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A possible structural basis for the extracellular release of acetylcholinesterase

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[Plates 8–17]

The ultrastructural localization of acetylcholinesterase and non-specific cholinesterase activity has been studied in sections of ox adrenal medulla by cytochemical methods. Non-specific cholinesterase activity, identified by using butyrylthiocholine as substrate and ethopropazine as inhibitor, occurs intracellularly in some adrenaline-containing chromaffin cells: the reaction end-product is deposited within the cisternae of the endoplasmic reticulum and in the nuclear envelope. Reaction end-product of non-specific cholinesterase also occurs in the endoplasmic reticulum of pericytes, around sinusoids and capillaries and within smooth muscle cells.

Acetylcholinesterase activity, identified by using acetylthiocholine as substrate and BW 284C51 as inhibitor, occurs in both the splanchnic nerve and in chromaffin cells. Reaction end-product is found at the following sites

(i) around myelinated and unmyelinated non-terminal axons of splanchnic nerve, between the axolemma and the Schwann cell membrane;
(ii) within the cisternae of axonal smooth endoplasmic reticulum; sometimes these cisternae appear to be connected to the axolemma;
(iii) between the axolemmas of preterminal axons and the plasma membranes of chromaffin cells;
(iv) between the axolemmas of nerve terminals and the plasma membranes of chromaffin cells, including the synaptic cleft;
(v) within cisternae of rough and smooth endoplasmic reticulum, and also within the nuclear envelope, of both adrenaline- and noradrenaline-containing chromaffin cells;
(vi) between the plasma membranes of adjacent chromaffin cells, but only when one or both of these cells contain reaction product within the cisternae of its endoplasmic reticulum; these cisternae sometimes appear to be connected to the plasma membrane.

These observations raise the question whether the acetylcholinesterase activity released from the perfused adrenal gland might originate from the cisternae of the endoplasmic reticula of splanchnic nerve and/or chromaffin cell.

Introduction

Acetylcholinesterase (AChE) is known to occur not only in cholinergic neurons (Lewis & Shute 1966), but also in non-cholinergic neurons (Brzin, Tennyson & Duffy 1966) and even in non-nervous tissue (Glinos & Bartos 1974).

Results reported in a previous paper (Chubb & Smith 1975*b*) have provided evidence that this enzyme can be released into extracellular fluids. It was found that one of the soluble isoenzymes of AChE present in the adrenal medulla could be released into the perfusate from the gland. The question of the origin of the released isoenzyme could not, however, be resolved by biochemical experiments (Chubb & Smith 1975*a*). This is because the structure(s) containing the soluble isoenzymes appear to be too fragile to withstand the preparative procedures required for the biochemical analysis of subcellular particles. A cytochemical study of bovine splanchnic nerve axons and adrenal medulla has, therefore, been carried out to obtain some information on the distribution of AChE activity within these tissues.

Materials and methods

Cattle (*Bos taurus* L.) adrenal glands from three oxen about two years old, and from two calves aged approximately 200 days were used for cytochemical purposes. The ox adrenal glands were obtained from the slaughter-house, while the calf glands were removed from animals, anaesthetized with sodium pentobarbitone and then bled to death, in the Department of Physiology, Cambridge.

Approximately 15–25 min after the death of the animal 1–2 mm^3 blocks of adrenal medulla from at least four different parts of the gland were cut with a razor blade, and placed into ice-cold fixative solution. Fixation was carried out for 2–4 h at 4 °C, with constant agitation by immersion of the blocks in a solution containing 2 g/100 ml glutaraldehyde and 2 g/100 ml formaldehyde buffered to pH 7.3–7.4 with 0.1 M sodium cacodylate buffer. After fixation, 40–50 μm thick sections were cut using a Sorval TC-2 Smith-Farquhar tissue chopper. Sections containing large intramedullary nerve trunks were selected and washed by shaking in 0.1 M sodium cacodylate buffer pH 7.3–7.4 containing 0.2 M sucrose, at 4 °C for 2–3 h. The above washing solution was used throughout the procedure.

For demonstration of cholinesterases the thiocholine technique (Koelle & Friedenwald 1949) was used as modified for electron microscopy by Kása & Csillik (1966). The thick sections were incubated for 30, 50 or 90 min at room temperature in media containing 73 mM sodium acetate buffer pH 5.5, 13.3 mM glycine, 6.6 mM $CuSO_4$, 0.2 mM $Pb(NO_3)_2$, together with either 8 mM *S*-acetylthiocholine iodide or *S*-butyrylthiocholine iodide (8 mM or 15 mM).

To establish the specificity of the reaction, the following incubations were carried out

(1) Incubation without substrate.

(2) Incubation in the presence of 10^{-4} M ethopropazine hydrochloride 10-(2-diethyl-amino-*n*-propyl) phenothiazine hydrochloride

(3) Incubation in the presence of 10^{-4} M BW 284C51 1,5,-bis-(4-allyldimethyl-ammoniumphenyl) pentone-3-one-dibromide

(4) Incubation in the presence of 10^{-4} M BW 284C51 and 10^{-4} M ethopropazine hydrochloride.

(5) Incubation in the presence of 1.5×10^{-5} M eserine sulphate.

When inhibitors were used, specimens were preincubated for 30 min at room temperature in a washing solution containing the appropriate inhibitor.

After incubation specimens were washed for 2×5 min and transferred into 2 g/100 ml $Na_2S.9H_2O$ dissolved in 0.2 M sodium acetate (pH adjusted to between 7.0 and 7.5 with acetic acid) for 4–5 min; washed again 2×5 min and postfixed for 8 min in 0.5% OsO_4, following the method of Palade (1952).

The sections were dehydrated in alcohol and embedded into either Durcupan (Fluka A.G.) or Epon-Araldite (TAAB Laboratories Ltd). Thin sections were stained for 30 min with saturated aqueous uranyl acetate followed by lead citrate (Reynolds 1963) for 2 min and examined in Metropolitan-Vickers E.M.6 or Phillips 200 electron microscopes.

To study the effect of fixation on the activity of the individual isoenzymes in adrenal medulla, electrophoresis on polyacrylamide gels of a high-speed supernatant from an adrenal medulla homogenate was carried out as previously described (Chubb & Smith 1975*a*). After the run, the gels were washed with water $2 \times$ 5min and the fixed with the cytochemical fixative for either 10, 20 or 40 min. After a 30 min wash, the gels were incubated to reveal the AChE isoenzymes (Chubb & Smith 1975*a*).

Acetylthiocholine iodide was obtained from B.D.H. Chemicals Ltd, butyrylthiocholine iodide and eserine from Sigma Chemical Corp., BW 284C51 from Wellcome Reagents Ltd, ethopropazine hydrochloride from May & Baker Ltd, glutaraldehyde, formaldehyde and OsO_4 from TAAB laboratories.

Results

Specificity of the cytochemical reaction

By the use of inhibitors and appropriate controls, it is possible to establish the specificity of the enzyme reaction. After incubating without substrate, or in the presence of eserine, we could not detect any reaction end-product. This result shows that under our experimental conditions not only are there no detectable levels of esterases other than cholinesterases in the tissues studied, but also that, since the end-product of the reaction is a metal sulphide, there is very little non-specific binding of the metal ion.

The reaction product obtained with either of the substrates was absent when both BW 284C51 (at low concentrations a specific inhibitor of AChE) and ethopropazine (at low concentrations an inhibitor of non-specific ChE) were incorporated into the incubation media. This was so even with extended (up to 90 min) incubation times. When BW 284C51 alone was used with acetylthiocholine

as the substrate, no end-product was visible after a 90 min incubation period. From all of these various controls we concluded that by using acetylthiocholine as the substrate in the presence of 10^{-4} M ethopropazine we would reveal sites of AChE activity only, while use of butyrylthiocholine together with 10^{-4} M BW 284C51 would only reveal non-specific ChE positive sites.

Finally, we examined the possibility that the fixative might selectively inhibit some of the AChE isoenzymes. We incubated, first in fixative then in AChE incubation medium, polyacrylamide gels on which soluble adrenal medullary extracts had been run. The fixative caused marked and progressive inhibition of enzyme activity, but there was no selective inhibition of any of the AChE isoenzymes.

Localization of acetylcholinesterase activity

Since there was no apparent difference in the localization of the cholinesterases in tissues obtained from adult or from young animals, results from both will be described together.

Intramedullary axons

Intramedullary nerve trunks, consisting of up to several dozen axons, contain myelinated and unmyelinated fibres. Cytochemistry revealed reaction end-product due to AChE activity between the plasma membranes of Schwann cells and axons (figure 1).† High magnification micrographs showed that the precipitate is present between the outer membrane leaflets (figure 4). Intra-axonally, end-product was found in tubules of smooth endoplasmic reticulum (figures 2 and 3), although only a minority of the axons contained them. In some of these axons, only one or two enzyme-containing tubules occurred, while in others there were many (figure 3). In the same axon not all the cisternae were active and sometimes the end-product was only present for part of the length (figure 2). When these tubules lie very close to the plasma membrane, the end-product within the cisternae seems to be continuous with that deposited outside the plasma membrane (figure 5).

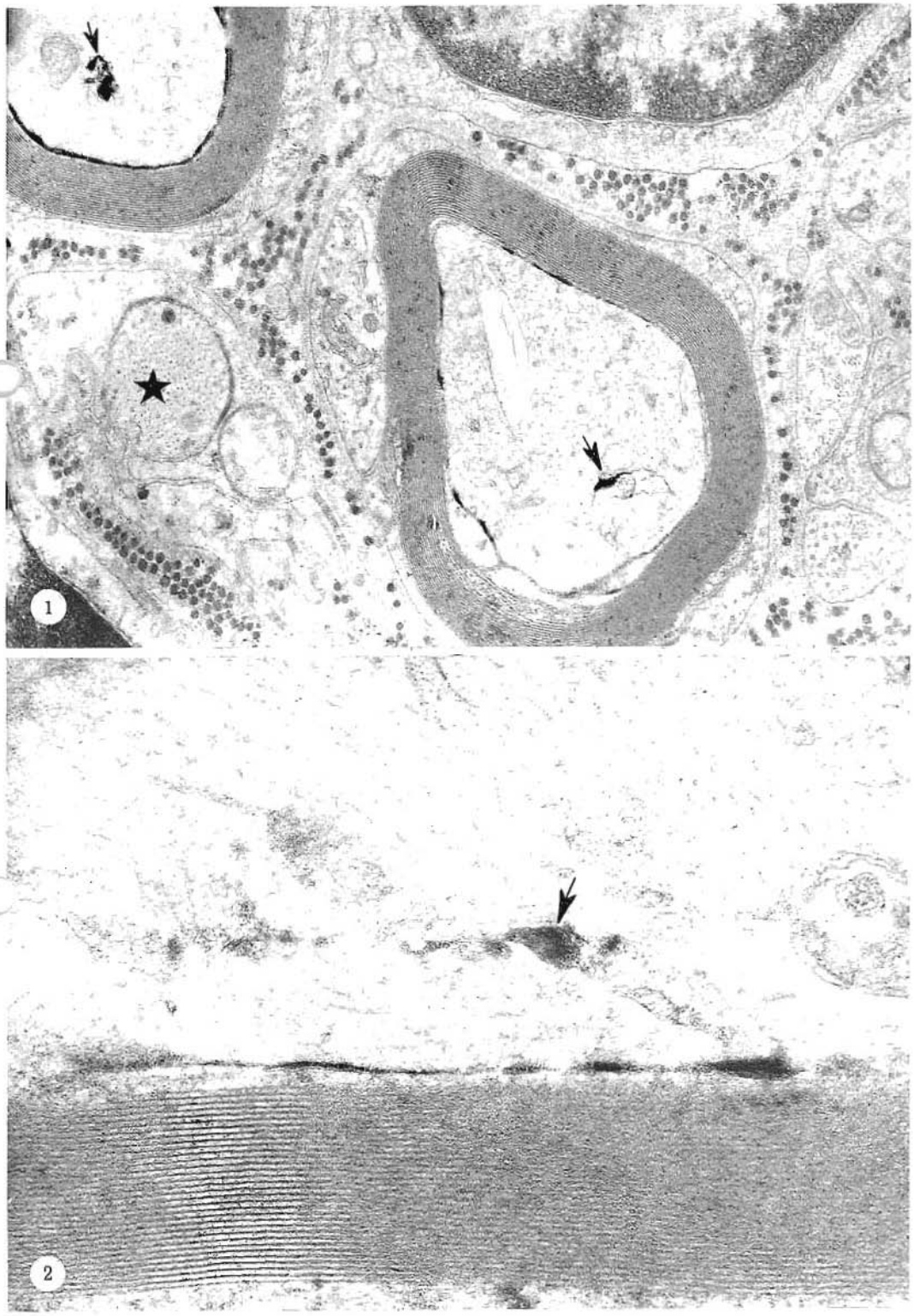
† Figures 1–21 appear on plates 8–17.

Description of plate 8

AChE reaction

Figure 1. Part of an intramedullary nerve trunk with two myelinated axons and one unmyelinated axon (asterisk). End-product due to AChE activity can be seen around all of the three axons and associated wih rmembranous structures within the axoplasm (arrow). (Magn. × 25000.)

Figure 2. AChE activity in a short segment of axonal smooth endoplasmic reticulum (arrow) near to a region of reactive axolemma. (Magn. × 68000.)



FIGURES 1 AND 2. For description see opposite.

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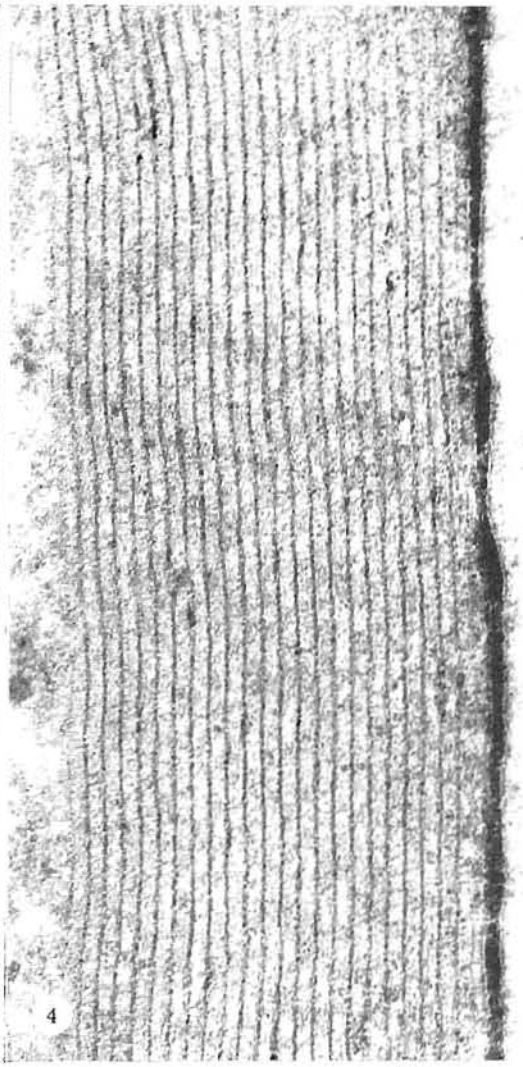

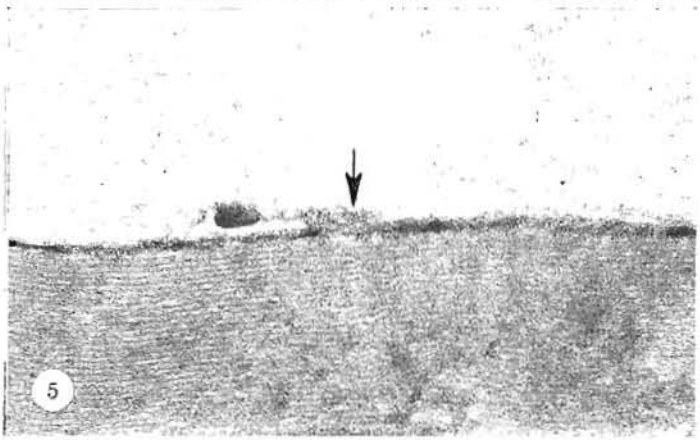

FIGURES 3–5. For description see opposite.

Nerve terminals

Unmyelinated preterminal axons running between chromaffin cells, either individually or in groups and with or without a Schwann cell sheath, are surrounded by end-product. The product is localized between the axolemma and the plasma membranes of the chromaffin cells. Since it is present when the chromaffin cells show no reaction intracellularly (see below) we conclude that the end-product is associated with the axon itself. Similarly, reaction end-product is always found around nerve terminals and within the synaptic gap (figures 14 and 15). When adjacent chromaffin cells display AChE activity, the end-product associated with the membrane of the nerve terminal is continuous with that around the chromaffin cells. In contrast to the nerve axons, intracellular reaction product was never found within nerve terminals, even after prolonged incubation (up to 2 h) with substrate.

AChE activity associated with chromaffin cells

In both adrenaline- and noradrenaline-containing chromaffin cells (identified by the characteristic appearance of the noradrenaline storage granules after fixation in glutaraldehyde as described by Coupland, Pyper & Hopwood 1964), reaction end-product was found within the cisternae of both rough and smooth endoplasmic reticulum and in the nuclear envelope (figures 6–16). Elements of the endoplasmic reticulum were often found running parallel to the plasma membrane and frequently they approached it like sub-surface cisternae (figure 16). Occasionally, these cisternae appear to be attached to the plasma membrane and then the intra- and extracellular reaction product appears to be continuous (figures 11 and 12). Transitional elements of the endoplasmic reticulum, around the Golgi apparatus, contain the end-product, but it can only rarely be observed within the parallel saccules of the Golgi system itself (figure 8). Activity can also be demonstrated between adjacent chromaffin cells, but this is only found when either or both of the cells also show intracellular activity (figures 6, 11 and 12).

Of the two types of chromaffin cell, AChE activity is more characteristic of the adrenaline-containing cell. Not only did we find that a higher proportion of the adrenaline cells were active, but also that they showed a stronger reaction than

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Description of plate 9

AChE reaction

Figure 3. Longitudinal section of a myelinated fibre containing several AChE active smooth endoplasmic reticulum profiles (arrows) in a comparatively small area. (Magn. × 18000.)

Figure 4. High power micrograph showing that the end-product is localized between the outer leaflets of the Schwann cell plasma membrane and the axolemma. (Magn. × 154000.)

Figure 5. End-product within a tubule of smooth endoplasmic reticulum is continuous with that around the axon (arrow). (Magn. × 48000.)

the noradrenaline cells when in the same section. Examples of enzyme activity in noradrenaline-containing cells are shown in figures 9 and 10. Within the same adrenal gland we found that the AChE-containing cells are situated in groups. This is unlikely to be due to lack of penetration of the substrate since we found this pattern at any level in the one thick section.

The adrenaline-storing cells are polarized. They have an endoplasmic reticulum-rich nuclear pole and it is in this region that the nerve axons terminate – usually very close to the nucleus. Thus, the area rich in AChE-containing endoplasmic reticulum is close to the nerve terminals.

Capillaries in the noradrenaline-cell area

We also found AChE activity which was apparently not associated with neurons or chromaffin cells. This activity was associated with a certain type of capillary blood vessel which was only found in the noradrenaline cell areas (figures 18 and 19). These vessels consist of a layer of endothelial cells which, in contrast to the sinusoids, are not fenestrated. Pericytes can be seen partially or entirely surrounding the endothelial cells. The space between the chromaffin cells and these blood vessels is filled out with a varying amount of connective tissue, but sometimes both the endothelium and the pericytes are separated from the noradrenaline-cells by just a single basal lamina.

The reaction product first appears between the basal lamina of either the pericyte, the endothelial cell or the chromaffin cell and the respective plasma membrane of these cells (figure 18). When the reaction is stronger (longer incubation time) the end-product covers all the basal laminae filling out the space among pericyte processes. Since the pericyte layer is not continuous, the end-product occasionally fills the space between the endothelium and the noradrenaline containing cells (figure 19).

Localization of non-specific cholinesterase (ChE) activity

When acetylthiocholine was used as substrate, in conjunction with BW 284C51 as inhibitor, we were unable to detect any enzyme activity, even after prolonged (90 min) incubation times. However, when butyrylthiocholine was used, also in the presence of 10^{-4} M BW 284C51, we were able to observe end-product which we attributed to the activity of non-specific cholinesterase (ChE).

In contrast the AChE, non-specific ChE was only localized intracellularly; it

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DESCRIPTION OF PLATE 10

AChE reaction

FIGURE 6. Survey picture of the nuclear region of an adrenaline-containing chromaffin cell area. AChE activity is evident in the endoplasmic reticulum including the nuclear envelope while the Golgi system (G) shows very faint activity. End-product is also present between the chromaffin cells. (Magn. × 10000.)

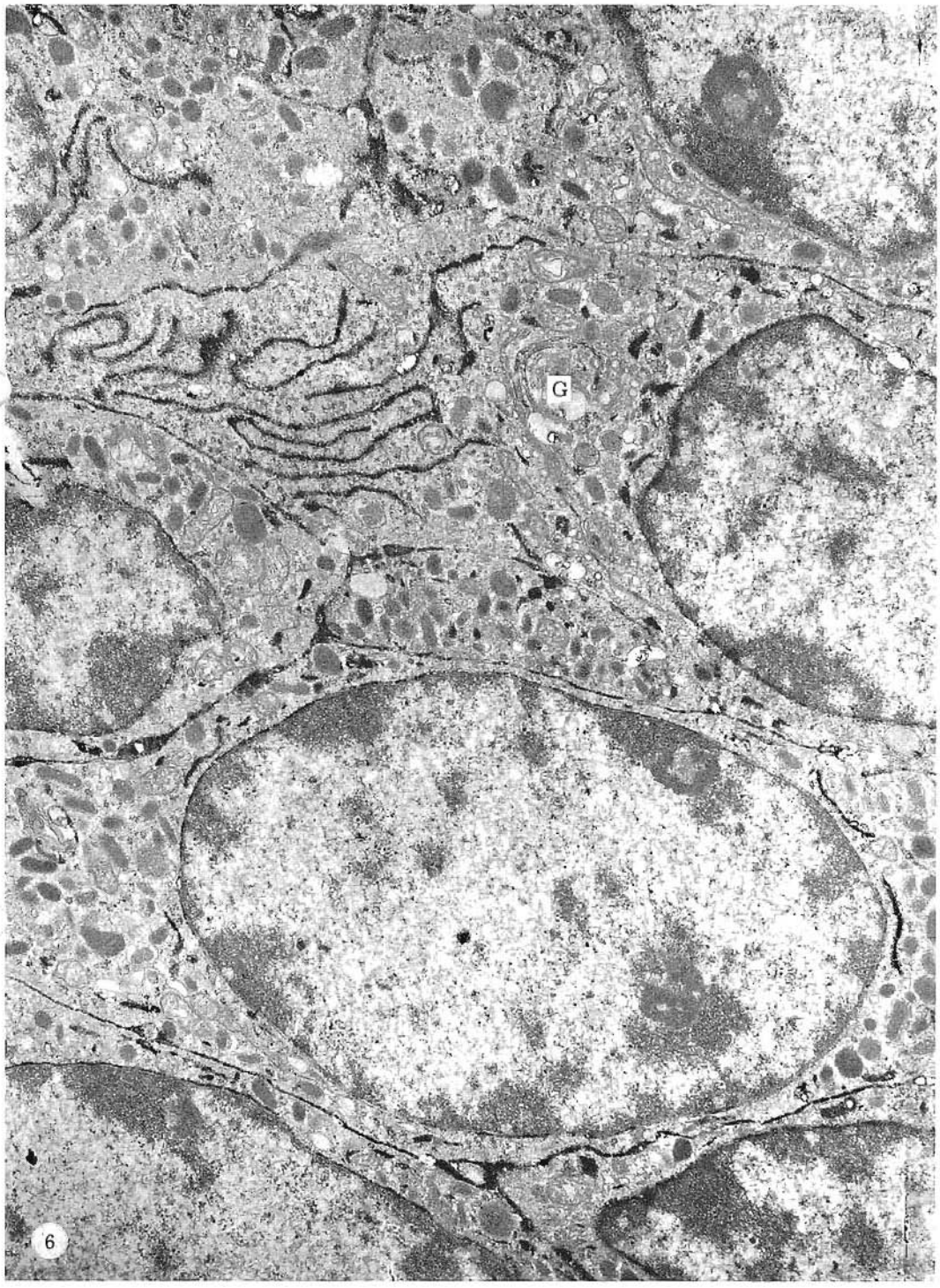

FIGURE 6. For description see opposite.

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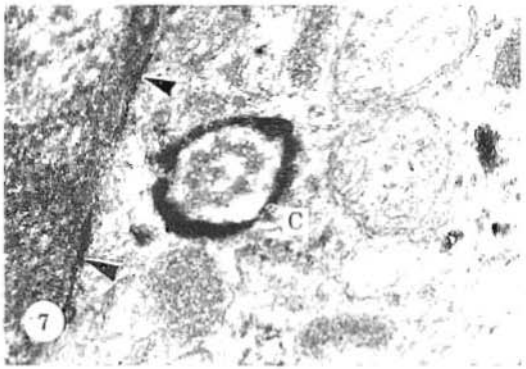

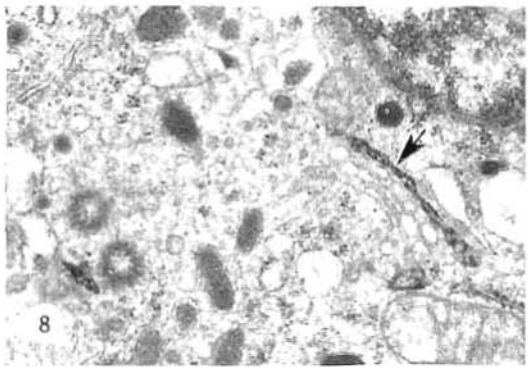

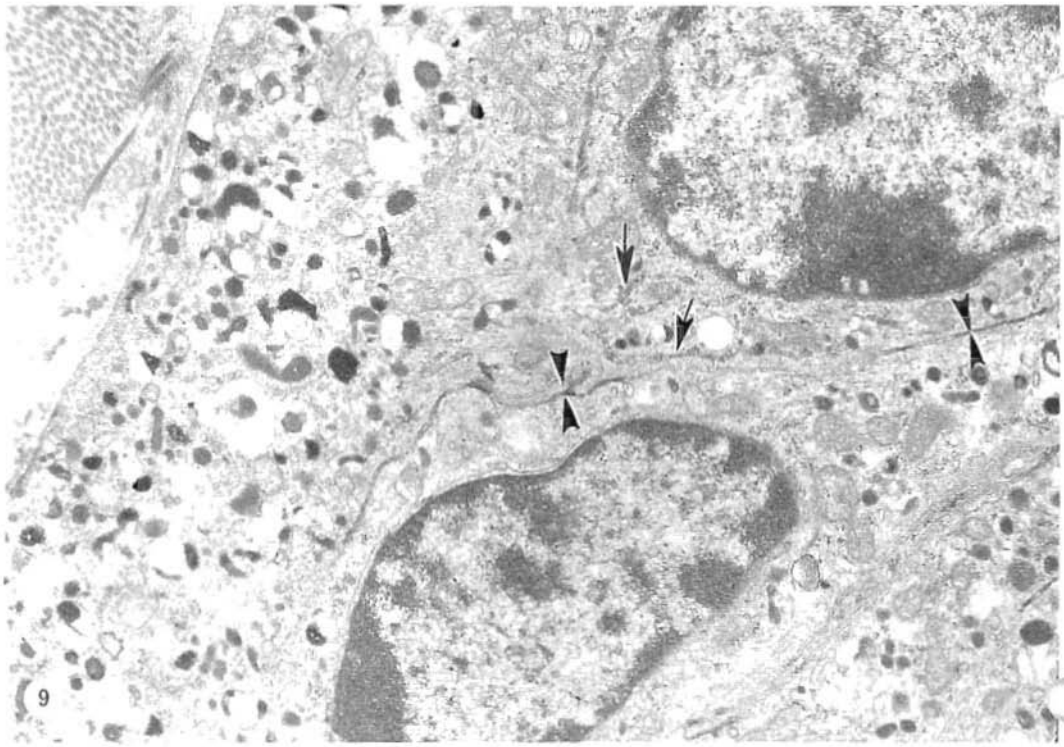

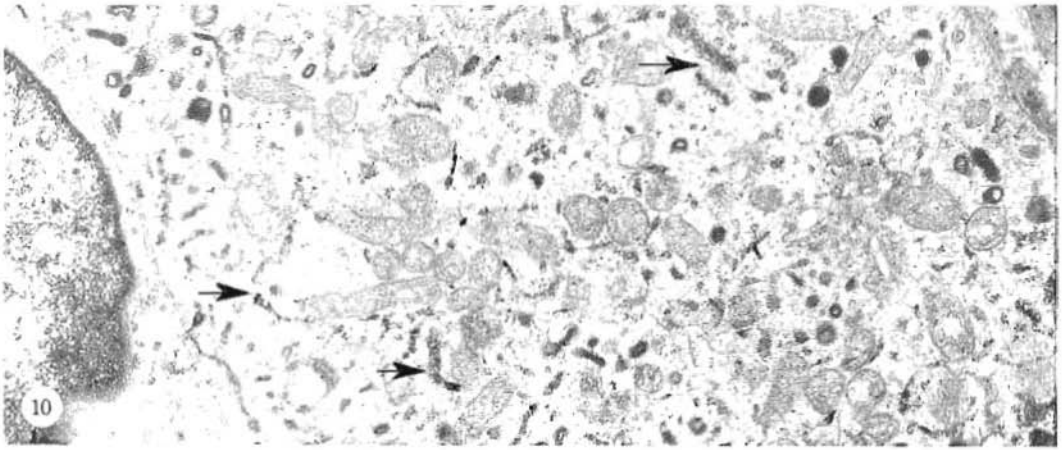

FIGURES 7–10. For description see opposite.

was found in the cisternae of the endoplasmic reticulum and nuclear envelope of adrenaline-containing cells (figure 21). In case we had not revealed any extracellular ChE activity because it was only present in small amounts, sections from the same gland were incubated in a medium containing higher concentrations of substrate (15 mM; the ChE activity in homogenates increases at high substrate concentration). However, we could only observe intracellular ChE activity whatever conditions were used. The distribution of ChE was similar to that of AChE in one respect in that only certain groups of cells were active but these cells had no particular structural characteristics when compared with inactive cells.

We also detected activity of ChE in cisternae of endoplasmic reticulum of pericytes (figure 20), around sinusoids and capillaries as well as in smooth muscle cells around larger blood vessels (figure 17).

Discussion

Distribution of AChE in the adrenal medulla

Cholinesterases in the mammalian adrenal gland have been the subject of both light and electron-microscopic studies ever since techniques for their localization were developed. By using light microscopic methods, their localization in the adrenal glands of the following species has been studied: cat (Koelle 1950, 1951; Coupland & Holmes 1958; Palkama 1964), rat (Coupland & Holmes 1958; Eränkö 1958; Eränkö, Hopsu & Räisänen 1959; Drews 1965), hamster (Eränkö, Hopsu & Palkama 1962; Palkama 1964), dog (Palkama 1964), rabbit (Coupland & Holmes 1958; Antopol & Glick 1940), pig (Antopol & Glick 1940) and ox (Antopol & Glick 1940; Palkama 1964). However, the localization of cholinesterases has only been studied by electron microscopic cytochemistry in the adrenal medulla of the rat (Lewis & Shute 1966, 1969; Palkama 1967).

From the earliest studies with the light microscope, it is apparent that the bulk of the demonstrable cholinesterase activity in the gland is localized in the medulla rather than the cortex. The distribution of AChE (Koelle 1951; Eränkö 1958; Coupland & Holmes 1958; Palkama 1964) and the loss of the activity after denervation (Eränkö *et al.* 1959; Eränkö *et al.* 1962) led to the conclusion that the enzyme is primarily associated with the nerve supply to the medulla.

Description of plate 11

AChE reaction

Figure 7. Extracellular activity around a cilium (C) which invaginates into the cell, and intracellular activity in the nuclear envelope (arrowhead). (Magn. × 49000.)

Figure 8. The outermost cistern (arrow) of the Golgi apparatus shows some activity in an adrenaline-containing cell. (Magn. × 22000.)

Figures 9 and 10. Noradrenaline-containing cells exhibiting activity in the endoplasmic reticulum (arrow); end product is also present between the cells (arrowhead). (Magn. 9, × 19000; 10, × 18000.)

The existence of AChE in chromaffin cells is more controversial. Koelle (1950) reported that cat chromaffin cells contained cholinesterases but later (Koelle 1951) attributed the staining to a diffusion artifact. In cat, rabbit and rat adrenals, reaction in the chromaffin cells was also attributed to a diffusion of the end-product (Coupland & Holmes 1958), but in studies on the hamster it was concluded that the cells did contain the enzyme (Eränkö *et al.* 1962). The use of the electron microscope has not clarified the position. Whereas Lewis & Shute (1969) could find intracellular reaction product in adrenaline-containing rat chromaffin cells, Palkama (1967), using the same species, could only find activity associated with the plasma membranes of noradrenaline-containing cells.

Our results show that, in bovine adrenal glands, AChE is associated with the nerve supply and with the chromaffin cells, both the adrenaline- and noradrenaline-containing types. The activity in these tissues is both intracellular and extracellular; the latter probably associated with the outer leaflets of the plasma membrane. However, we must emphasize that our results only apply to bovine glands, the evidence summarized above and that of Palkama (1964) strongly suggest that in different species AChE is distributed differently within the adrenal gland.

Our results confirm earlier light microscopic observations that adrenaline- and noradrenaline-containing cells in the bovine adrenal medulla differ in several respects such as location, vascularization, cell orientation and shape (Palkama 1964) and add to them the fact that AChE is more characteristic of the adrenaline-con-

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Description of plates 12–14

AChE reaction

Figure 11. (*a*) Two adrenaline-containing cells with end product throughout their endoplasmic reticulum as well as associated with their plasma membranes. At one point (arrow) end-product within the endoplasmic reticulum is continuous with that between the two cells. This area is shown, at higher magnification, in serial sections in figures 11*b*, *c*. (Magn. 11*a* × 15000; 11*b*, *c* × 34000.)

Figure 12. Adrenaline-containing cell. Another example of continuity of end-product within the endoplasmic reticulum and the extracellular space. (Magn. × 36000.)

Figure 13. Adrenaline-containing cells are oriented towards a sinusoid which is surrounded with fenestrated endothelial cells (arrows). Endoplasmic reticulum is not abundant in this part of the cell but, when present, it also exhibits AChE activity (asterisk). End-product can also be seen for short distances between the cells (arrowhead). (Magn. × 7700.)

Figure 14. Longitudinal section of a nerve ending lying between three adrenaline-containing cells. Several synaptic contacts, indicated by vesicle clusters, can be seen (arrowheads). AChE activity occurs around the entire terminal and also in profiles of endoplasmic reticulum of chromaffin cells. (Magn. × 23000.)

Figure 15. Higher power magnification picture of a terminal between two chromaffin cells (C_1 and C_2). The end-product around the terminal is continuous with that between the two chromaffin cells. Notice activity in smooth membraneous structures (arrow) near the terminal in C_2. (Magn. × 56000.)

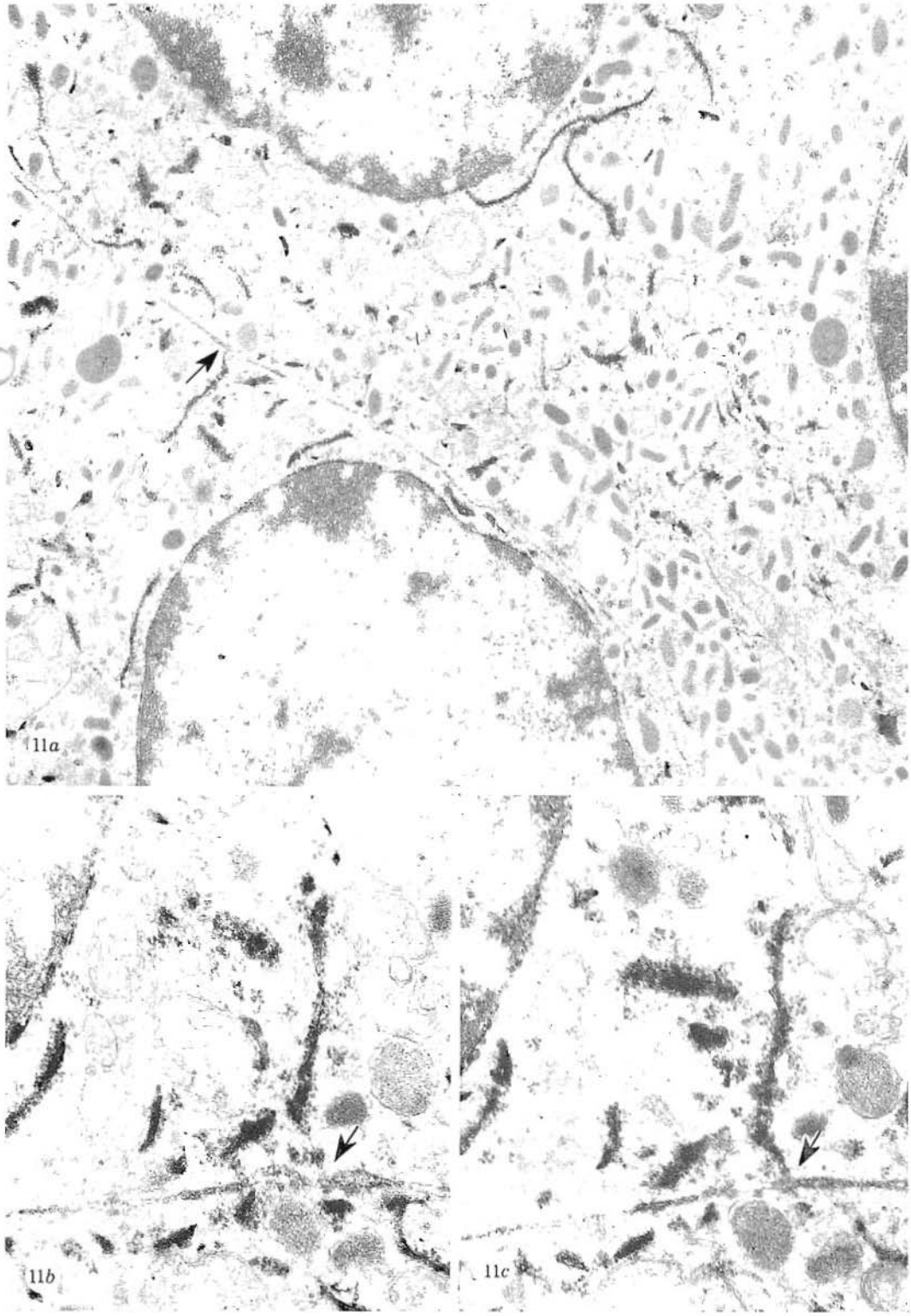

FIGURE 11. For description see opposite.

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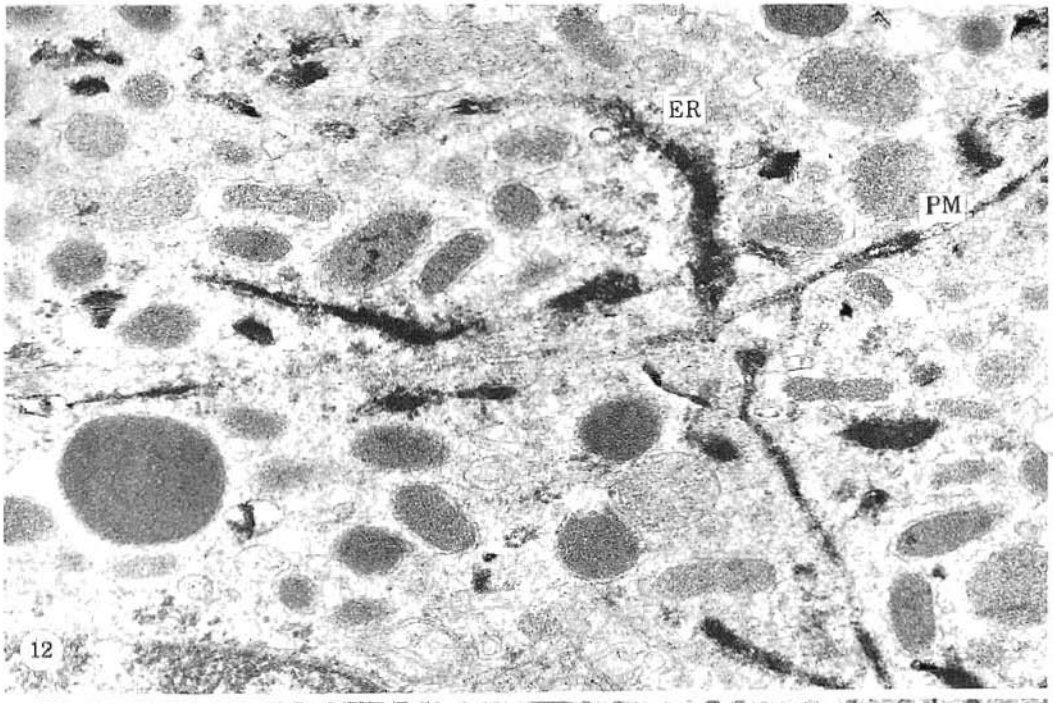
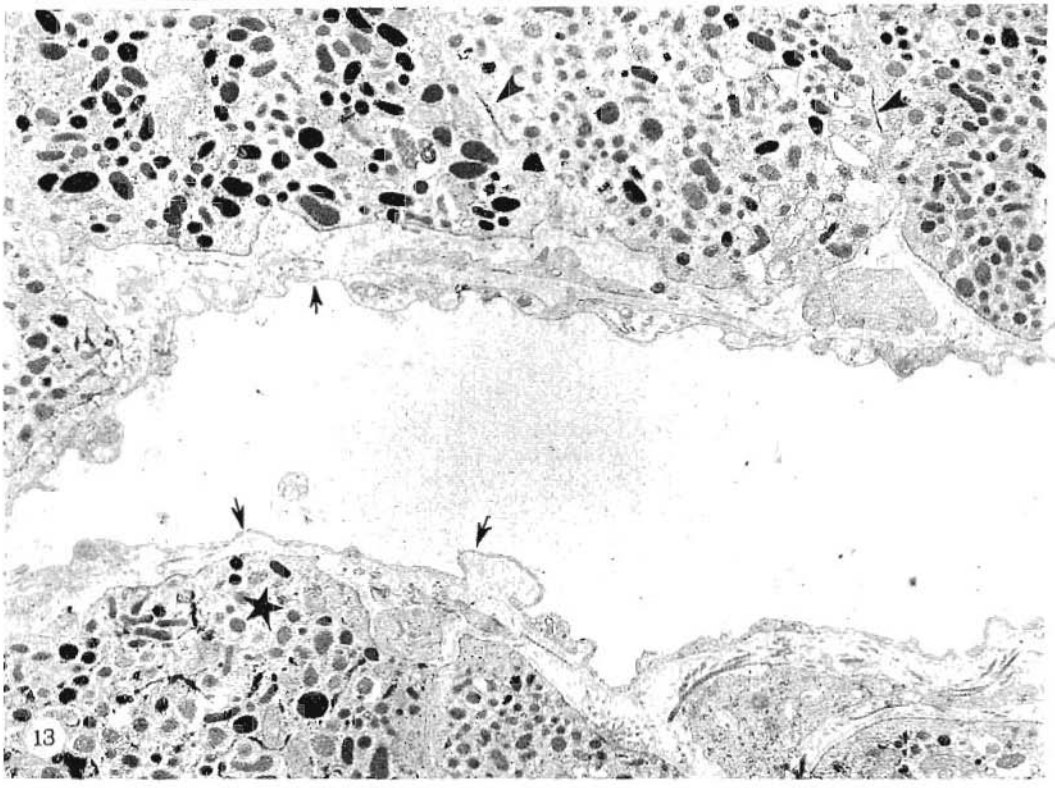
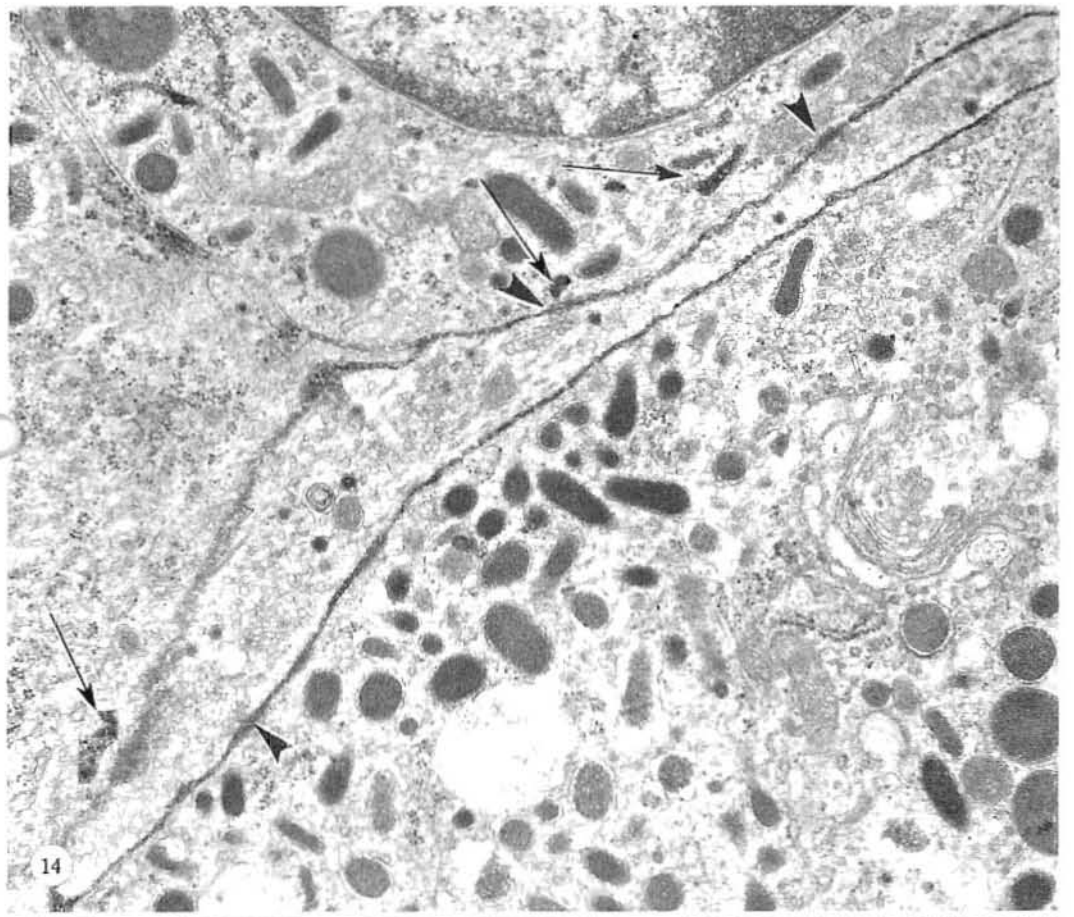
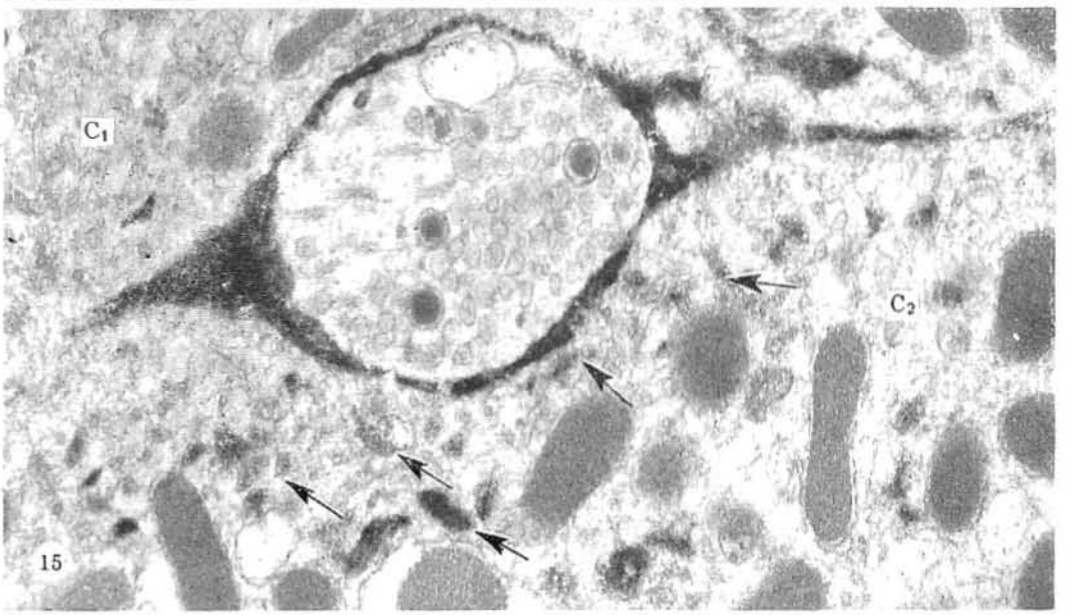


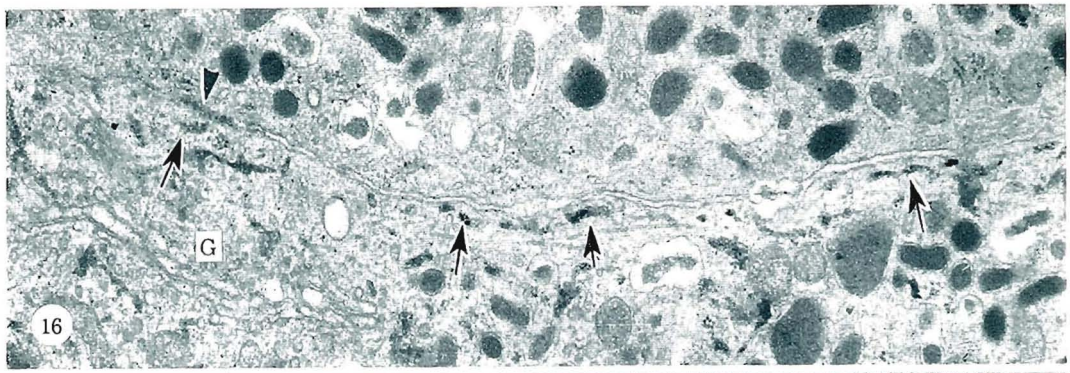


FIGURES 12 AND 13. For description see page 278.





FIGURES 14 AND 15. For description see page 278.



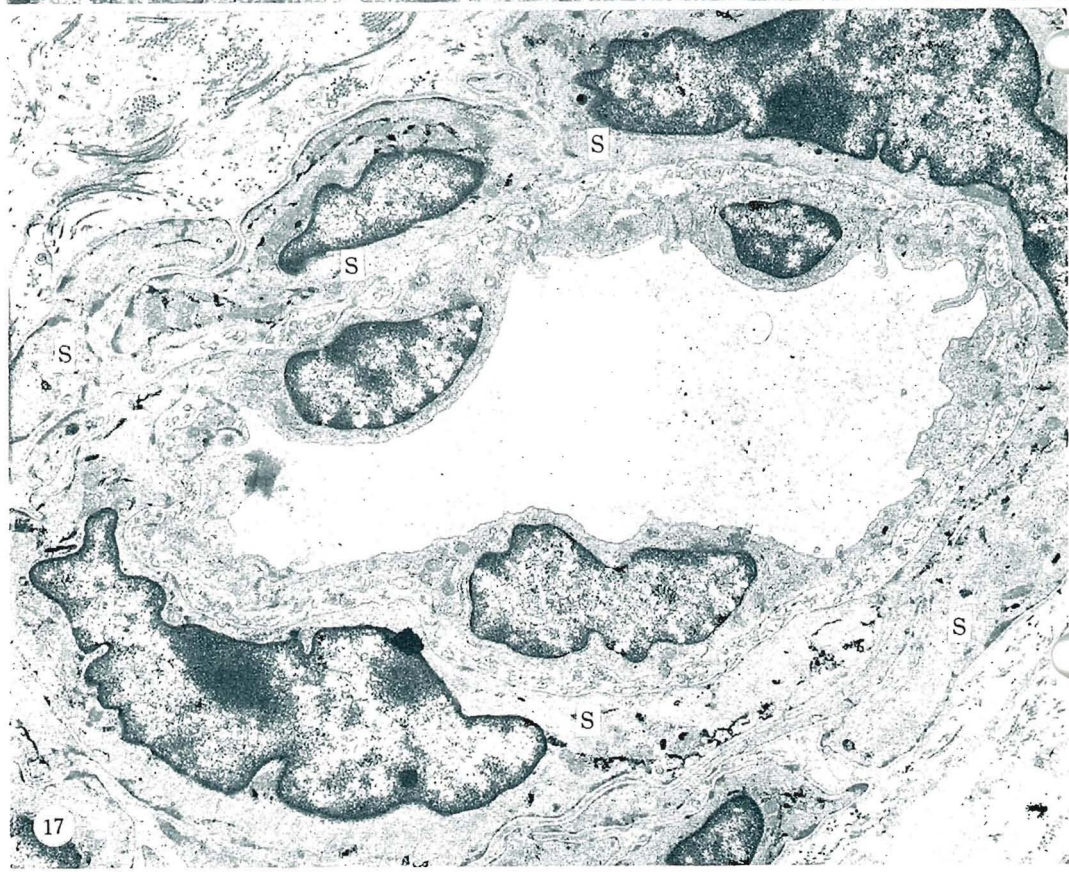


FIGURE 16. Smooth endoplasmic reticulum which contains end-product due to AChE activity runs parallel with the plasma membrane of a chromaffin cell (arrow). The Golgi region (G) is inactive. Reaction product is present between a short segment of the plasma membranes (arrowhead). (Magn. × 24000.)

FIGURE 17. Non-specific cholinesterase (butyrylthiocholine as substrate) in smooth muscle cells (S) in the wall of a large intramedullary blood vessel. Activity is demonstrated throughout the endoplasmic reticulum. (Magn. × 4500.)

taining cells. We have no explanation for the much rarer and weaker reaction of the noradrenaline-containing cells.

AChE activity associated with capillaries

AChE and butyrylcholinesterase have been reported to occur in capillary walls in the central nervous system of several mammalian species (Koelle 1954; Croock 1963; Joó & Csillik 1966; Kása & Csillik 1966; Gwyn & Wolstencroft 1968; Flumerfelt, Lewis & Gwyn 1973; Kreutzberg & Tóth 1974). Our results show that AChE can also occur in the capillaries in a peripheral tissue. Although we have found activity associated with the basal laminae of either pericytes or endothelial cells, the exact site of the activity remains uncertain due to the possibility of some diffusion of the end-product. Equally, the origin of the enzyme is obscure since there is no intracellular activity in either of these cells. It has been suggested that AChE activity in basement membrane and pinocytotic vesicles indicates transport of the enzyme, secreted by neurons, through the endothelial cell (Kreutzberg & Tóth 1974). A similar transport process for enzyme secreted from either the splanchnic nerve or the chromaffin cell could give rise to the results obtained. On the other hand, transport in the reverse direction, i.e. from blood plasma, must not be discounted. Bovine plasma contains AChE (see Augustinsson 1963) and endothelial pinocytotic vesicles incorporating plasma could thus trap AChE activity. However, we did not observe reactive pinocytotic vesicles.

Localization of non-specific cholinesterase

Although so little is known about the function of non-specific ChE, a great deal of work has been done on the localization of the enzyme.

In the adrenal medulla of the rat, this enzyme was found in the endoplasmic reticulum of chromaffin cells (Lewis & Shute 1966, 1969; Palkama 1967). Palkama (1967) also reported that the enzyme was associated with membranes of non-myelinated axons and with plasma membranes of chromaffin cells. Lewis & Shute (1966, 1969) did not, however, observe extracellular reaction-product and concluded that non-specific ChE was more characteristic of the endoplasmic reticulum of supporting elements such as Schwann cells and endothelial cells. Our findings in bovine glands also show that non-specific ChE is only demonstrable within cells. Even after prolonged incubation periods and with high substrate concentrations we could not detect any extracellular reaction product.

Can intramedullary axons or the chromaffin cells be the origin of the released AChE?

The intramedullary axons can be characterized by their having a dual localization of AChE, one at the axon surface and another within the axonal smooth endoplasmic reticulum. This pattern seems to be a general characteristic of all nerves exhibiting AChE activity (see, for example, Novikoff, Quintana, Villaverde & Forschirm 1966; Schlaepfer & Torack 1966; Kása 1968; Tennyson, Brzin & Duffy 1968; Kása *et al.* 1973).

Kása (1968) has shown that AChE-rich smooth endoplasmic reticulum accumulates above ligations placed around sciatic nerve axons. He has also shown that reactive tubules of endoplasmic reticulum can, occasionally, be connected to the axon surface and that, when they are, the end-product of the AChE reaction is continuous within and without the axon. Our observations on sections of the splanchnic nerve show the same phenomenon (see figure 5). If these results mean that the AChE on the axon surface is supplied from within the axon, via the endoplasmic reticulum, then it might be expected that a proximodistal (decreasing) gradient of AChE-rich endoplasmic reticulum could be seen. Unfortunately, there are too few active cisternae to allow us to determine whether such a gradient does occur in these axons. It is interesting in this context, though, that we have never been able to show AChE in any structure within the nerve terminals. Thus, there could be an extraordinarily rapid removal of the enzyme from the terminal or, alternatively, it may never arrive there.

If our results really mean that there is no AChE within the terminal, and negative cytochemical experiments must always be interpreted with caution, then it seems unlikely that the axon is the origin of the released AChE. Although there is a structural basis for suggesting that release could occur, and we and Kása (1968) might have observed the process, it is likely that the diffusion of AChE released from any axonal site other than from the terminal would be so limited by the Schwann cell or myelin sheath that it would only slowly appear in the perfusate.

Chromaffin cells

The other major AChE-containing element of the ox adrenal medulla is the chromaffin cell, and it is apparent that the same structural basis for release is present in these cells as is present in the axon i.e. tubules rich in AChE. Furthermore, it seems very likely that the AChE associated with the plasma membranes (the end-product between adjacent chromaffin cells) is synthesized within one of the two cells. We have never found activity between cells unless one of them contained AChE-rich endoplasmic reticulum. The mechanism by which the intracellular enzyme gets between the cells could be as shown in figure 11 where we were able to

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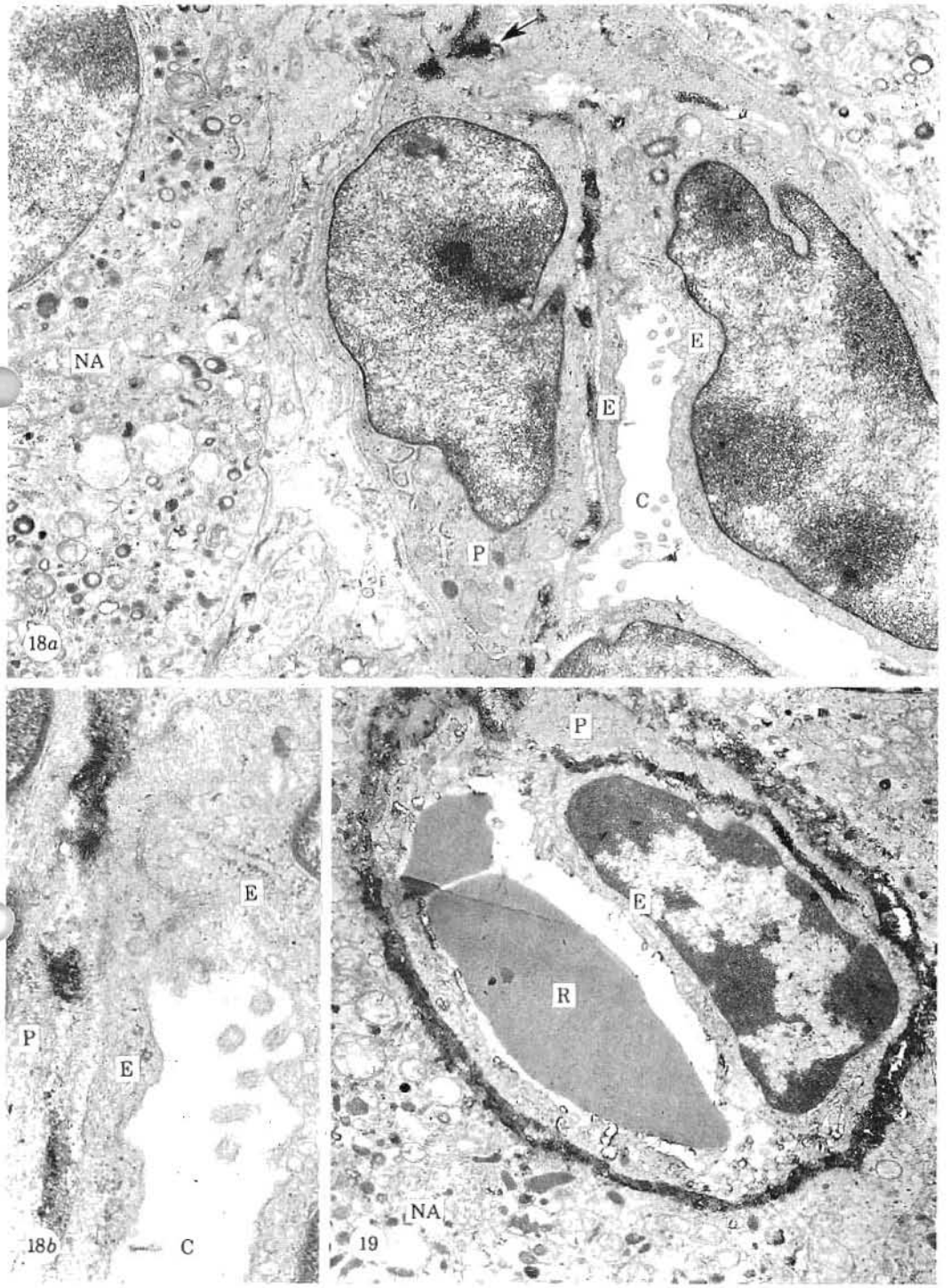
DESCRIPTION OF PLATE 16

AChE reaction

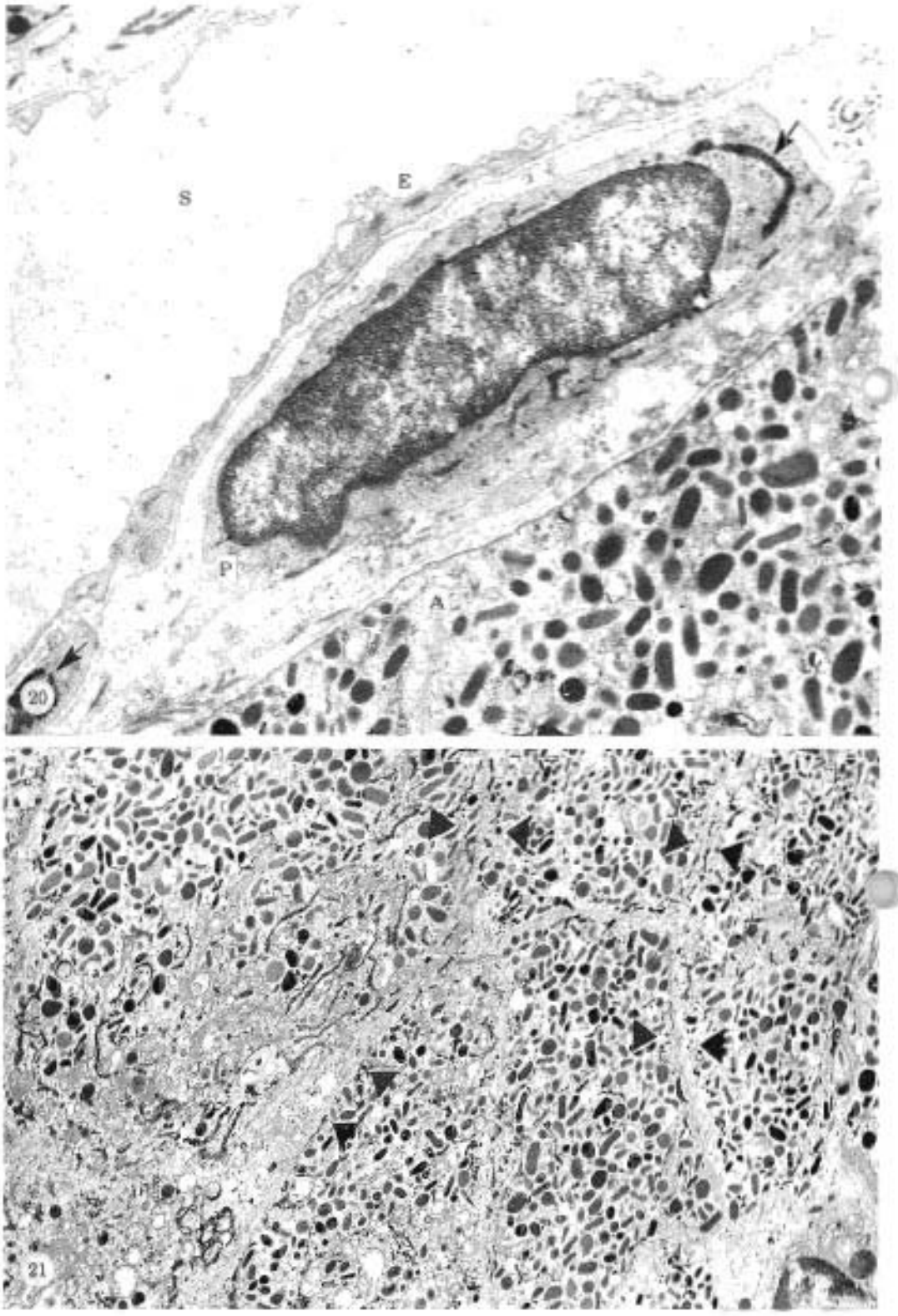
FIGURE 18. (*a*) After a 30 min incubation period, end-product can be seen between a pericyte and an endothelial cell, as well as between processes of pericytes (arrow). (*b*) At higher magnification it can be seen that the reaction occurs between the basal lamina and the plasma membrane. (Magn. (*a*) × 14000; (*b*) × 30000.)

FIGURE 19. After prolonged incubation (70 min) the end-product fills the extracellular space. Because of the long incubation time, some of the end-product has crystallized and other has diffused. (Magn. × 8400.) P, pericyte; E, endothelial cell; NA, noradrenaline-containing cells; C, capillary lumen; R, red blood cell.



FIGURES 18 AND 19. For description see opposite.

(*Facing p.* 289)



FIGURES 20 AND 21. For description see opposite.

observe an apparent joining of the plasma membrane and a tubule of the endoplasmic reticulum. These 'joins' could represent temporary connexions between the extracellular space and the space within the tubule. If the AChE within the tubule is soluble then part of it would, presumably, be free to diffuse out of the tubule and into the extracellular space whereupon it could diffuse through the intercellular space (with the aid of canaliculi?, see Grynszpan-Winograd 1971; Benedeczky 1967) and eventually, through the fenestrations of the endothelial cells, into the blood capillaries.

Thus, there are reasons for supposing that AChE could be released from both the splanchnic axons and from the chromaffin cells which they innervate. However, the temporal relation with the release of catecholamines (Chubb & Smith 1975*b*) suggests that, at least in the isolated adrenal preparation, the chromaffin cells are the primary source of the AChE in the perfusate.

Chromaffin cells are well known secreting cells, releasing catecholamines and protein from the chromaffin granules by exocytosis (see Smith & Winkler 1972). It now seems reasonable to attribute to this cell another property, that of protein secretion directly from the endoplasmic reticulum. Both types of secretion require depolarization of the cell and the presence of Ca^{2+} ions but there the similarities seem to end. The chromaffin granule is formed in the Golgi apparatus, a system which very rarely shows any AChE activity. Most of the intracellular stored granules are in the supranuclear cytoplasm at the apical pole of the cell whereas AChE-rich endoplasmic reticulum is around the nucleus and along the lateral plasma membranes. The bovine chromaffin cell is, therefore, specialized not only in the types of protein that it secretes but also in the spatial arrangement of the different secretory proteins within the cell as well as having quite distinct storage organelles for them.

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Description of plate 17

Non-specific ChE reaction

Figure 20. With butyrylthiocholine as substrate, end product can be seen in the endoplasmic reticulum (arrow) of a pericyte. (Magn. × 16000.)
P, pericyte; E, endothelial cell; A, adrenaline-containing cell; S, sinusoid lumen.

Figure 21. An adrenaline-containing cell area where non-specific ChE can be shown throughout the endoplasmic reticulum but not on the cell surfaces. Note that the reaction end-product emphasizes the parallel arrangement of endoplasmic reticulum along the lateral plasma membranes (triangles). (Magn. × 4900.)

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