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SOME ASPECTS OF PROTEIN HISTOCHEMISTRY IN THE PARATHYROID GLAND OF THE RABBIT

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ABSTRACT

A series of chemocytological observations have been made on the parathyroid gland of the rabbit. In the cytoplasm of the parathyroid cells there has been confirmed the presence of three types of protein granules which are reactive for coupled tetrazonium, DDD diazo blue B, and HNAH diazo blue B respectively. These granules appear identical in chemocytological features and cytophysiological significances to corresponding those reported previously in the parathyroid cells of other animal species. In the cytoplasm of the parathyroid cells observed here, five other types of protein granules have been newly detected. These include granules exhibiting positive 1) alkaline tetrazolium, 2) Ninhydrin-Schiff, alloxan-Schiff or chloramine T-Schiff, 3) DMAB-nitrate, 4) Sakaguchi, and 5) DNFB H acid reactions respectively. The alkaline tetrazolium reactive granules are thought to be comparable in nature to DDD diazo blue B reactive ones, while the true cytophysiological significances of the rest of the granules remain to be known and await further experimental studies.

Since we reported some chemocytological features of the rat parathyroid cells (Hara and Yamada[10]), it has been noted that different intracellular granules are demonstrated by certain methods of protein histochemistry. In the light of the physiologically and biochemically known functions of the endocrine gland, attemts have been made to elucidate the cytophysiological significances of these granules in a series of subsequent studies on the glandular cells under varying conditions (Yamada[22)23)], Hara and Yamada[12)], Yamada[24)25)], Hara and Hotta[9)]). The most rewarding concept derived from the attempts is a possible correlation of the morphology of sulfhydryl group containing granules with the secretory activity of the parathyroid cells.

The purpose of the present communication is to report observations on some aspects of protein histochemistry in the parathyroid gland of the rabbit and to extend our knowledge concerning the chemocytology of proteins in mammalian parathyroid glands.

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MATERIALS AND METHODS

Eleven adult rabbits of both sexes were sacrificed by ether anesthesia and bilateral parathyroid glands with adjacent organs were quickly dissected out and fixed in the following solutions; 1 per cent trichloracetic acid in 80 per cent ethanol, Zenker's and Carnoy's fluid. Paraffin sections of the fixed materials were cut at 6 to 8 μ and stained with routine and special techniques. As the routine techniques four methods were employed; hematoxylin-eosin, Heidenhain's azan, periodic acid-Schiff (PAS) (McManus[19]) in conjunction with amylase digestion for glycogen (Pearse[20]), and pyronin methylgreen together with ribonuclease-digested controls for ribonucleic acid (RNA) (Casselman[8]). The majority of sections were, however, stained by the following special methods of protein histochemistry.

(1) Coupled tetrazonium method for proteins in general (Pearse[20]).

(2) Ninhydrin-Schiff, alloxan-Schiff and chloramine T-Schiff methods for proteins with amino groups (Yasuma and Ichikawa[26], Burstone[7]).

(3) P-dimethylaminobenzaldehyde (DMAB) nitrate method for protein with tryptophane residues (Adams[1]).

(4) Sakaguchi reaction for protein with arginine residues (Pearse[20]).

(5) 2, 2′-Dihydroxy-6, 6′-dinaphthyl disulfide (DDD) diazo blue B (Barrnett and Seligman[4]) and alkaline tetrazolium using *p*-nitroblue tetrazolium (Modification of Pearse[20]) for proteins with sulfhydryl groups. The former method was occasionally conducted after treatment of sections with (a) 0.5 M thioglycolate adjusted to pH 8.0 by addition of 0.1 N sodium hydroxide for 1 hour at 50°C (Barrnett and Seligman[5]) and (b) *N*-ethyl maleimide (0.1 M *N*-ethylmaleimide in 0.1 M Sörensen's phosphate buffer pH 7.4 for 4 hours at 37°C)-potassium cyanide (10 per cent aqueous potassium cyanide pH 11.0–11.5 for 2 hours at 37°C) sequence for proteins with disulfide groups. Performic acid alcian blue method (Adams and Sloper[2,3]) was also used for demonstrating the disulfide group containing proteins.

(6) Dinitrofluorobenzene (DNFB) H acid method for proteins with tyrosine residues and amino and sulfhydryl groups (Burston[7]).

(7) 2-Hydroxy-3-naphthoic acid hydrazide (HNAH) diazo blue B method for proteins with carboxyl groups (Barrnett and Seligman[6]) conducted at times following prior treatment of sections with (a) 10 per cent sodium hydroxide for 15 minutes at 26°C or (b) 90 per cent ethanol for 24 hours at room temperature.

OBSERVATIONS

(*1*) *General histology.* From observations on the hematoxylin-eosin and azan stained preparations the general histology of the rabbit parathyroid gland is well recognized. This gland consists of masses of parenchymal cells which

are invaded by highly vascular interstitial layers originating from a vary thin connective tissue capsule (Fig. 1). Within each parenchymal mass the interstitial layers invade farther between cell cords as a thin membranous sheet which is characterized by an extreme abundance of blood capillaries (Fig. 1).

The parenchymal cells are generally polygonal in shape and contain a relatively large round or oval nucleus (Fig. 2). The cytoplasm of these cells is finely granular and varies considerably in staining intensity with individual cells. The nucleus is limited by a distinct nuclear membrane, filled with a variable amount of chromatin granules and provided with small round nucleoli. In the interstitial layers fibrous and cellular elements of connective tissues are found in addition to abundant blood vessels.

In the preparations stained by PAS and hematoxylin the cytoplasm of parenchymal cells is found to contain PAS reactive granules of different sizes, which vary in amount and distribution pattern with individual cells (Fig. 3). In some cells these granules are acccumlated at juxtanuclear and peripheral areas of the cytoplasm, while their distribution is relatively uniform throughout the cytoplasm of other cells (Fig. 3). Still other parenchymal cells are devoid of the PAS reactive granules. These granules can be digested by prior treatment of sections with saliva or β-amylase, indicating that they are of glycogen in nature. In the interstitial tissues the endothelial cytoplasm of blood vessels exhibits a varying intensity of PAS reaction and fibrous elements are weakly reactive for PAS (Fig. 3).

If the parathyroid tissues are stained by the methylgreen pyronin method, the cytoplasm of parenchymal cells displays diffuse pyroninophilia varying in intensity with individual cells (Fig. 4). Besides such stainability, the cytoplasm is loaded with clumps of further strongly pyroninophilic granules of different sizes (Fig. 4). The intracellular location of these clumps is varied, depending upon each cell. Some clumps occur at the periphery of the cytoplasm, whereas others are in its perinuclear areas. In the nucleus of parenchymal cells the nuclear membrane is colored with methylgreen and encloses clumps of similarly stained chromatin granules. Contrary to these, the nucleoli are variously pyroninophilic. The majority of the pyroninophilic structures in the parenchymal cells are digested by prior RN-ase treatment and conceived, thus, to contain RNA. The cytoplasm and nucleus of the vascular endothelial cells are stained moderately or weakly with pyronin and methylgreen respectively. The majority of fibrous and cellular components of the interstitial connective tissues are feebly reactive for the nucleic acid stains.

(*2*) *Protein histochemistry.* According to the coupled tetrazonium techniques, both the cytoplasm and nucleus of parenchymal cells are reactive in varying intensities (Fig. 5). Within the weakly or moderately tetrazonium reactive ground substance of the cytoplasm, the presence of brownish red granules of

various sizes is noted (Fig. 5). Such granules are disseminated sparsely throughout the cytoplasm of most cells, but in some cells they are concentrated in particular sites of the cytoplasm, such as those close to the nucleus and the plasma membrane facing the perivascular spaces (Fig. 5). The nuclear membrane of the parenchymal cells is distinctly reactive for the tetrazonium and within the nucleus are seen a variable number of moderately or intensely reactive granules and globules (Fig. 5). Some of these globules are presumed to be nucleoli from their localization within the nucleus. Of the interstitial tissue constituents, the vascular endothelial cytoplasm and red blood corpuscles reveal the most pronounced tetrazonium reaction, and the rest of the constituents are not distinguished in coloration.

When the ninhydrin-Schiff method is used, the cytoplasm and nucleus of parenchymal cells exhibits positive reactions of different intensities (Fig. 6). The ground matrix of the cytoplasm presents a diffuse staining which is weak in intensity throughout. In such matrix are imbeded a variable amount of relatively fine granules. In most cells, these granules are stained moderately and disseminated uniformly (Fig. 6). In some cells, however, their aggregations are detected here and there within the cytoplasm. In the nuclear matrix enclosed by a well stained nuclear membrane there occur a variable number of coarse granules exhibiting moderately positive reaction. The ninhydrin-Schiff reaction of the interstitial tissue elements is generally weak except for the rather intense reaction of the vascular endothelial cells (Fig. 6). In the alloxan-Schiff and chloramine T-Schiff stained preparations all the parathyroid tissue components are similar in stainability to those in ninhydrin-Schiff stained specimens.

Treatment of sections with the DMAB-nitrate reagents discloses that the reaction of the cytoplasmic matrix is feeble or moderate in the parenchymal cells and the nucleus is of feeble or doubtful reaction (Fig. 7). However, in the cytoplasm of occasional cells moderately reactive coarse granules are accumulated in peripheral or perinuclear areas (Fig. 7). In the interstitium of the parathyroid gland the vascular endothelial cell cytoplasm is the only structure that is distinguished by its strongly positive DMAB-nitrate reaction, and the rests of the interstitial elements are practically non-reactive.

In most of the parenchymal cells the Sakaguchi reaction of the cytoplasmic matrix is faint or negative, but in some cells it is moderate (Fig. 8). In the cytoplasm of a substantial number of parenchymal cells, further, granules of different sizes are disseminated which are moderately reactive for the Sakaguchi stain and vary in number with individual cells (Fig. 8). Nearly all the nuclear components of the parenchymal cells are feebly or hardly reactive for the Sakaguchi stain even though a few number of intranuclear coarse granules are often pronounced in stainability. The interstitial tissue constituents are faintly positive or negative for the reaction, with, however, the exception that

the vascular endothelium shows a rather intense staining.

In the DDD diazo blue B stained preparations, the cytoplasm of parenchymal cells is found to contain varying amounts of reactive granules which are colored purple (Fig. 9). These protein granules are nearly uniform in size and are, in most cells, accumulated at particular cytoplasmic loci such as the juxtanuclear areas and those right beneath the plasma membrane (Fig. 9). It is of further note that this type of granules is often observed within the extracellular viz. perivascular connective tissue spaces (Fig. 9). The nuclear marix exhibits usually a weak DDD diazo blue B reaction, but in it are enclosed a few number of rather intensely reactive small globules (Fig. 9). In the interstitial layer, the cytoplasm of vascular endothelium is intensely or moderately reactive for the DDD diazo blue B, and no other interstitial elements are so marked in stainability as the endothelium. Reduction of sections by means of thioglycolate treatment does not increase significantly the number and staining intensity of the DDD diazo blue B reactive granules in the cytoplasm of parenchymal cells. Likewise, in specimens which have undergone prior *N*-ethyl-maleimide-potassium cyanide sequence, the DDD diazo blue B method does not reveal any appreciable amount of reactive granules in the cytoplasm. In consistent with these results, the performic acid alcian blue technique can not demonstrate any strongly reactive entities in the cytoplasm.

In the parathyroid tissues of the rabbit the alkaline tetrazolium method discloses a histological and cytological pattern of reactive components nearly identical with that observed following the application of the DDD diazo blue B method (Fig. 10). A finding worthy of mention is that in the cytoplasm of parenchymal cells the tetrazolium reactive granules tend to be concentrated much more densely at particular cytoplasmic loci than DDD diazo blue B reactive granules.

If the DNFB-H acid technique is performed, the cytoplasmic matrix of parenchymal cells is diffusely colored purplish red (Fig. 11). In the matrix the presence of intensely DNFB-H acid reactive fine and coarse granules is noted (Fig. 11). These granules are varied in amount and distribution pattern depending upon each cell. In most cells, however, they tend to surround the nucleus or to be clustered at the peripheral cytoplasm (Fig. 11). Some of such granules are occasionally seen outside the cytoplasm. The nuclear membrane is apparently reactive for the DNFB-H acid reagent and encloses weakly reactive nuclear matrix containing a small number of intensely stained globules (Fig. 11). The fibrous elements of the interstitial connective tissues show a faint or moderate reaction, whereas the vascular endothelium and red blood corpuscles are very pronounced in stainability.

In the parenchymal cells both the nuclear and cytoplasmic matrices react faintly to the HNAH diazo blue B (Fig. 12). In the cytoplasmic matrix, there occur a variable amount of vividly HNAH diazo blue B reactive granules of

uniform size (Fig. 12). In contrast with the DDD diazo blue B reactive and other protein granules, the HNAH diazo blue B granules are distributed evenly throughout the cytoplasm, and their accumulations can hardly be seen (Fig. 12). The extracellular localization of this type of protein granules can be demonstrated, but it is not so frequent as in the case of the DDD diazo blue B reactive granules. Within the nuclear matrix there exist a small number of HNAH diazo blue B reactive coarse granules which are similar in coloration to those found in the cytoplasm. As in the cases of most other stains used in the present study, the vascular endothelium cytoplasm is most prominent in HNAH diazo blue B reaction, of all the interstitial elements. Prior incubation of sections in either sodium hydroxide or ethanol has hardly any effect upon the HNAH diazo blue B staining intensity of reactive granules in the cytoplasm of parenchymal cells.

DISCUSSION

As the general histology of the parathyroid gland in the rabbit shows, the parenchymal and interstitial tissues are common in features to those in other mammalian species such as the mouse (Yamada[25]) and rat (Hara and Yamada[10], Hara, Yamada and Hotta[11], Yamada[22]). The presence of glycogen granules and RNA particles in the cytoplasm of the rabbit parathyroid cells is, likewise, consistent with similar findings described previously in the gland of other mammals (Hara, Yamada and Hotta[11], Yamada[24]). On the basis of various experimental studies on the parenchymal cells of the parathyroid gland, glycogen has been advocated to be an energy source necessary for the secretory and metabolic activities of the cells (Yang[27], Hara, Yamada and Hotta[11], Hotta[15]). These experimental studies have also elucidated an important role played by the cytoplasmic RNA in protein synthesis for the same activities of the parenchymal cells (Hara, Yamada and Hotta[11], Hotta[15]). Such cytophysiological significances of the two cytoplasmic substances are supposed to hold true in the parathyroid cells of the rabbit.

In the parathyroid gland of the rabbit coupled tetrazonium reactive granules of various sizes are demonstrated in the cytoplasm of parenchymal cells. In the parathyroid gland of the monkey, similar granules were described and presumed to present a pattern of mitochondrial proteins (Hara and Yamada[12]), in view of their abundance in the cytoplasm of oxyphil cells in which mitochondrial concentration was confirmed by electron microscopy (Trier[21], Lange[18], Holzmann and Lange[14]). The coupled tetrazonium reactive granules observed here, may likewise, be comparable to mitochondrial proteins, inasmuch as their preponderant distribution is intracellular and sparse throughout the cytoplasm.

In the present study three types of protein granules have been newly demonstrated in the cytoplasm of the rabbit parathyroid cells. They are 1)

ninhydrin-Schiff, alloxan-Schiff and chloramine T-Schiff reactive granules, 2) DMAB nitrate reactive granules and 3) Sakaguchi reactive granules. In accorrdance with different residues of proteins responsible for the three histochemical reactions, the three types of granules are varied in cytological properties, such as size and distribution with one another, and are therefore independent. Until studies are made in the future on the response of these granules to experimental stimulation and supression of the parathyroid activity, however, their cytophysiological significances remain to be known.

As in the cytoplasm of the parathyroid cells of other vertebrate species such as the toad (Yamada[22]), Hara and Yamada[13]), quail (Yamada[23)24)]), mouse (Yamada[25)]), rat (Hara and Yamada[10)]) and monkey (Hara and Yamada[12)]), DDD diazo blue B reactive granules are demonstrated in the cytoplasm of the rabbit parathyroid cells. The effects of bilateral nephrectomy (Hara, Yamada and Hotta[11)]), and parathormone administration (Hara and Hotta[9)]) upon the chemocytological features of DDD diazo blue B reactive granules in the rat parathyroid cells have led us to the concept that these granules deserve a cytological indicator by which the secretory activity of the cells is assessed. In the parathyroid cells of the rabbit the DDD diazo blue B reactive granules are identical, in chemocytological characteristics such as stainability and distribution pattern, with those described in the cells of the rat. Therefore, it is likely that in the rabbit the secretory activity of the parathyroid cells may be parallel with the amount and stainability of this type of protein granules. Since prior treatment of sections by thioglycolate does not increase significantly the staining intensity of these granules, protein bound sulfhydryl groups are primarily responsible for the DDD diazo blue B reaction. This is endorsed by the effects of N-ethylmaleimide potassium cyanide sequence upon the reaction, indicating that protein bound disulfide groups are nearly absent in the granules. Moreover, the negative or doubtful performic acid alcian blue reaction in the cytoplasm of the parathyroid cells speaks for the absence of any appreciable number of protein bound disulfide groups in the granules. The alkaline tetrazolium method for the histochemical demonstration of protein bound sulfhydryl groups has disclosed the presence of reactive granules which are nearly identical in chemocytological properties to the DDD diazo blue B reactive granules. Thus, the responsibility of protein bound sulfhydryl groups for the staining of both the DDD diazo blue B and alkaline tetrazolium reactive granules is evident.

The DNFB-H acid technique for the demonstration of proteins with tyrosine residues and amino and sulfhydryl groups has revealed the presence of reactive fine and coarse granules in the cytoplasm of the present parathyroid cells. In view of the protein bound groups responsible for the reaction, some of these granules must be of the same cytophysiological meaning as that described for the DDD diazo blue B and tetrazolium reactive granules. However, the

plurality of the responsible groups of proteins makes it difficult to elucidate the true natures of the DNFB-H acid reactive granles.

In the parenchymal cells of the rabbit parathyroid gland HNAH diazo blue B reactive granules are observed which have chemocytological characteristics common to those of similar granules in the toad (Hara and Yamada[13]), quail (Yamada[23)25)]), mouse (Yamada[25)]), rat (Hara and Yamada[10)], Hara, Yamada and Hotta[11)]) and monkey (Hara and Yamada[13)]). Based upon the fact that in the monkey parathyroid gland the HNAH diazo blue B reactive granules are densely packed in the cytoplasm of oxyphil cells containing an abundance of mitochondria (Trier[21)], Lange[18)], Holzmann and Lange[14)]), the majority of these granules were concluded to be mitochondrial proteins. In addition, their extracellular localization has been provocative of their other cytophysiological significances such as a possible relation to the secretory activity of the parathyroid cells (Hara, Yamada and Hotta[11)]). The present cytological data on the HNAH diazo blue B reactive granules are not decisive enough to gain any insight into their true nature. However, the similarity of their chemocytological properties to those of similar granules in the monkey parathyroid gland lends support, at least in part, to the idea that their majority represent a pattern of mitochondrial proteins. The failure of the sodium hydroxide or ethanol treatment to alter the HNAH diazo blue B staining intensity of the granules indicates that the protein bound carboxyl groups responsible for the reaction are of side chain in nature (Karnovsky and Fasman[16)], Karnovsky and Mann[17)]). This result is in line with that reported for similar granules in the parathyroid cells of other animal species examined previously (Yamada[23)25)]).

Of the interstitial tissue components, the vascular endothelial cells are worthy of discussion because of their outstanding reactions for most of the tests of protein histochemistry. Together with the intense reaction for alkaline phosphatase described in the vascular endothelium of the rat parathyroid gland (Hara and Yamada[10)], Hara, Yamada and Hotta[11)]) the distinguished reactions for proteins of the present endothelial cells are interpreted to bespeak their importance in exchanges of substances between the secreting parathyroid cells and blood stream.

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EXPLANATION OF PLATE FIGURES

FIG. 1. Parathyroid gland of a rabbit. Masses of the parenchymal cells are invaded by highly vascular interstitial layers. Hematoxylin eosin. ×240.

FIG. 2. Part of the parathyroid gland of a rabbit. The parenchymal cells contain a large round or oval nucleus and have finely granular cytoplasm. Hematoxylin eosin. ×1,300.

FIG. 3. Part of the parathyroid gland of a rabbit. In the cytoplasm of parenchymal cells a varying amount of PAS reactive granules of different sizes are seen. PAS and hematoxylin. ×1,300.

FIG. 4. Part of the parathyroid gland of a rabbit. In the diffusely pyroninophilic cytoplasm of parenchymal cells is noted the presence of clumps of distinctly pyronin reactive granules. Methylgreen pyronin. ×1,300.

FIG. 5. Part of the parathyroid gland of a rabbit. Note distinctly coupled tetrazonium reactive granules in the cytoplasm of parenchymal cells and similarly reactive vascular endothelium and red blood corpuscles in the interstitial layers. Coupled tetrazonium. ×1,300.

FIG. 6. Part of the parathyroid gland of a rabbit. The cytoplasm of parenchymal cells is loaded with a variable amount of ninhydrin-Schiff reactive fine granules. Ninhydrin-Schiff. ×1,300.

FIG. 7. Part of the parathyroid gland of a rabbit. In some parenchymal cells, moderately DMAB-nitrate reactive coarse granules are accumlated in peripheral or perinuclear areas. DMAB-nitate. ×1,300.

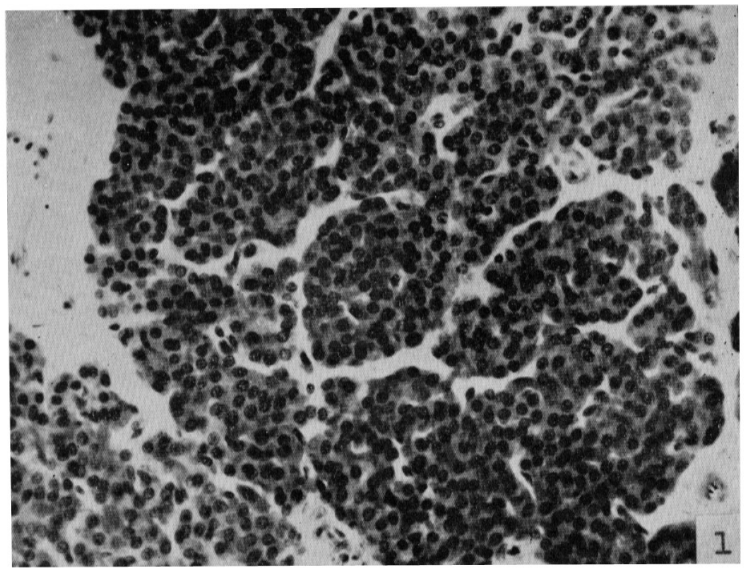
FIG. 8. Part of the parathyroid gland of a rabbit. In the cytoplasm of a number of parenchymal cells, moderately Sakaguchi reactive granules of different sizes are disseminated. Sakaguchi reaction. ×1,300.

FIG. 9. Part of the parathyroid gland of a rabbit. DDD diazo blue B reactive granules are characteristically accumlated at juxtanuclear and peripheral loci of parenchymal cells and can be seen in the extracellular speces. DDD diazo blue B. ×1,300.

FIG. 10. Part of the parathyroid gland of a rabbit. In the glandular tissues, the distribution of alkaline tetrazolium reactive granules is nearly identical with that in the previous figure. Alkaline tetrazolium. ×1,300.

FIG. 11. Part of the parathyroid gland of a rabbit. In the cytoplasmic matrix of parenchymal cells, intensely DNFB-H acid reactive fine and coarse granules are imbedded. DNFB-H acid. ×1,300.

FIG. 12. Part of the parathyroid gland of a rabbit. Throughout the cytoplasm of parenchymal cells vividly HNAH diazo blue B reactive granules of a uniform size are demonstrated which vary in amount with individual cells. HNAH diazo blue B. ×1,300.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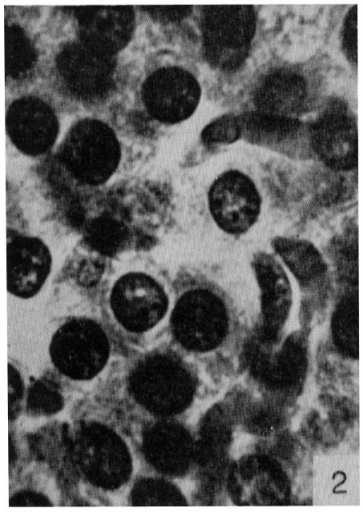
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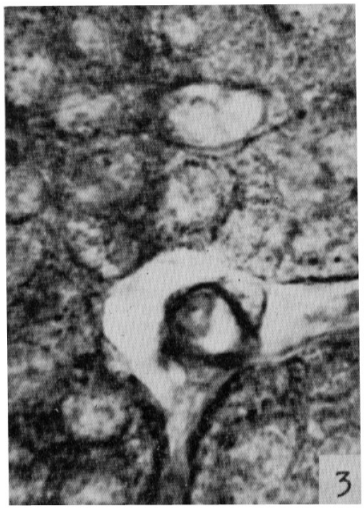
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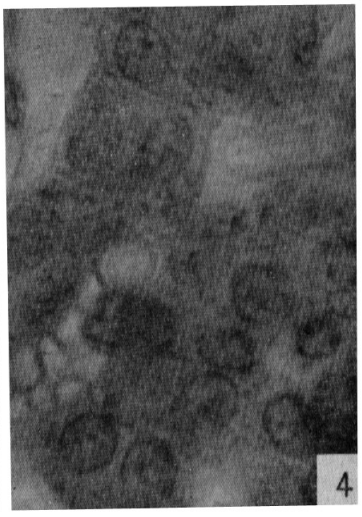
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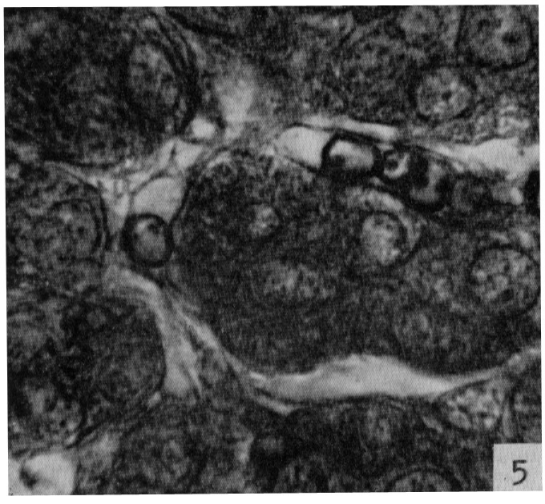
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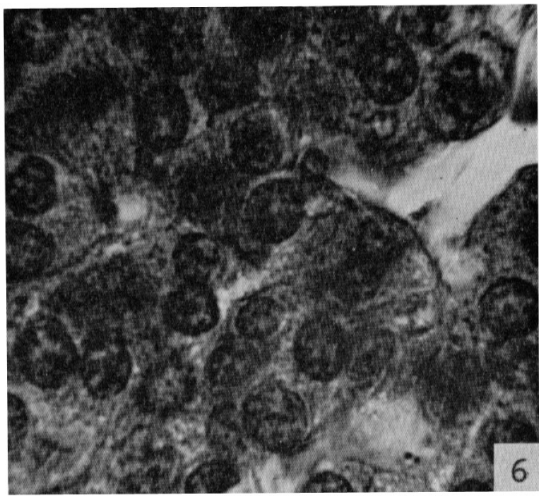
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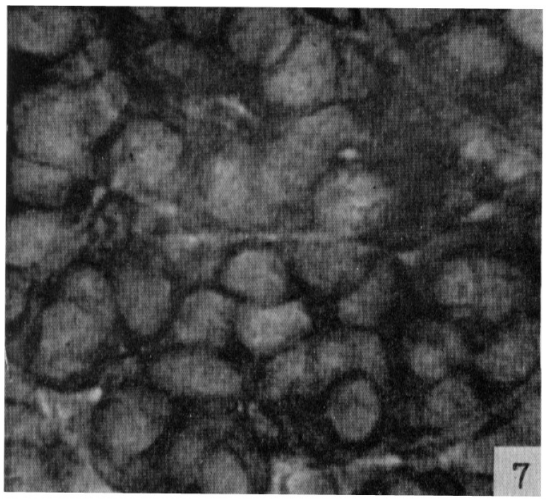
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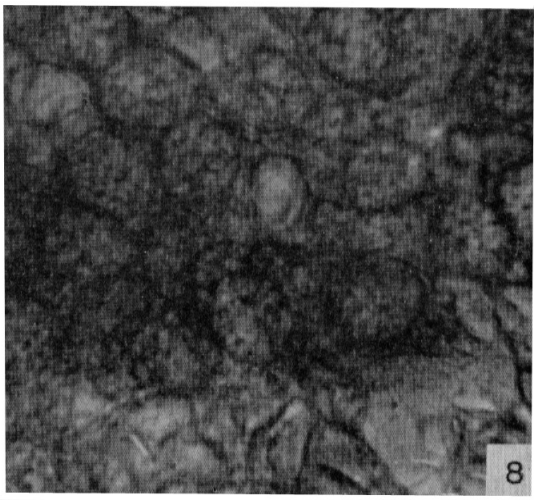
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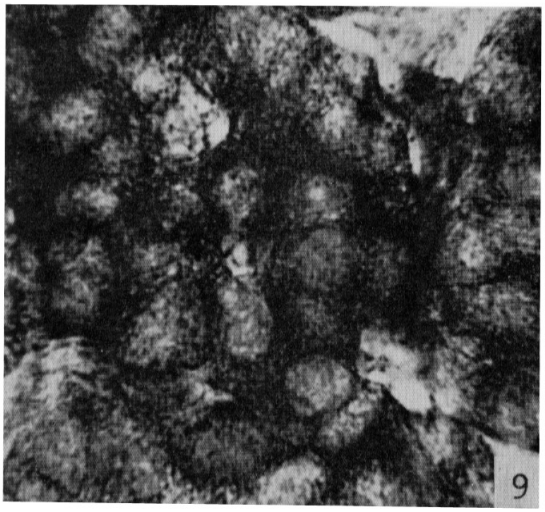
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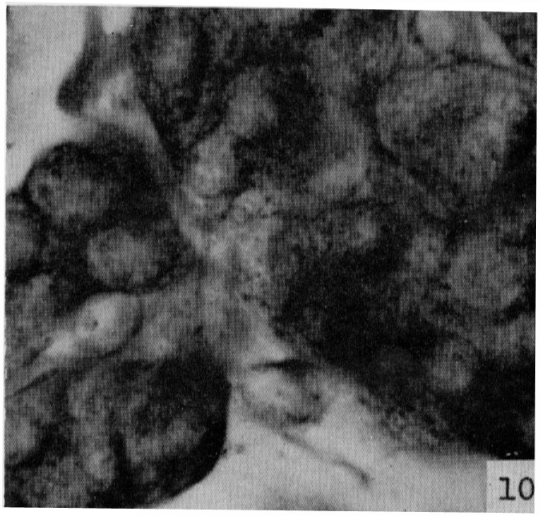
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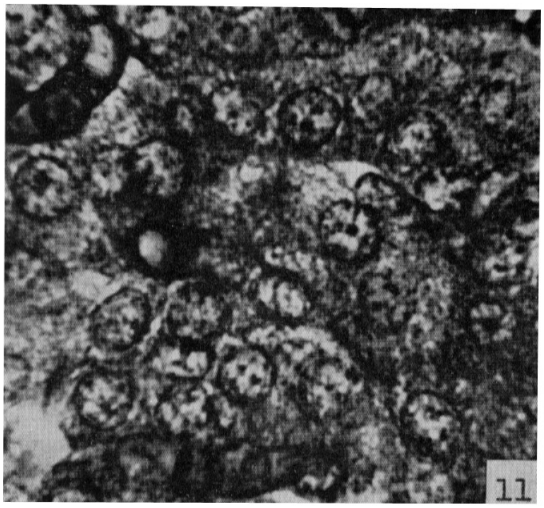
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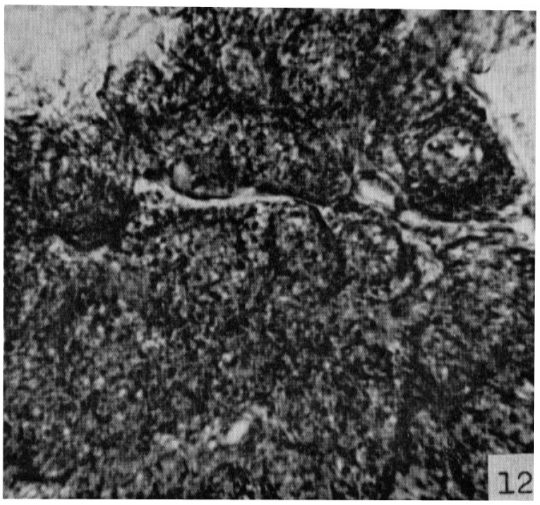
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