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Histochemical Investigations on the Human Fetal Subcommissural Organ

II. The "Large Granules"*

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Summary. A histochemical and ultrastructural study was carried out on subcommissura organs from 42 human embryos and fetuses in order to characterize some "large granules"

Typical "granules" make their appearance in the rostral hypendymal region of the subcommissural organ (SCO) in fetuses of about 50 mm CRL. Although they appear in other SCO-regions later, the highest number of "granules" is always located towards the pineal gland.

Typical "granules" are of spherical shape with a diameter of about 2 microns. The various histochemical reactions reveal a reactivity which differentiates the shell of the "granules" from the "granule" interior. Nucleoproteins are present in the shell together with phospholipids and/or lipoproteins. The interior of the "granules" can contain different materials such as glycogen or lipid or "neurosecretory substance". Ultrastructural observations show that a "granule" consists of whorls of endoplasmic reticulum sparsely studded with ribosomes surrounding an interior containing either lipid or lipoprotein inclusions, large amounts of glycogen or simply cytoplasm.

It is suggested that the concentric lamellar organelle (CLO) is a morphological entity that might be involved in secretory processes rather than being the secretory granules themselves.

Introduction

In a previous study of the human fetal subcommissural organ (SCO) (Møllgård, 1972) the rostral part seemed to show 1. a distinct activity of alkaline phosphatase, 2. a stronger PAS-positivity than the rest of the organ, 3. a pronounced metachromasia in a perivascular position and 4. a number of "large granules".

A more recent investigation (Møllgård, in press) lists data that indicate that the rostral part of SCO may demonstrate some secretory mechanism. The "large granules" seen by light microscopy in the active rostral area of SCO and often very close to an extended perivascular space, represent tempting candidates for secretory granules. However, it is pointed out in the same paper that: "the precise nature and significance of the granules is enigmatic".

The purpose of the present study is to solve this enigma by histochemical means as far as possible. Furthermore, we attempt to give a four-dimensional description of the so-called granules (the distribution of the particles in space and time), since both their location and their number are significant in reference to the developmental stage of the SCO.

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Material and Methods

The material examined was supplied by way of legal abortions and comprises subcommissural organs from a total number of 42 human fetuses of both sexes. It includes fetuses of the following crown-rump-length (CRL) (all measurements being made on the unfixed material): 20, 24, 27, 29, 30, 32, 35, 50, 54, 55, 56, 57, 62, 66, 70, 74, 75 (2), 76, 88, 90, 92 (2), 94, 95, 100, 102, 103, 109, 110, 116, 132, 146, 156, 157, 162, 167, 168, 176, 178, 186, 196 mm. The fetuses used for lipid investigations measured 95, 103, 109, 146, 157, 167, 168 mm. The postmortem interval preceeding fixation was less than 30 min.

Fixation and Preparations. Ice-cold fixation was performed with the following fixatives: 1. Formol-calcium (pH = 5.3) (Pearse, 1968). Fixation time 24 hrs. With regard to amino acid reactions a fixation's time from 4–12 hrs was used. Lipid investigations were carried out following 48 hrs. of fixation in formol-calcium. — 2. Ethanol-formalin-acetic acid (pH = 3.7) (Lillie, 1965). Fixation time 24 hrs. — 3. Glutaraldehyde 2.5% in a 0.1 M sodium cacodylate buffer (pH = 7.4). Fixation time 6–8 hrs. This material was postfixed for 2 hrs in 2% OsO_4 in the same buffer, stained *en bloc* in 0.5% aqueous uranyl acetate for 1 hr and embedded in Epon after dehydration.

Serial paraffin sections of 3–5 micron were cut in sagittal or frontal planes. Following fixation the tissue intended for lipid investigations was stored in ice-cold gumarabic-sucrose until sectioned. After initial cooling in a CO_2-expansion cooler with a covering device, 4–6 micron sections were cut on a Pearse-Slee cryostat (type HR) at —25° C. From the Epon-embedded material 1 micron survey sections were obtained. Thin sections (500–600 Å) were used to compare the histochemical results with the fine structure. These sections were post-stained by uranyl acetate and lead citrate.

Histochemical Methods

I. Carbohydrates and Mucosubstances. 1. Alcian blue (0.05%) (G.T. Gurr) — critical electrolyte concentration (Scott and Dorling, 1965; Scott and Stockwell, 1967; Scott *et al.*, 1968). — 2. Periodic acid-Schiff. The PAS-method was used with de Tomasi's as well as with Barger and de Lamater's Schiff reagent (Pearse, 1968). Periodic acid was used as 0.5% as well as 1% aqueous solutions. Prior to the treatment with periodic acid, some sections were coated with celloidin in order to avoid loss of glycogen due to the periodic acid. As a control some sections were digested by Maltase (Merck) (Pearse, 1960) before staining. Other control sections were stained omitting the periodic acid oxidation. — 3. Metachromatic staining with 0.1% toluidine blue 0 (Merck) in 30% ethanol (Kramer and Windrum, 1955). Staining time 1–10 min.

II. Proteins. 4. Bennett's 1–4 (4-chloromercuriphenylazo)-2-naphthol (Mercury Orange) method for sulphydryl groups (Bennett and Watts, 1958). A saturated solution i dimethylformamide was used. Staining time 2–16 hrs. Some sections were stained for S-S groups after thioglycollate reduction (Pearse, 1968). — 5. Performic acid-Alcian blue (PFA-AB) method for SH and S-S groups (Adams and Sloper, 1956). Control sections were stained by Alcian blue (3%) in 2 N H_2SO_4 for 1 hr thus omitting the performic acid oxidation. — 6. The Adams (1957) DMAB-nitrite method for tryptophan (Pearse, 1968). — 7. The Yasuma and Itchikawa ninhydrin-Schiff method for proteinbound NH_2 (Pearse, 1968). Control: No deamination before treatment with Schiff's reagent. — 8. The Bensley and Gersh Millon-reaction for tyrosine (Pearse, 1968) modified by Andersen (1964). Control: Iodination of the tyrosine side-chain. — 9. The Sakaguchi reaction for arginine (Baker's modification, Pearse, 1968).

III. Nucleoproteins. 10. Feulgen reaction for DNA (Pearse, 1968). — 11. Chromalum-gallocyanin reaction for nucleic acids (Einarson, 1951; Pearse, 1968). — 12. Methyl green-pyronin reaction for nucleic acids (Pearse, 1968). Methyl green-pyronin was also used with $MgCl_2$ or in a sodium acetate buffer as suggested by Scott (1967). — 13. The previously mentioned staining with toluidine blue was also used to detect the presence of nucleoproteins. — 14. Sections digested by ribonuclease (Fluka) (1 mg/ml dist. water, pH = 5.6) for 1–2 hrs were used as a control in each of the above-mentioned staining methods for nucleoproteins.

IV. Reactions for "Neurosecretory Substance". 15. Bargmann's Chrom Hematoxylin Method (Pearse, 1968). — 16. Bock's Chromalum Gallocyanin Method (Bock, 1966). — 17. The reaction for cystine (PFA-AB) was compared with abovementioned reactions for "neurosecretory substance". — 18. Sections digested by ribonuclease (Fluka) (1 mg/ml dist. water, pH = 5.6) for 1–24 hrs were used as a control in each of the above-mentioned staining methods.

V. Lipids and Lipoproteins. 19. Sudan Black B staining of paraffin sections as well as of frozen sections (Pearse, 1968). — 20. Hori's acid hematein method (Pearse, 1968). — 21. Adams' Osmium tetroxide-Alpha naphthalamine method (Pearse, 1968). — 22. Lillie's Oil Red method (Pearse, 1968). — 23. Schultz Method for cholesterol and its esters (Pearse, 1968). — 24. The Okamoto method for cholesterol and its esters (Pearse, 1968).

Observations

In a recent paper (Møllgård, 1972) a description of the general morphology of the human fetal subcommissural organ has been given. The same study includes a number of different histochemical reactions. These data will not be mentioned in the following except for a few observations of relevance to the present study. Apical and basal cytoplasm is defined in the same paper.

The SCO can be divided into four areas from a topographical point of view namely, pars supracommissuralis, pars precommissuralis, pars subcommissuralis and pars retrocommissuralis. The subdivision is indicated in Fig. 1 (for a more extensive description of the subdivision cf. to Palkovits, 1965). In each area, throughout the organ, it is possible to distinguish between an ependymal layer and a hypendymal layer. The hypendyma consists of glial cells, SCO cells and neurons.

Morphology and Distribution of the Granules at Different Developmental Stages

It was not possible to observe any difference between granules from male and female fetuses.

The granules are in sections stained with toluidine blue first seen in human fetuses of about 50 mm CRL. They are found in the basal cytoplasm of cells of the pars supracommissuralis and often in very close relation to large capillaries. The granules are of spherical shape with a diameter usually ranging from 0.5 to 4 microns. They demonstrate a weakly stained interior surrounded by a fine shell of a red-violet metachromatic material. Since the elements have the same appearance in frontal and sagittal sections, and since it is possible to make them disappear by focusing up and down within the sections, they are considered to be spherical particles and not cross-sectioned tubules.

The number of granules observed increases very much with increasing maturation of the SCO partly because of the growth of the organ but especially because the granules tend to get more closely packed i.e. the actual concentration increases. At about 100 mm CRL the granules are abundant in the hypendyma of pars supracommissuralis and pars precommissuralis (see Fig. 2). At the same stage of development very few granules can be distinguished in the ependyma of pars subcommissuralis. With further development granules get more closely packed in the hypendyma (in one single paraffin section several hundred granules were counted in the SCO of a fetus of CRL = 167 mm). Thus the granules are first found widely spaced in the hypendymal region of pars supracommissuralis, and although they appear in other parts of SCO later, the pattern remains the same, the highest number of granules always being seen towards the pineal gland.


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Histochemical Reactions of the Granules

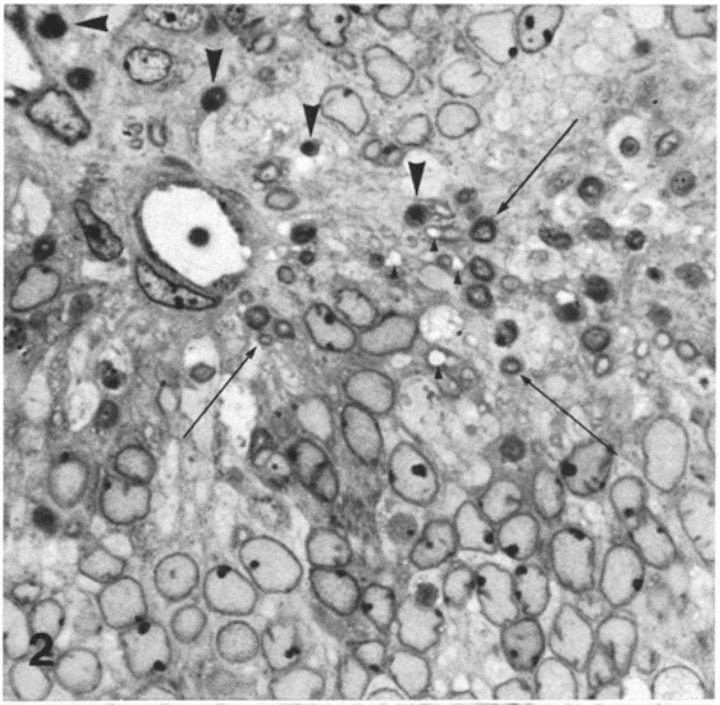
It should be emphasized that this study deals exclusively with the description of the granules. Most of the methods performed demonstrate a reactivity located in other parts of the SCO as well (cf. Møllgård, 1972). In particular, reactions for lipids and "neurosecretory substance" are prominent outside the granules, but these results will not be dealt with here.

I. Carbohydrates and Mucosubstances. A negative reaction of the shell as well as of the interior of the granule is found when the sections are stained according to the Alcian blue-CEC method, and no red metachromasia is seen following toluidine blue staining. A very pronounced PAS-positivity is seen in the hypendyma (Fig. 3). At high magnification it is possible to identify numerous granules. The shell can be seen as an unstained ring surrounding a strongly PAS-positive interior (indicated with arrows in Fig. 3). Granules with a PAS-negative interior are also found. The basal cytoplasm surrounding the granules is frequently strongly PAS-positive. The strongest reactions are obtained when 1% periodic acid is used after a celloidin-coating of the sections (cf. Møllgård, 1972). Following maltase digestion the PAS-positivity of the granule-interior is largely diminished.

II. Proteins. The Mercury Orange method for sulphydryl groups demonstrates a strong reaction in the periphery of the granules. The reaction product can also be found in the interior of the granules. Previous reduction with thioglycollate accelerates the reaction, but reveals no qualitative changes. Although the PFA-AB method for S-S groups is destructive to the vulnerable fetal material, a positive reaction in the shell is evident. In some of the granules a reaction product is found in the granule-interior as well. The DMAB-nitrite method, the Millon reaction, the Sakaguchi reaction and the ninhydrin-Schiff reaction show positive reaction products mainly confined to the shell. Control reactions (cf. Material and Methods) are negative.

III. Nucleoproteins. The Feulgen reaction for DNA shows a distinct nuclear reaction while the granules remain unstained. After chromalum-gallocyanin staining a nuclear reaction as well as a strong reaction of the shells is observed (Fig. 4a). The shell-stainability is not seen if the sections are digested by ribonuclease prior to the staining reaction (Fig. 4c). The shell is stained red with methyl green-pyronin and shows a red-violet metachromasia after toluidine blue staining. Both of these staining reactions are abolished after digestion by ribonuclease. Following a prolonged digestion an interesting phenomenon is clearly revealed by the disappearance of all cytoplasmic basophilia. A stainability is located to the granule-interior while the shell is non-reactive (Fig. 4c). This effect is particularly pronounced after chromalum-gallocyanin staining, though it is also observed with toluidine blue.

Fig. 1. Sagittal section through the entire subcommissural organ which is subdivided into pars supracommissuralis (1), pars precommissuralis (2), pars subcommissuralis (3), and pars retrocommissuralis (4). This subdivision is based on topographic relations rather than on histological differences from one region to the next, since a smooth transition exists histochemically from the most rostral to the most caudal part of the SCO. *ca* cerebral aqueduct; *mr* mesocoelic recess; *P* pineal gland; *pr* pineal recess; CRL 168 mm; chromalum-gallocyanin. × 78



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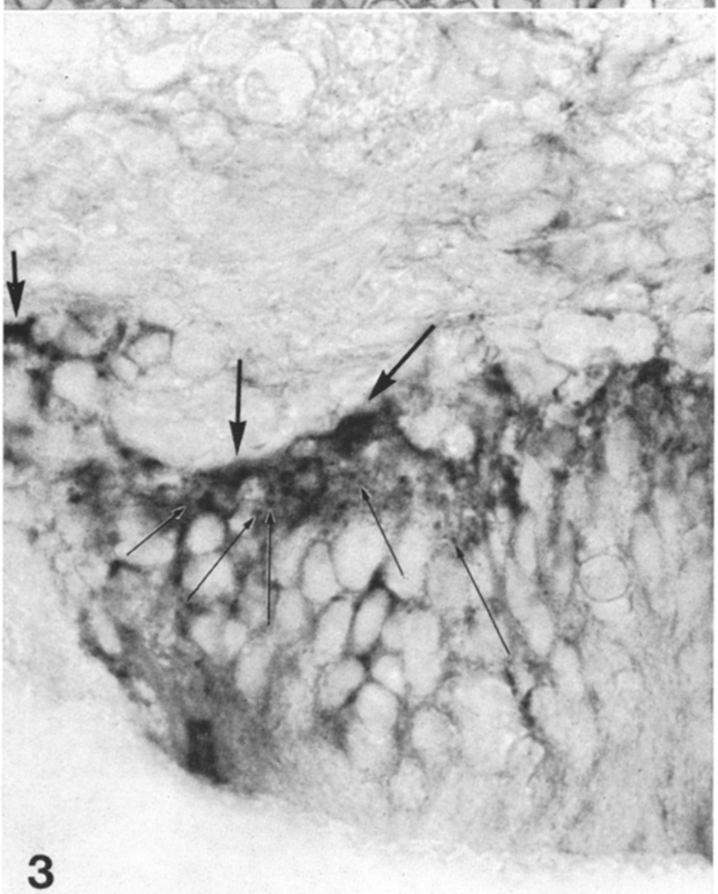

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IV. Neurosecretory Substance. In older fetuses the methods for neurosecretory substance reveal numerous hypendymal granules (Fig. 5). In the younger fetuses the granules are visualized better by the method of Bock than by the method of Bargmann. Stainability of the granules is also found in sections which have been digested with ribonuclease. The reaction product is mainly confined to the interior of the granules. The PFA-AB reaction is described in the section on proteins.

V. Lipids and Lipoproteins. The shell of the granules is stained intensively by Sudan Black B (Fig. 6) in paraffin sections as well as in frozen sections and also by Hori's acid hematein. Following the OTAN reaction the shell of all the granules is stained orange-red while the interior of some of the granules turns black. The interior of some of the granules stains weakly with Sudan Black B and Hori's acid hematein. However, it is not possible to exclude the possibility that this reaction is caused by parts of the shell lying deeper in the section.

No positive reactions are observed following the Oil Red method. The Okamoto reaction is very destructive to the fetal material and though a slight blue-green reaction is present in the hypendymal area it is impossible to localize the reaction products to any particular structure. The Schultz method is better suited to the vulnarable fetal material. Small green particles can be distinguished in the hypendymal area, and by phase-contrast microscopy it is possible to observe the green reaction products in some typical granules. Other granules are non-reacting.

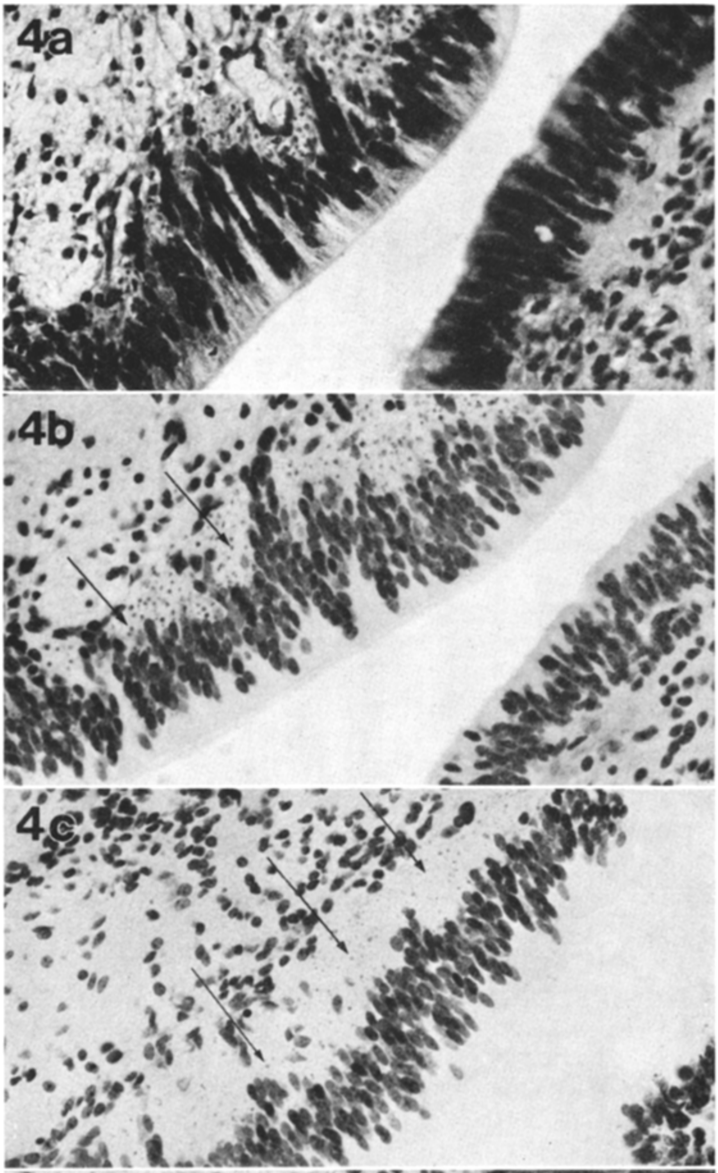
VI. Ultrastructural Observations. Only observations relating to the granules will be dealt with here. Different types of granules are shown in Fig. 7a—d. A typical granule consists of whorls of concentric endoplasmic reticulum membranes sparsely studded with ribosomes. The interior can contain lipid or lipoprotein inclusions, large amounts of glycogen, lysosomes and degradation products or eventually what appears to be cytoplasm.

Discussion

The term granule is used to describe various small particles. Whether a particle should be considered of small size or not depends how the actual observation is made. If histochemistry at a light microscopic level is the method employed one could designate particles with a diameter from 0.2 to a few microns as granules, whereas, at the electron microscopic level, the term "granules" usually describes particles falling in the range of 40–300 nm (400–3000 Å).

Fig. 2. Numerous "large granules" are characteristic constituents of the hypendymal region of pars supracommissuralis in human fetuses from about 100 mm CRL. The dense stained shells usually surround a more lucid interior (small arrows), but occasionally the granule interior contains strongly stained material (large arrowheads), or appears totally empty (small arrowheads). CRL 116 mm; toluidine blue. A one micron section of Epon embedded material. $\times 1120$

Fig. 3. A very pronounced PAS-positivity is present in pars supracommissuralis. Note the unstained shells (indicated by small arrows) which often surround a strongly PAS-positive interior. A strong reaction is also prominent between the granules. Large arrows indicate the border between the SCO and the pineal gland. Sagittal section through the rostral part of pars supracommissuralis; CRL 167 mm; PAS method without maltase digestion. $\times 1500$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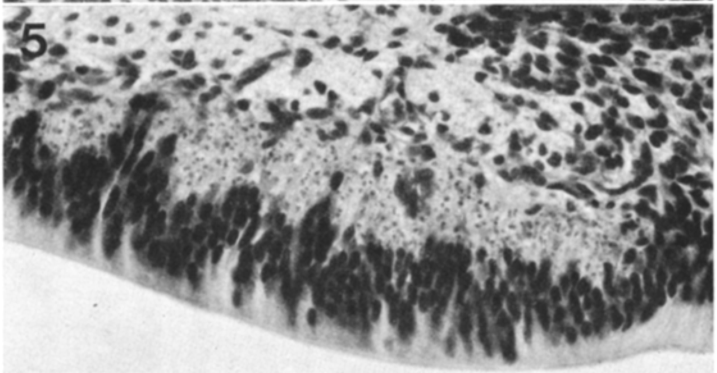
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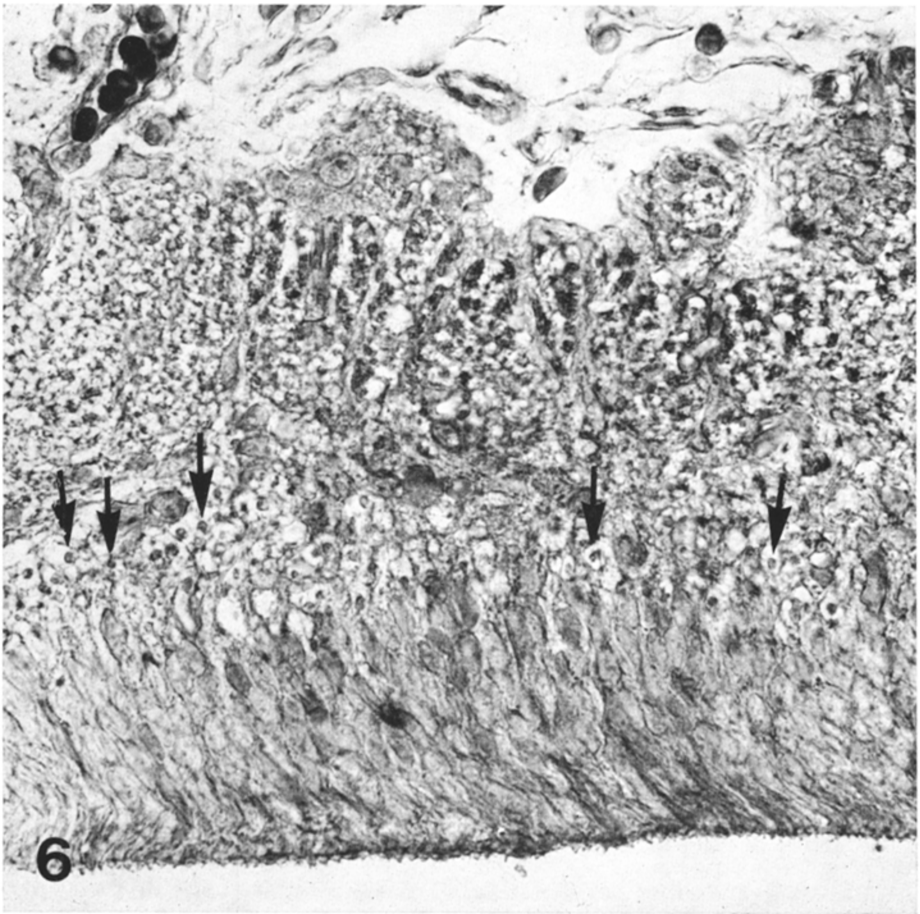

Fig. 6. A distinct reactivity is present in the shells of the granules (indicated by arrows) following the Sudan Black B reaction. The weak stainability which is sometimes seen in the granule interior is difficult to demonstrate on a photograph. Sagittal paraffin section through pars subcommissuralis. CRL 167 mm. × 600

Fig. 4a—c. Chromalum-gallocyanin staining without previous ribonuclease treatment (Fig. 4a), after one hour (Fig. 4b) and after six hours (Fig. 4c) of ribonuclease digestion prior to the staining reaction. Fig. 4a, b and c are from the same series of sagittal sections through the pars supracommissuralis. The strong shell stainability which can be observed in Fig. 4a is somewhat diminished in Fig. 4b but is on the other hand more easy to observe since most cytoplasmic basophilia is now removed. Typical granules are still recognizable (two are indicated by arrows). After a prolonged RNA-ase digestion (Fig. 4c) a surprising phenomenon is revealed. The only stainable structure in the cytoplasm seems now to be the granule interior, whereas the shells can be identified as unstained rings (arrows). CRL 168 mm. × 375

Fig. 5. A sagittal section from the same series as demonstrated in Fig. 4a—c stained with Bargmann's chrome hematoxylin method. This picture demonstrates that the "large granules" are also stained by a method which has been considered specific for "neurosecretory substance". CRL 168 mm. × 375

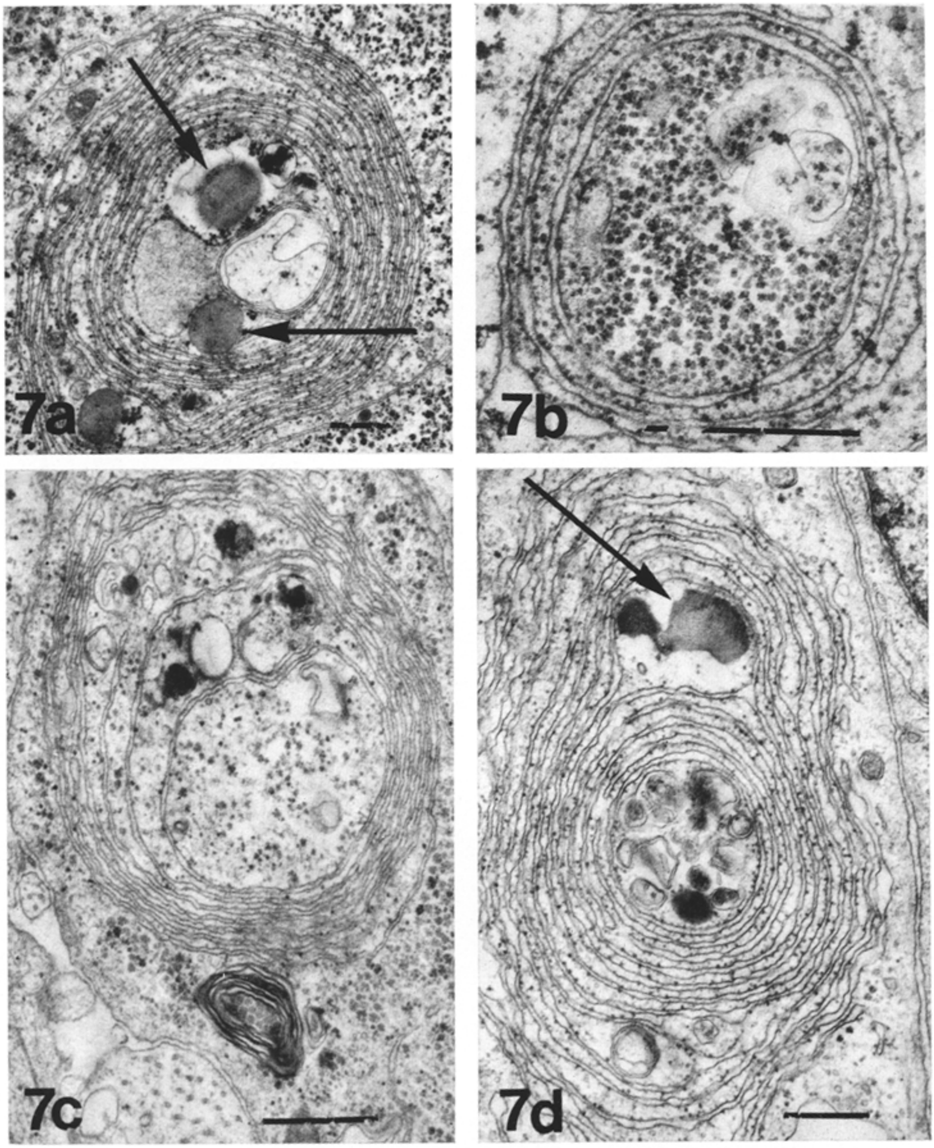

Fig. 7a—d. SCO cells from the hypendymal layer contain whorls of concentric endoplasmic reticulum membranes sparsely studded with ribosomes. Lipid or lipoprotein inclusions (arrows) are seen in Fig. 7a and d. The membranes in Fig. 7b enclose an accumulation of glycogen. Note the difference between the number of ribosomes associated with the membranes in Fig. 7c in comparison to Fig. 7d. The membranes of Fig. 7d enclose what appear to be degradation products. The bar indicates 0.5 μ.

Fig. 7a. CRL = 102 mm, $\times$ 16000
Fig. 7b. CRL = 102 mm, $\times$ 57000
Fig. 7c. CRL = 54 mm, $\times$ 30000
Fig. 7d. CRL = 178 mm, $\times$ 22000

It should be realized that most of the histochemical methods used in the present study are pushed to their limit since we want to distinguish between those histochemical reaction products located primarily in the shell and those characteristic of the granule interior which is often less than 1 micron in diameter. Prior to discussion on the spatial distribution of granules through developmental stages it seems useful to summarize the morphological and histochemical data that allow us to define the different constituents of a typical granule. The granule seems to be a hollow sphere, the histochemical reactions of which can be confined to the whole sphere, the shell or the interior.

The very strong stainability of the shell following toluidine blue or chromalum-gallocyanin staining, together with the lack of stainability after previous RNA-ase digestion, indicates that ribonucleic acids are present in the shell. The various positive amino acid reactions demonstrate that protein is also present in the shell. The Sudan Black B reaction, both in paraffin and in frozen sections, and the acid hematein test demonstrate a strong reaction likewise confined to the shell which points to the presence of phospholipids and/or lipoproteins. The presence of ribonucleoproteins as well as phospholipids indicates that the shell may consist of circularly arranged cisternae of membranes, probably of the rough-surfaced type. However, the possibility that smooth membranes together with free polyribosomes give the same reaction also exists.

The granule interior is often found to be PAS-positive. In most cases the PAS-positivity disappears after maltase digestion. However, it is possible to find granules without any activity. It is concluded that glycogen is often present in the granule interior. In some of the granules there is a definite reaction for different amino acids in the interior, but since the methods are somewhat destructive to the vulnerable fetal material, and since the granule interior often has a size close to the resolution power of the light microscope as mentioned previously, a further discussion on this subject is not given. "Neurosecretory substance" is often present in the interior of the granules as can be clearly demonstrated after RNA-ase digestion. The stainability with chromalum-gallocyanin after prolonged RNA-ase digestion is difficult to explain at present, but the phenomenon is probably caused by polyanionic substances of non-nucleic acid nature. The lipid reaction localized to the interior indicates the presence of lipoid substances. The Oil Red reaction is negative which indicates that the lipid in question is not neutral fat, but the positive Schultz reactions point to the presence of cholesterol or cholesterol esters in the interior of some granules. This observation is supported by the Okamoto and OTAN reactions. A further description must await extraction experiments combined with more extensive lipid histochemical investigations. In conclusion, the granule interior may contain glycogen, amino acids as found in normal cytoplasm, "neurosecretory substance" and lipids in any combination.

When a comparison is made between the above-mentioned histochemical results and the ultrastructural observations, it appears that what has been termed "granules" until now should be considered organelles consisting of concentric membranes with varying inclusions.

When the typical shape, size and distribution of the socalled granules are kept in mind, they can be identified in numerous studies of the SCO, though under various names. A short account of different findings in various subcommissural organs that probably all can be traced back to "the concentric lamellar organelle" (CLO) described in the present study, will be given in the following.

Argentophil granules seen following Bodian's Protargol method described by Leonieni (1969) in different mammals have the same size and localization as the granules described in the present study. We have used the same method, and found a stainability of the granule-shell (unpublished results), but we have never seen contact between "granules" and nerve fibers as mentioned by Leonieni (1969).

Neurosecretory Material. The SCO from various species has been extensively studied with methods considered selective for neurosecretory material, since Stutinsky (1950) first demonstrated Gomori-positive material in the organ. With our present knowledge in mind the "granules" can be identified in most studies on the SCO where these "selective stains" for neurosecretory material have been used (see e.g. the description and the distinct figures in Bargmann and Schiebler, 1952; Wislocki and Leduc, 1952; Wingstrand, 1953). The human fetal SCO has also been stained for "neurosecretory material" and again it is easy to identify the CLO (e.g. Wislocki and Roth, 1958 [Fig. 3]; Olsson, 1961 [Fig. 2]).

In hypendymal endfeet Herrlinger (1970) described dark blue secretion granules after aldehyde thionin staining, the stain which she considers the best and most specific for neurosecretory substance. According to our findings, Herrlinger's light microscopic dark blue granules (see e.g. her Fig. 1 b) cannot correspond to the granules she finds electron microscopically in the basal processes, but rather correspond to concentric lamellar organelles (CLO). We cannot exclude the possibility, however, that some of the "neurosecretory material" described by her is entrapped within the CLO.

"*Nebenkern*" (a German word for paranucleus—an old light microscopic term for a body resembling a nucleus found in the cytoplasm near the nucleus. For a review of the literature, see Haguenau, 1958).

The structure termed "Nebenkern" in light microscopic studies of SCO also corresponds to the CLO described in the present study. Isomäki *et al.* (1965) described "tightly packed spiral coils of endoplasmic lamellae" often containing osmiophilic droplets. These structures were considered to be the same as "Nebenkerne". This statement was later supported by Vigh *et al.* (1967) and by Papacharalampous *et al.* (1968).

Electron Microscopic Descriptions. The concentric lamallar organelles have been reported in a number of EM-studies under various names. The findings by Murakami *et al.* (1970) are of special interest for the present study. They reported that a concentric lamellar formation of granular endoplasmic reticulum occurred frequently in the basal part of SCO-cells in human fetuses. It was suggested that this structure has merely a phylogenetic significance in man. Barlow *et al.* (1967) described lamellar cytoplasmic lipid bodies in young lambs scattered throughout the deep layers of SCO, while they found tightly-coiled membranous whorls (fingerprints) in adult sheep. They proposed that these changes indicate involution. Oksche (1969) found whorl-like multilamellar bodies in dog, and Smith (1970) described "whorls of concentric endoplasmic reticulum membranes sparsely studded with ribosomes and containing lipid or lipoprotein inclusions" in the hypendymal layer of embryonic chicken. However, a more thorough discussion of the ultrastructure of the CLO and its relation to other related structures is not the purpose of the present histochemically oriented study.

It seems unlikely that the CLO described in the present study have merely a phylogenetic significance as suggested by Murakami *et al.* (1970). In the earliest stages investigated the organelles are not found, but at CRL = 50 mm these cellular structures begin to appear in the pars supracommissuralis, that region of the SCO which according to an earlier study (Møllgård, 1972) is known to be the site of extremely high and diversified metabolic activity.

A definite polarization of the SCO-cells is established by the fact that the granules tend to accumulate in the basal processes in close relationship to large capillaries. It seems unlikely that the presence of these granules should indicate degenerative changes since they appear at the initial stages of development of the SCO, and since they are definitely associated with SCO-regions containing the previously mentioned high metabolic activity.

Probably, the organelles are involved in a secretory process. They could add a specific component (cholesterol?), or they could function in concentrating and/or storing mechanisms. They might function in an inactivation of a surplus secretion. At present it is not possible to determine whether the "granules" which contain different material (e.g. glycogen or lipid) represent different functional stages of the same organelle or whether they represent a possibility of performing diversified functions.

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