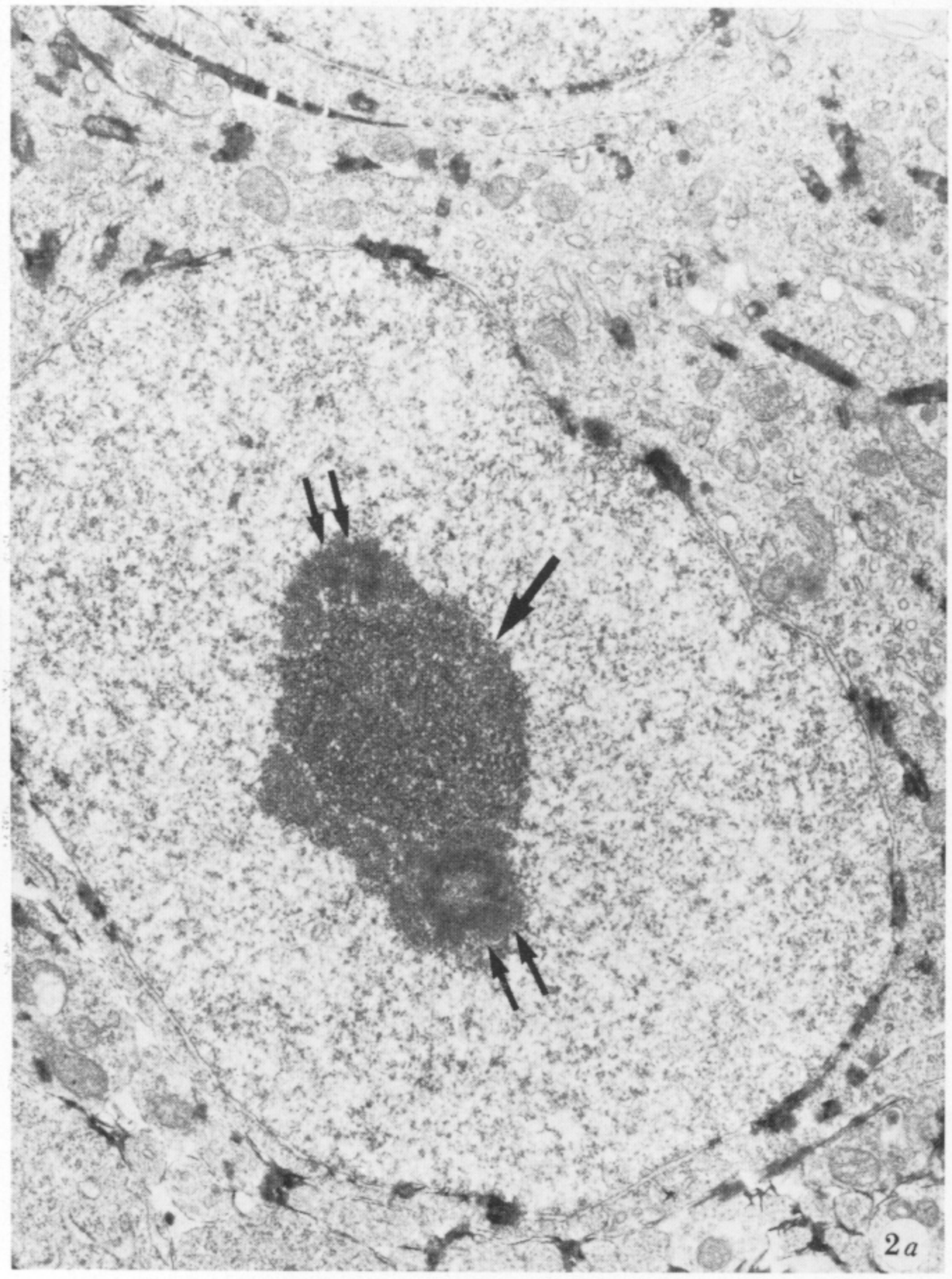
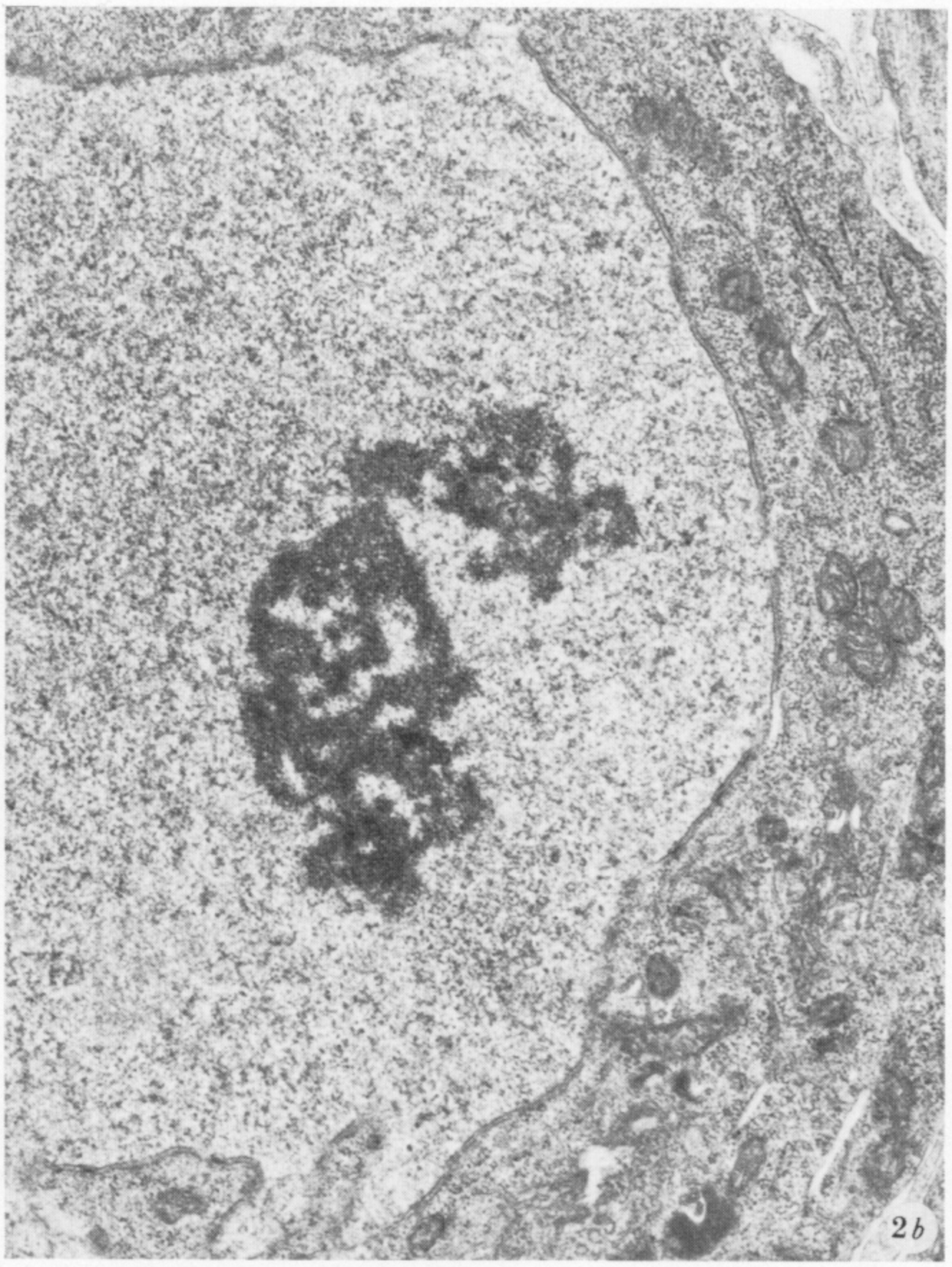


2a



2b

FIGURES 1 AND 2. For description see opposite.

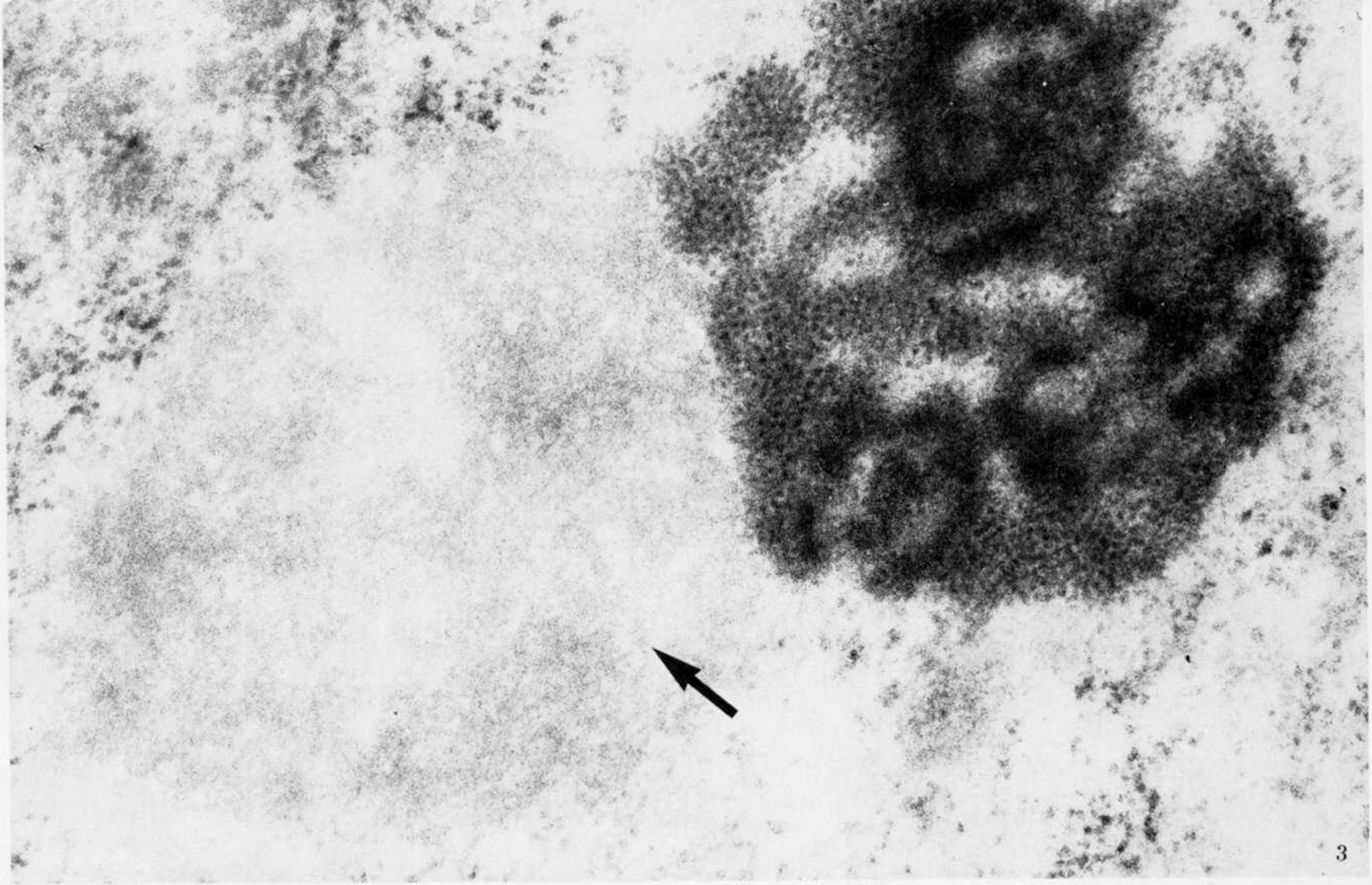
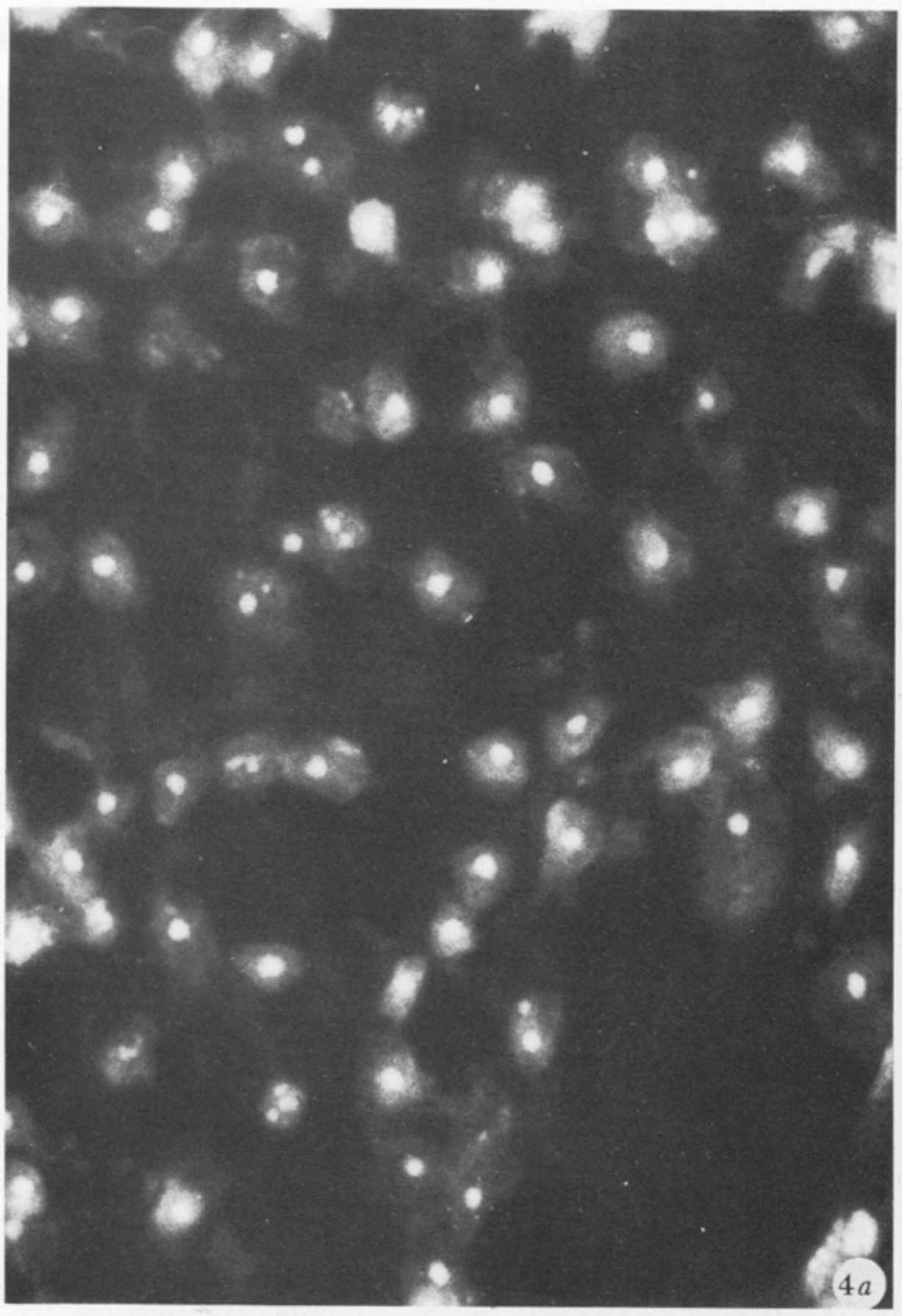
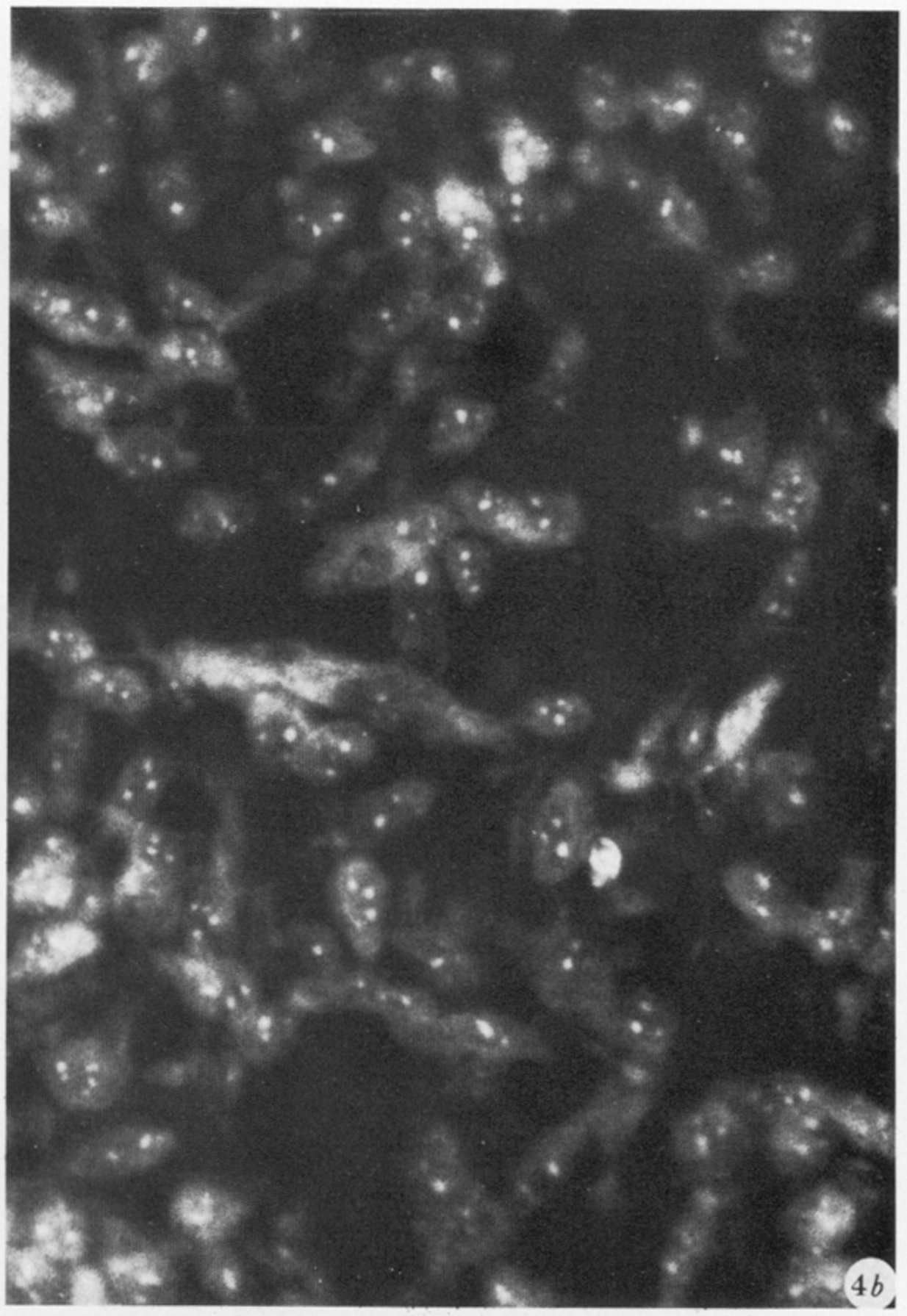


FIGURE 3. Quail adrenocortical cell nucleolus. After EDTA treatment the heterochromatin (arrow) and the intranucleolar strands of DNA are unstained while RNA fibrils and granules are contrasted. (Magn. ×72000.)



4*a*



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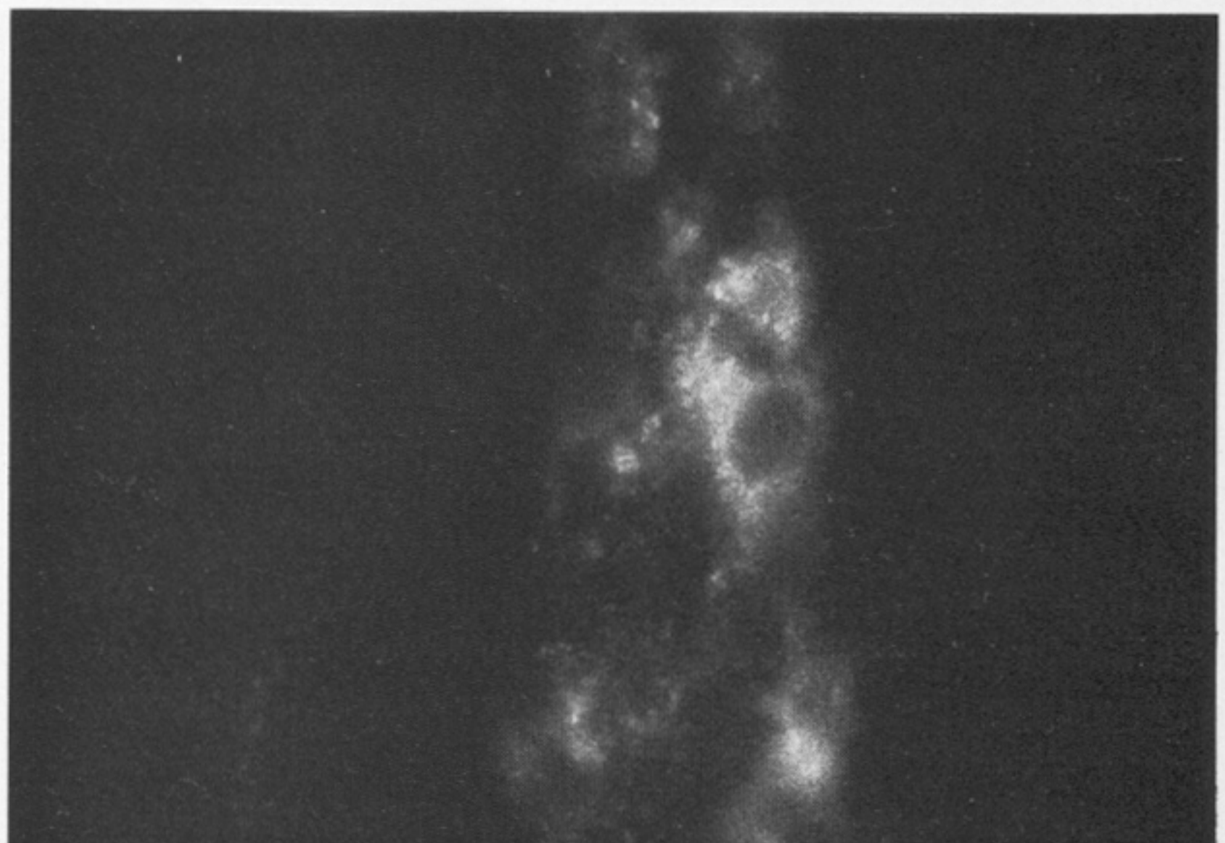
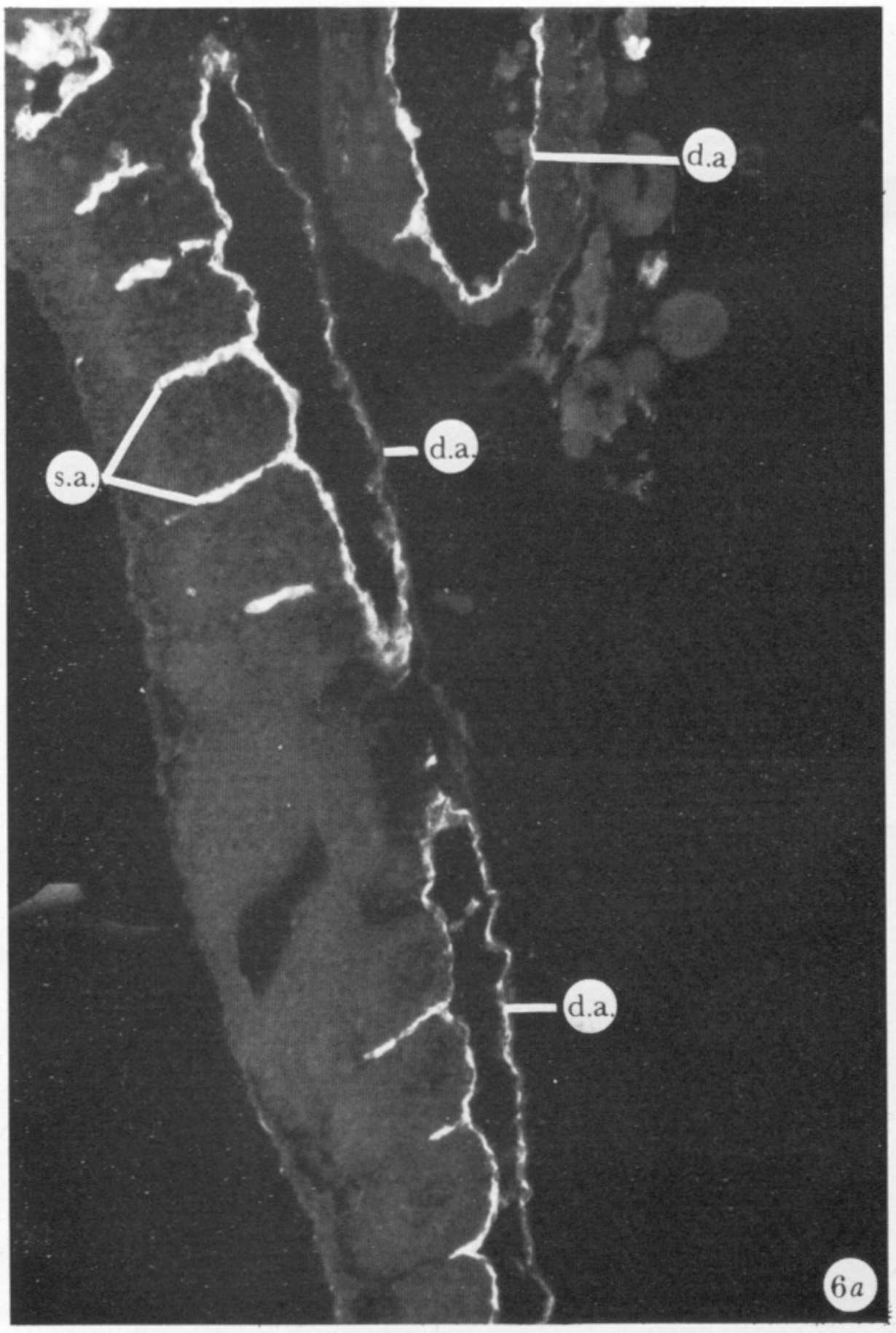
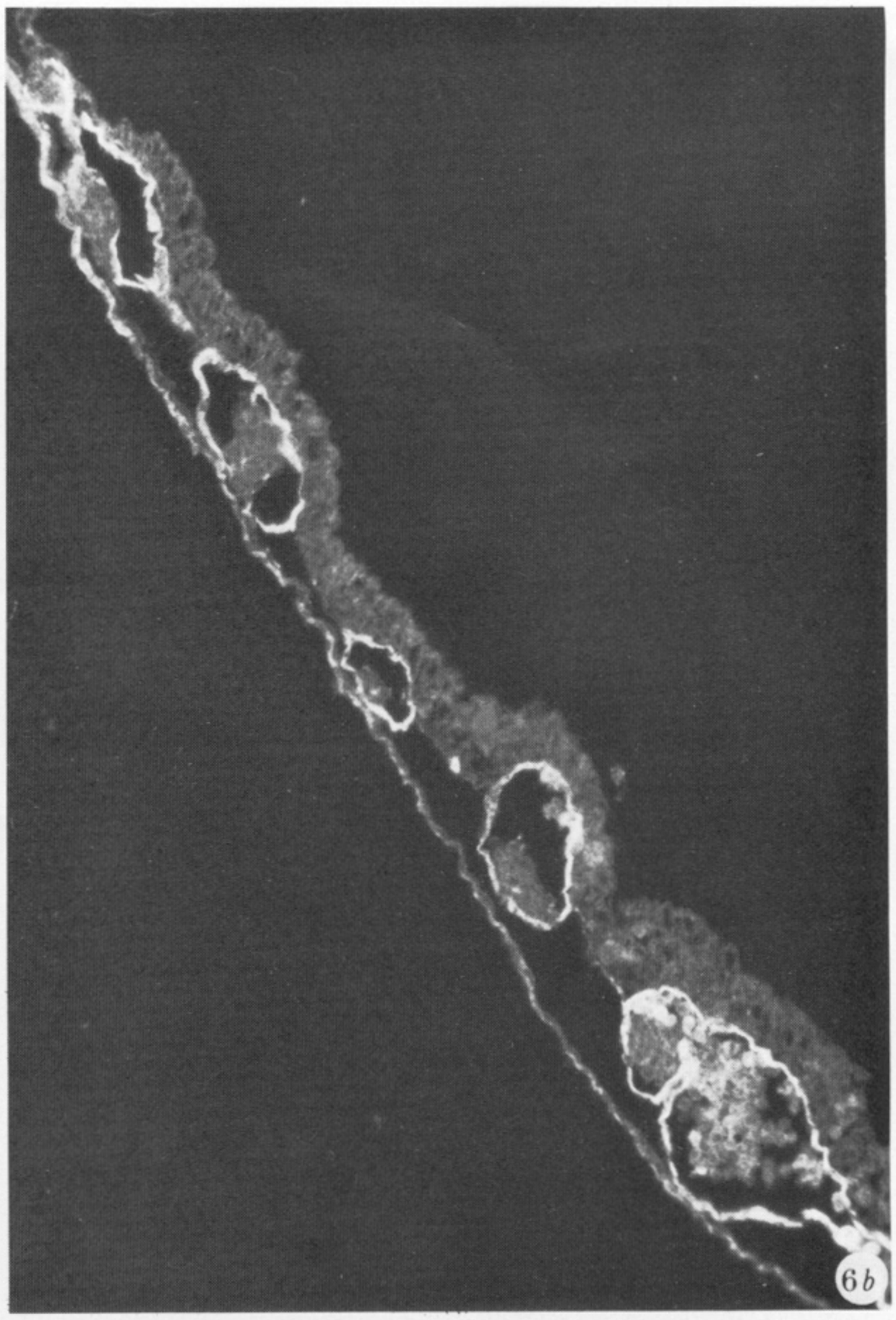


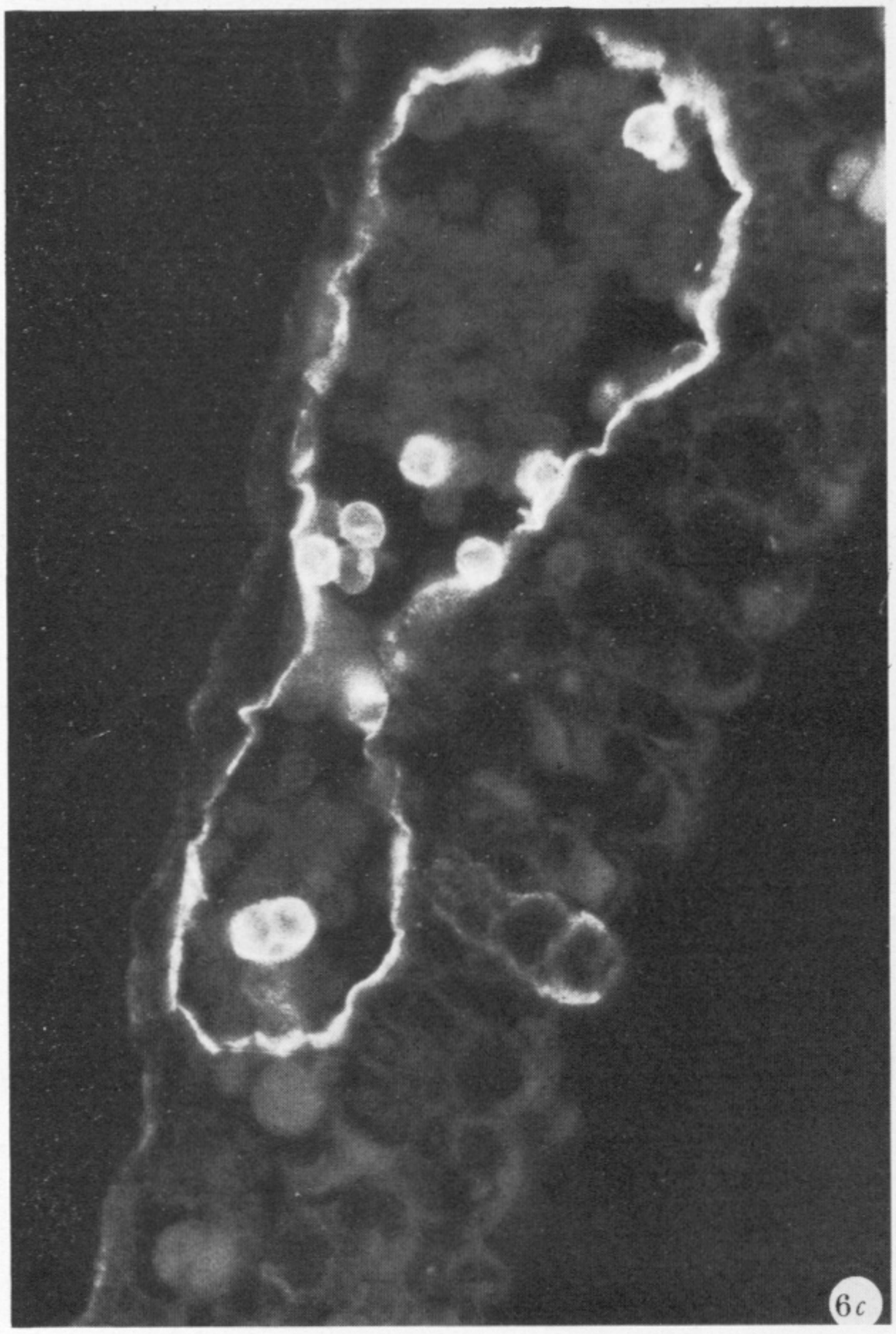
5*a*

5*b*

FIGURES 4 AND 5. For description see opposite.







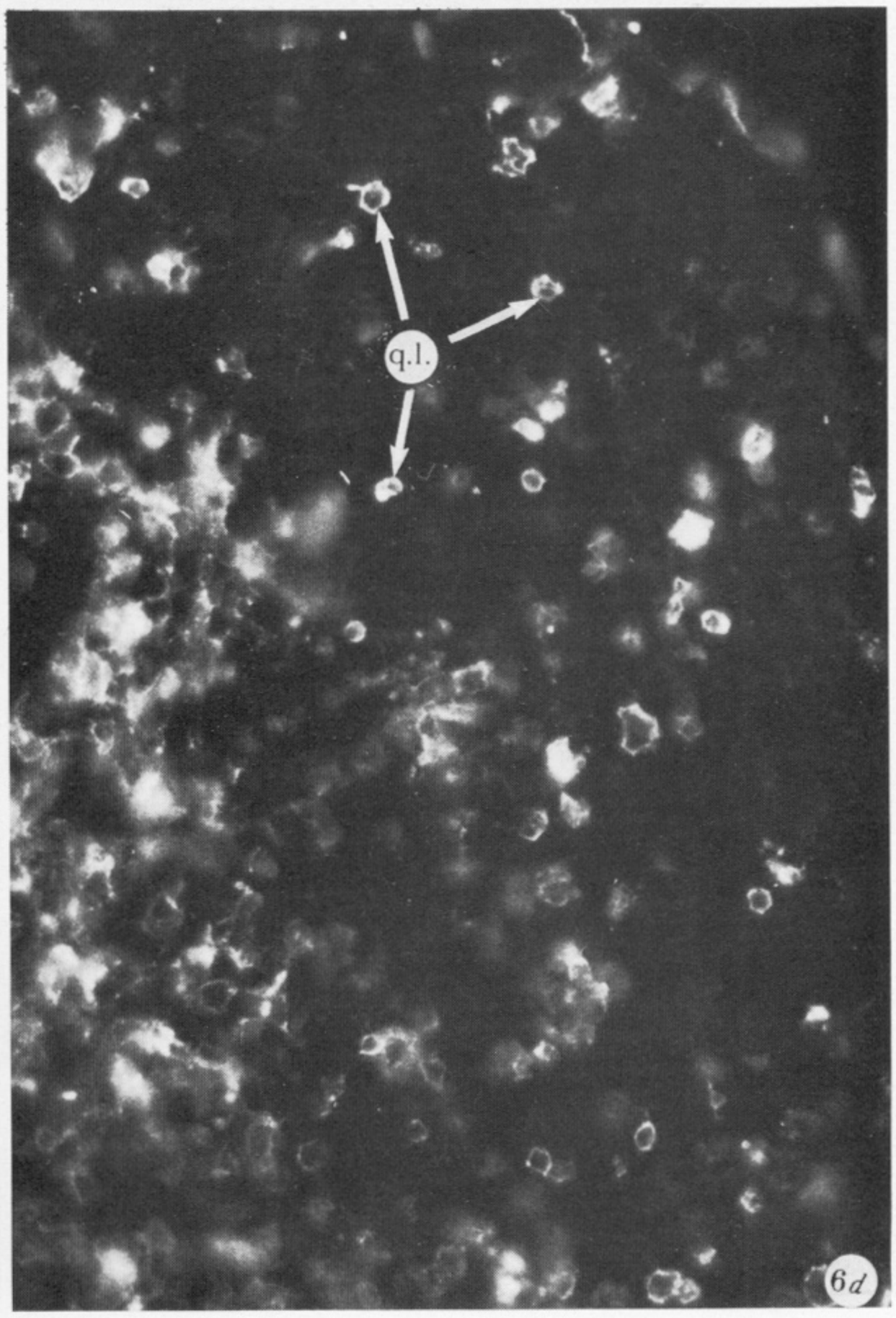
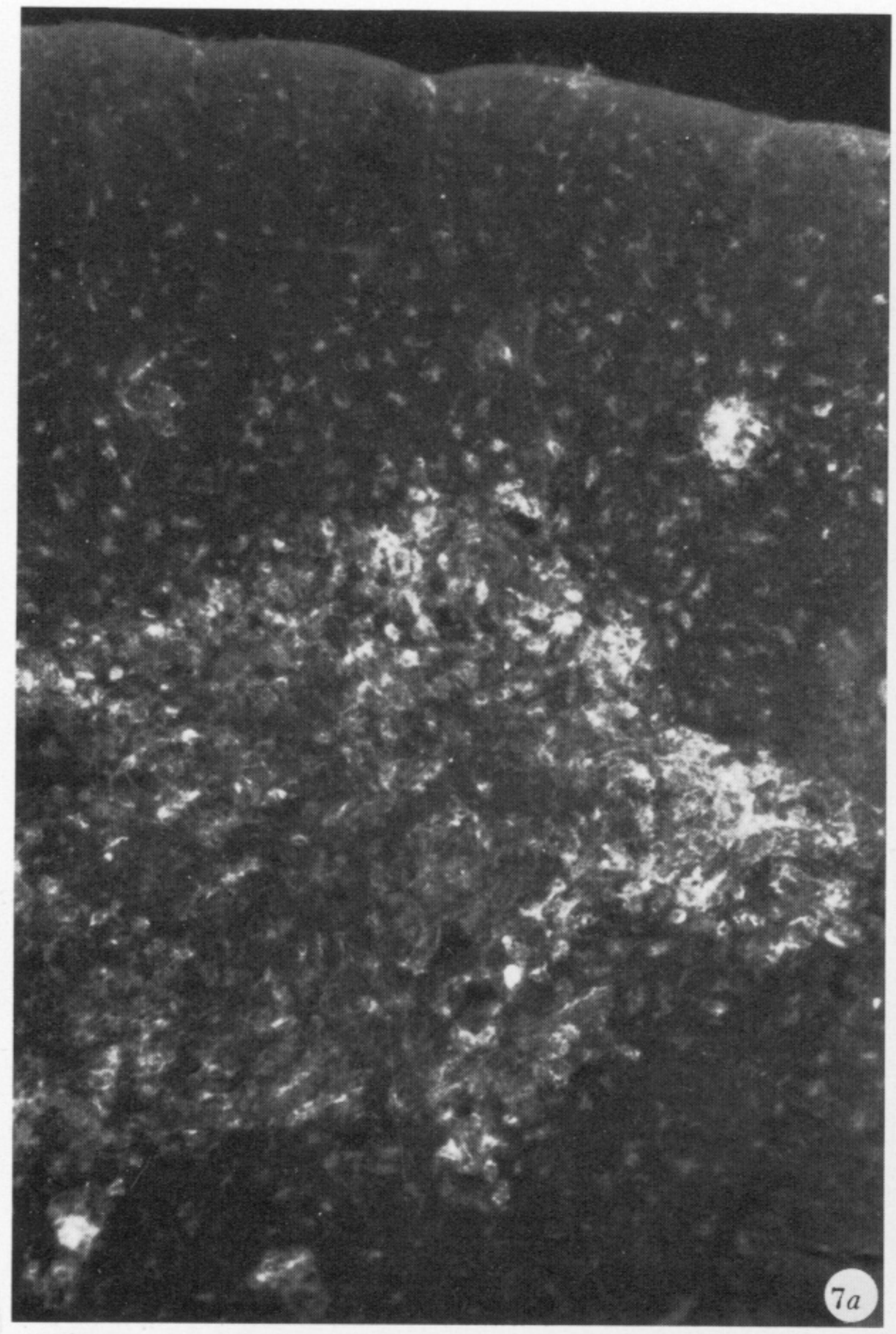
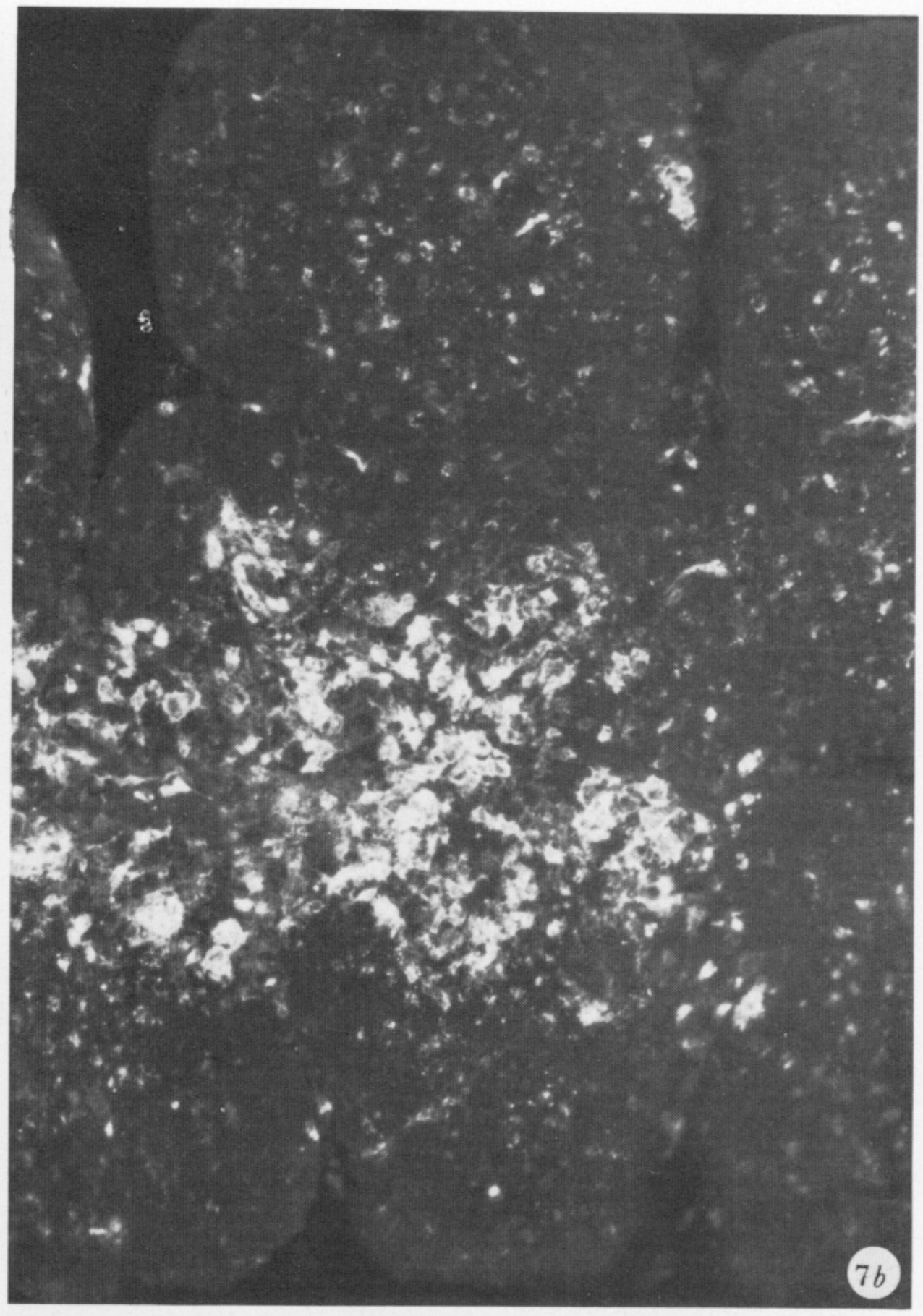


FIGURE 6. For description see page 155.



7a



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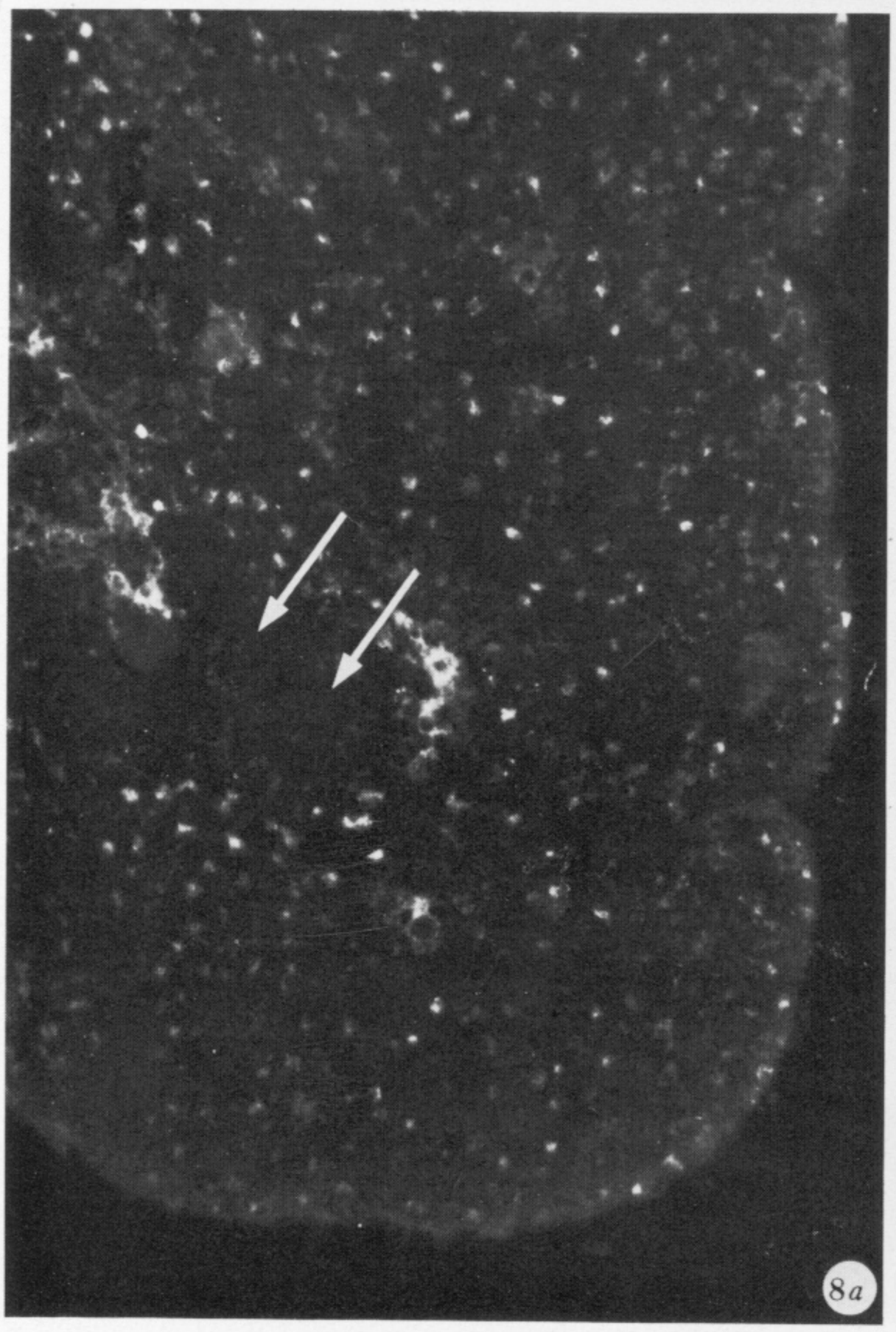

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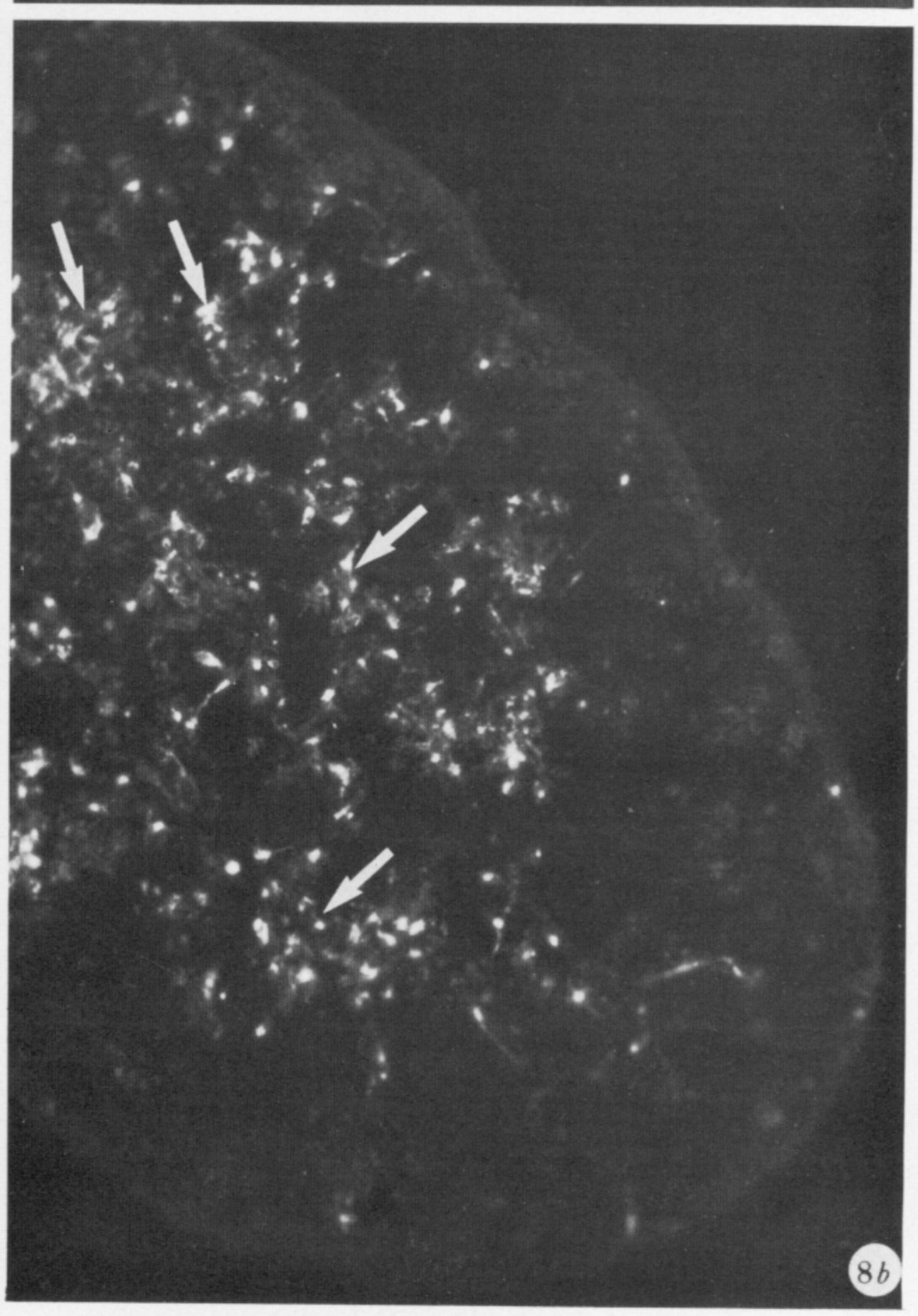

8b

FIGURES 7 AND 8. For description see page 155.

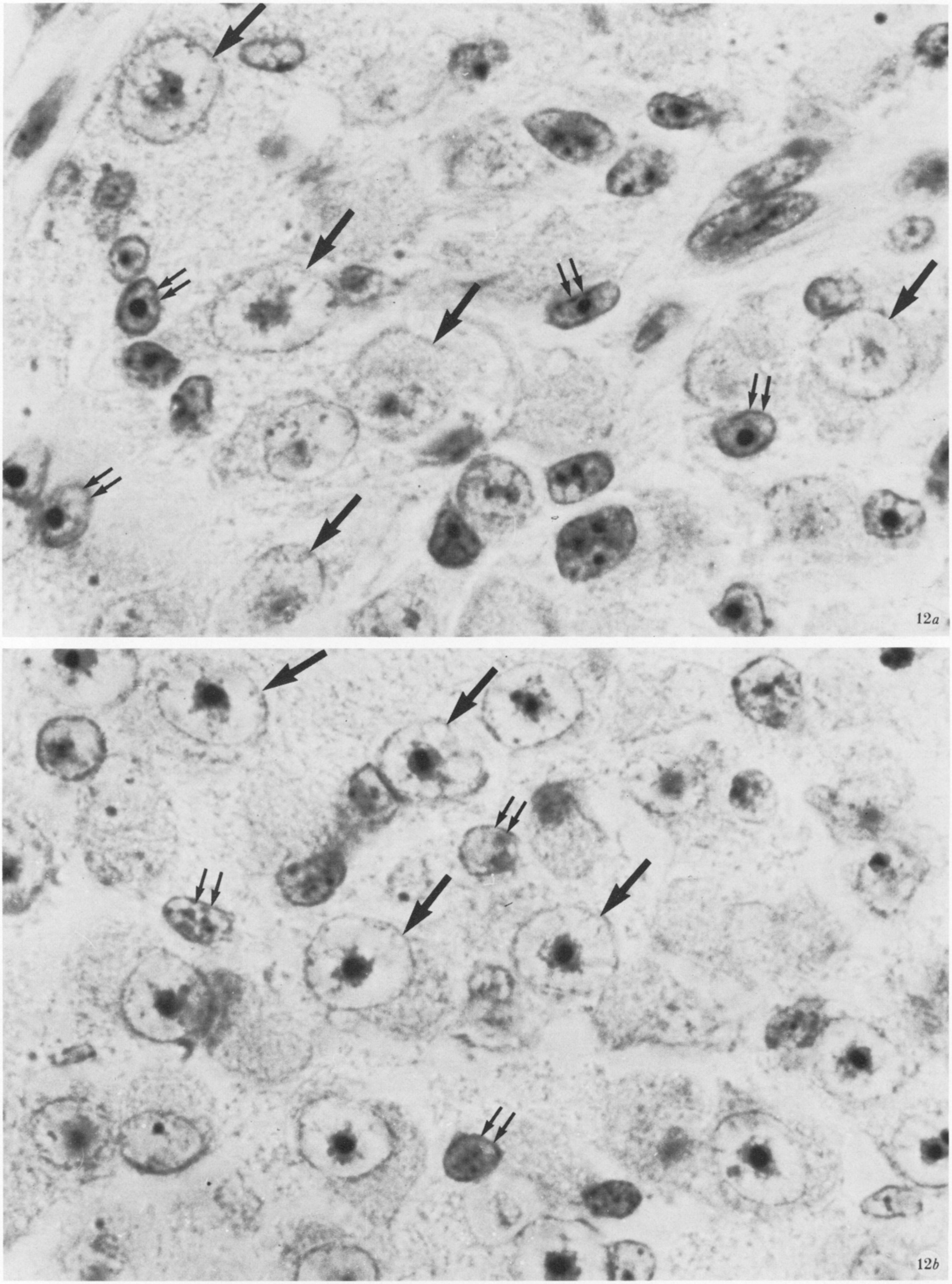

FIGURE 12. For description see opposite.