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IMMUNOHISTOCHEMICAL LOCALIZATION OF PROTEIN COMPONENTS OF CATECHOLAMINE STORAGE VESICLES

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SUMMARY

1. The distribution of specific proteins in sympathetic neurones has been examined by immunofluorescent histology using antibodies prepared against soluble protein components of the catecholamine storage vesicles of the adrenal medulla.

2. Two antigen preparations were separated by ion exchange chromatography of the soluble proteins released on osmotic lysis of catecholamine storage vesicles which had been isolated by centrifugation from homogenates of sheep adrenal medulla. One fraction (AgDH) had high dopamine-β-hydroxylase activity, while another (AgCB), consisting of the bulk of the protein, had some capacity to bind catecholamines. On disk gel electrophoresis the antigens ran as single bands with very different mobilities.

3. Antisera (AsDH) and (AsCB) produced in rabbits to the two antigens were shown to react specifically with their antigens by immunodiffusion and electrophoresis in agarose.

4. Indirect immunofluorescent staining of tissue sections was achieved by layering first the rabbit anti-sera, followed by goat anti-rabbit globulin serum which had been conjugated with fluorescein isothiocyanate.

5. The adrenal medulla and the cell bodies of sympathetic ganglia showed the most intense green fluorescence with the immune rabbit sera, and hardly stained when pre-immune serum from the same animal was used. The reactivity of the antisera could be abolished by incubation with the corresponding antigen.

6. The preterminal and terminal axons of sympathetic nerves also stained specifically but less intensely with both antisera. When the nerves were ligated for up to 24 hr, the portion immediately proximal to the constriction showed an enhanced reaction to the antisera.

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7. The results provide evidence that sympathetic neurones contain proteins immunologically identical to those involved in the synthesis and storage of noradrenaline in the adrenal medulla, and support the concept that granular vesicles are synthesized in the perikaryon of the neurone and are transported somatofugally in the axon.

INTRODUCTION

The granular vesicles in adrenal medullary cells and those in sympathetic neurones have many properties in common, including a high concentration of catecholamines complexed in stoichiometric proportions with ATP (Schumann, Schnell & Philippu, 1964). In 1960, Hillarp had suggested that the catecholamines were held within the vesicles by forming a non-diffusible complex with protein and ATP-Mg^{2+}. Recently specific proteins, called chromogranins by Blaschko, Comline, Schneider, Silver & Smith 1967) which are released by osmotic lysis of isolated adrenal medullary vesicles, have been purified and characterized (Smith & Kirshner, 1967; Smith & Winkler, 1967*a*, *b*). The main component of the chromogranins (termed 'S_1' by Smith & Kirshner (1967) and 'chromogranin A' by Schneider, Smith & Winkler (1967) has been found to bind catecholamines (Smith & Kirshner, 1967) while the other protein to be identified thus far, dopamine-β-hydroxylase, catalyses the final step in noradrenaline synthesis.

While there is good evidence that the granular vesicles in sympathetic nerves contain dopamine-β-hydroxylase (see Potter, 1967), they have not as yet been shown to contain a specific protein involved in the binding of noradrenaline. We have therefore produced antibodies in rabbits against both dopamine-β-hydroxylase, and the major chromogranin component (A or S_1), prepared from the chromaffin granules of sheep adrenal medullae. The reactivity of sheep sympathetic neurones to these antibodies has been examined on the assumption that the antigens in neural and medullary tissues would be immunologically compatible, as their common function and ontogenic origin might suggest. By the use of the specific and sensitive immunofluorescence technique, we have detected the presence of both these proteins in the cell bodies, axons and axon terminals of sheep sympathetic neurones as well as in the adrenal medulla. A preliminary report of this work has been given (Livett, Geffen & Rush, 1969).

METHODS

Preparation of antigens. Sheep adrenal medullae obtained from the abattoir were weighed, chopped into small pieces, and homogenized in ice-cold 0·3 M sucrose (1:5, w/v) with a Teflon glass homogenizer of clearance 0·09 mm by passing the pestle up and down five times at 2000 rev/min. Catecholamine storage vesicles were

prepared from the homogenate by ultracentrifugation of the large granule fraction through 1·6 M sucrose (Smith & Winkler, 1967*a*). The sediment of chromaffin granules was lysed by suspension for 30 min in 5 vol. ice-cold distilled water (Smith & Kirshner, 1967), a procedure which liberates catecholamines and large quantities of soluble protein. This solution was centrifuged at 50,000*g* for 20 min to remove insoluble material and the lysate was lyophilized to yield 300 mg protein dry weight. The protein was dissolved in 3 ml. ice-cold water and separated from catecholamines and other small molecules by dialysis against 300 ml. 0·001 M sodium phosphate buffer pH 7·4 for 2 hr with frequent changes of buffer.

The dialysed solution was centrifuged at 11,000 *g* for 5 min, the small amount of sediment discarded, and 9 ml. of the dialysate containing 250 mg of protein was chromatographed on a 1·7 × 23·1 cm column of DEAE-cellulose equilibrated with 0·01 M sodium phosphate, pH 7·4 (Smith & Kirshner, 1967). Elution was performed with 600 ml. NaCl (0–0·8 M) linear gradient in 0·01 M phosphate buffer pH 7·4, prepared by the method of Davis, Santen & Agranoff (1965). Fractions of 10 ml. each were collected and the protein elution pattern followed spectrophotometrically at 289 mμ. Dopamine-β-hydroxylase was assayed by the method of Levin, Levenberg & Kaufman (1960) using dopamine as substrate and the amount of noradrenaline produced during a 30 min incubation was determined fluorimetrically by the trihydroxyindole procedure using iodine as oxidant (Crout, 1961). The fractions exhibiting high dopamine-β-hydroxylase activity (AgDH), and those containing the bulk of the catecholamine binding protein (AgCB) (the S_1 fraction of Smith & Kirshner, 1967), were pooled separately, each pressure dialysed to obtain a final protein concentration of 1–2 mg/ml. and stored below −20° C in 0·5 ml. aliquots.

Disk gel electrophoresis of the two antigens was performed at 4° C in 7 % polyacrylamide according to the method of Davis (1964). In each run 0·1 mg of AgDH or AgCB was applied at a constant current of 6 mA/tube at pH 9·5 for 3 hr (Gibb, Spector & Udenfriend, 1967).

Production of antibodies. Rabbits of either sex, 6 months old and weighing about 2 kg, were bled from an ear vein to obtain about 20 ml. pre-immune serum. Each rabbit then received 1 mg of either antigen in an equal volume of complete Freunds adjuvant (C.S.L. Melbourne) together with 10 mg penicillin (1000 u.) and 50 mg streptomycin. The 2 ml. of solution was injected into four intramuscular sites, 0·67 ml. into each hind quarter and 0·33 ml. into each forequarter. Six weeks after the first injection the animals received a second identical injection, and 14 days later their sera were tested for production of the corresponding antibodies (AsDH and AsCB) by the Ouchterlony immunodiffusion method (see Weir, 1967).

Animals which produced only a low titre of antibody were given a third injection and bled out 1 week later, whereas the others were bled out immediately. Blood was collected in sterilized centrifuge tubes, which were incubated at 37° C for 60 min, and then kept at 4° C for 12 hr to allow the clot to contract. The serum was removed and stored below −20° C in the presence of 0·1 % merthiolate (w/v) in plastic vials. Goat anti-rabbit globulin serum labelled with fluorescein isothiocyanate (FITC) was purchased from Baltimore Biological Laboratories.

Immunodiffusion and immunoelectrophoresis. Ouchterlony plates were prepared using 0·5 % agarose (w/v) in 50 ml. solution containing 121 mg Tris (hydroxymethyl) aminomethane and 425 mg NaCl adjusted to pH 7·7 with hydrochloric acid. The antigens and antibodies were placed in appropriate wells together with pre-immune sera and saline controls. The diffusion patterns which formed after 24–48 hr were rendered visible by dark-field illumination or by staining with Ponceau S (0·2 g in 100 ml. 3 % trichloracetic acid (w/v)) or with amid oblack (0·5 g in a methanol : water : acetic solvent, 4 : 5 : 1) for 3–5 min.

Immunoelectrophoresis of the antibodies and antigens was performed on microscope slides using 1 % agarose in a field strength of 6 V/cm. for 45 min and the precipitation patterns produced after 24 hr diffusion were recorded.

Preparation of tissues. Adult ewes weighing about 30 kg were anaesthetized with sodium pentobarbitone solution (60 mg/ml.) I.V. Various sympathetic nerves in the sheep, including fibres from the superior cervical and coeliac ganglia, were ligated under anaesthesia after crushing them against a glass rod with surgical gut, and 5 days later the sympathetic ganglia, constricted nerves, spleen and adrenal glands were removed.

Fluorescence histology. Tissues were fixed in ice-cold 98 % alcohol (v/v) and blocked in paraffin. Occasionally fresh tissue blocks of ganglia and adrenals were snap frozen in a slurry of liquid nitrogen and isopentane, and frozen sections cut on a cryotome (see Nairn, 1969). Immunofluorescent staining was carried out promptly using undiluted serum as the middle layer followed by the goat anti-rabbit globulin serum conjugated with FITC (Holborow & Johnson, 1967). The reacted tissue sections were mounted in non-fluorescent phosphate buffered glycerol (9 parts glycerol to 1 part buffer).

The multiple layer or 'sandwich' technique was used rather than the direct staining method for immunofluorescence histology for two reasons. Fluorescent anti-rabbit globulin was available commercially in high titre, thus obviating the need to conjugate our rabbit anti-sera and thereby reduce their titres. Secondly, the multiple layer technique is up to ten times more sensitive, since there are many antigenic sites on each antibody molecule in the middle layer to react with the fluorescent anti-rabbit serum.

Sections were observed using a Zeiss Orthoplan fluorescence microscope with transmitted dark-field illumination and the results recorded on Agfacolor CK, 100 ASA, daylight colour film. Two or three primary filters (UG 1) were used, together with a pale yellow secondary filter (K 430).

The specificity of the staining was controlled by always photographing with identical exposure the native fluorescence of the sections alone, in the presence of the fluorescent goat serum without a middle layer of rabbit serum, with serum taken from the rabbit before immunization, and finally with the specific antiserum as the middle layer. Also it was possible to abolish the specific immunofluorescence by pre-incubation of the antiserum with its antigen, which Nairn (1968) has stressed is one of the best criteria for excluding non-specific staining. The diffusion of soluble antigens can be another cause of artefact, and we attempted to minimize this problem by fixing the tissue in ice-cold alcohol and avoiding flotation of sections on water. Nevertheless, since the antibodies were applied in aqueous solution, some diffusion may have occurred.

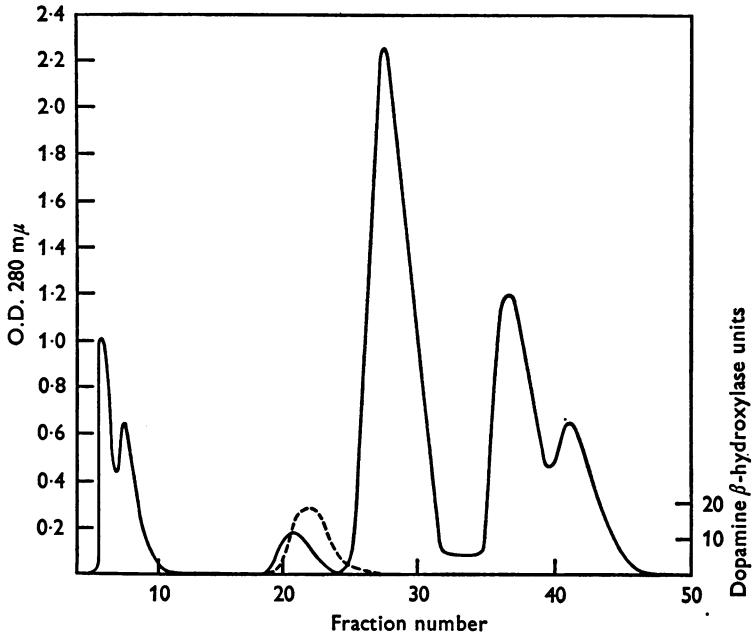
Catecholamine fluorescence was developed in freeze-dried sections treated with formaldehyde under the conditions used by Geffen & Rush (1968).

RESULTS

Separation and characterization of antigens

Chromatography. The elution pattern from chromatography on DEAE-cellulose of the soluble proteins from the chromaffin granules is shown in Text-fig. 1. The first peaks, appearing in fractions 7–11, consisted mainly of haemoglobin (as indicated by their red colour and a high absorbance at 410 mμ) and were not appreciably retarded by the resin. Following these

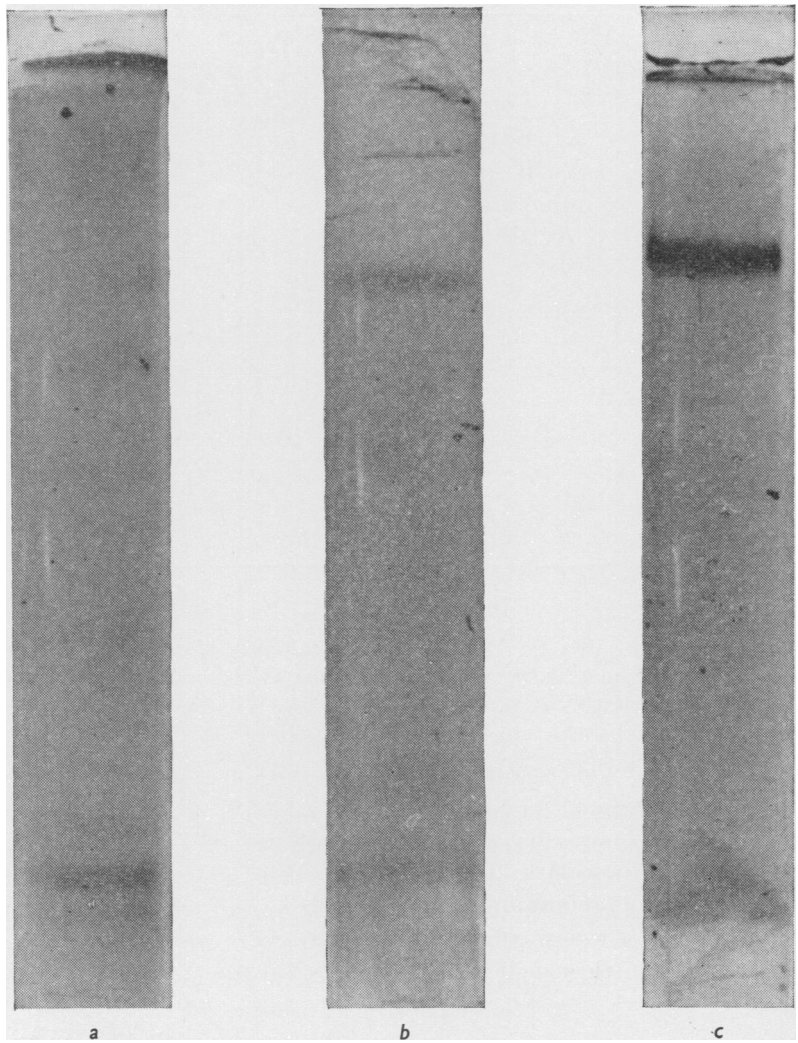
came a small protein peak (fractions 19 to 24) which exhibited a high dopamine-β-hydroxylase activity of 1·54 μ-moles noradrenaline formed per 30 min per mg protein in the peak tube. In more recent experiments we have found that if the chromaffin granules are lysed with a solution of 0·1 % Catscum or with 0·05 % Triton X-100, this peak is sharper, it contains up to three times the amount of protein and up to five times the amount of dopamine-β-hydroxylase activity. Further, it now appears in fractions 18–21 and is well separated from the bulk of the protein.



Text-fig. 1. Separation on DEAE-cellulose columns of soluble proteins from catecholamine storage vesicles isolated from sheep adrenal medullae. The optical density (O.D.) at 280 mμ (left ordinate) of the various fractions of eluate is plotted as a continuous line and the interrupted line indicates the location of dopamine-β-hydroxylase activity (right ordinate).

The next fractions to appear contained 80–90 % of the total soluble protein which was distributed between three peaks. The first contained approximately 70 %, the second 15 % and the third 5 % of the total protein. The relative amounts of the two latter peaks varied from preparation to preparation. Fractions 26–31 (AgCB) corresponding to the S_1 fraction of Smith & Kirshner (1967) and fractions 20–25 containing the dopamine-β-hydroxylase activity (AgDH) were pooled separately, dialysed against 0·01 M phosphate buffer pH 6·8, pressure dialysed and both then reconstituted with 0·001 M phosphate buffer pH 6·8 to give a final concentration of 1–2 mg/ml.

The capacity of AgCB to bind noradrenaline was investigated by equilibrium dialysis against 1 mM-L-[^{14}C]noradrenaline according to the method of Smith & Kirshner (1967). In these preliminary studies a concentration gradient (defined as ratio counts per minute (cpm)/ml. inside dialysis sack containing 50 mg AgCB to cpm/ml. dialysate) of 1·6 was obtained in the absence of ATP and Mg^{2+}, but further studies were not carried out due to the difficulty in obtaining large quantities of the antigen and the demand for AgCB for immunization procedures.



Text-fig. 2. Polyacrylamide disk gel electrophoresis of antigen preparations: (*a*) AgCB, (*b*) AgDH and (*c*) a highly purified preparation of bovine dopamine-β-hydroxylase. The origin is at the top.

Electrophoresis. Polyacrylamide disk gel electrophoresis of the two antigens showed only one main band for each, and their electrophoretic mobilities differed markedly (Text-fig. 2). The preparation of AgDH contained a main band with an electrophoretic mobility identical with that of a sample of highly purified bovine dopamine-β-hydroxylase, prepared according to the method of Friedman & Kaufman (1965) by A. Foldes in our laboratory. In addition, a minor protein band appeared further down the tube having the same electrophoretic mobility as that of AgCB.

Immunodiffusion and electrophoresis. Potent antisera were produced to both antigens in response to the second injection in most animals and in the others following a third injection. In double diffusion reactions with the antisera, a single line of precipitation was obtained between AgDH and AsDH and between AgCB and AsCB. No precipitin lines were formed between AgDH and AsCB, between AgCB and AsDH, or between the antigens and the pre-immune sera. A typical precipitin pattern is shown in Text-fig. 3*a* between AgDH and antisera prepared from three different rabbits.

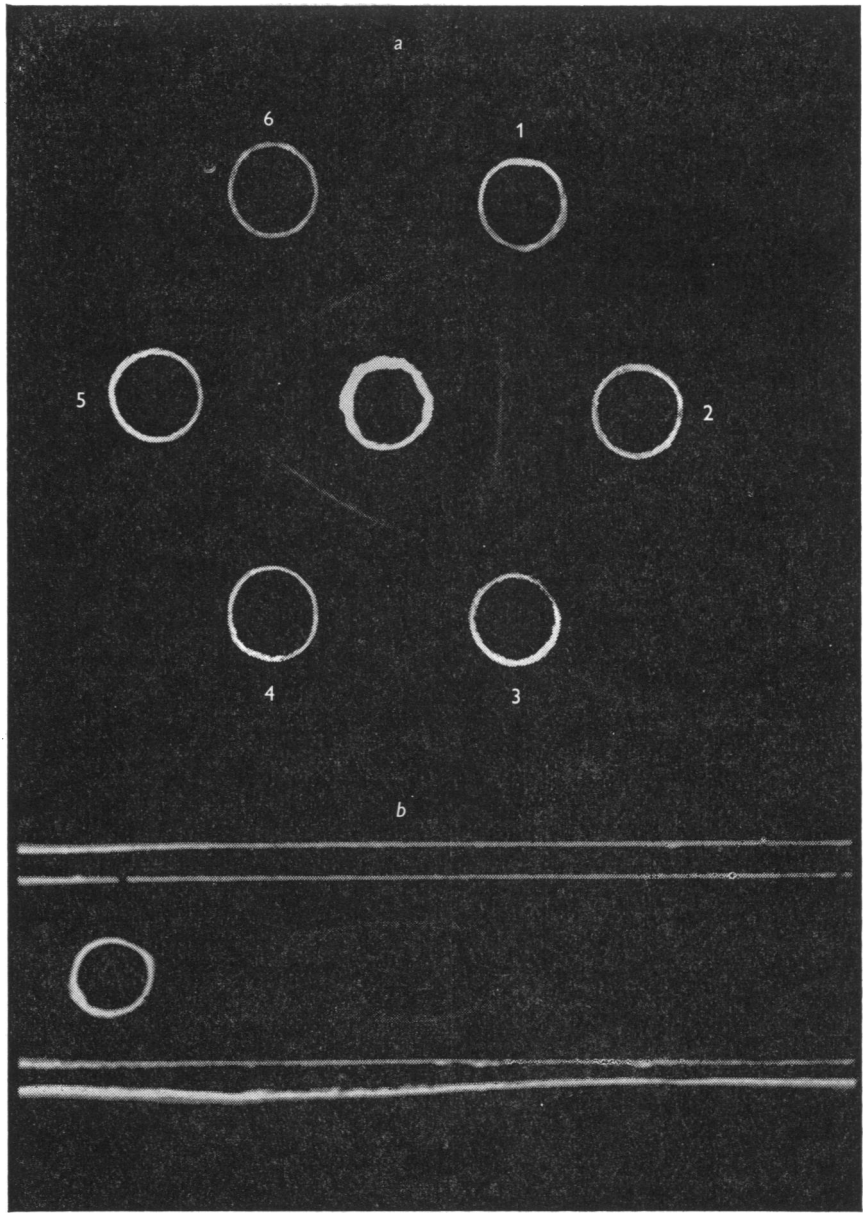
Immunoelectrophoresis in agarose confirmed that there was only a single reacting globulin in both the immune sera. Text-fig. 3*b* shows the single precipitin arcs produced by electrophoresis of AgCB followed by diffusion against AsCB placed in the troughs on either side.

Cross-reactivity of AsDH and AgDH was also shown by enzyme inhibition studies. Pre-incubation of both AgDH and purified bovine dopamine-β-hydroxylase with AsDH abolished enzymic activity (Table 1).

Fluorescence histology. Indirect immunofluorescent staining of the adrenal medulla demonstrated the presence of both antigens (Pl. 1). The blue native fluorescence of the large medullary cells, and of the smaller cells of the cortex, was hardly altered by the preimmune serum, but with immune sera the cells of the medulla showed a bright green fluorescence which contrasted with the unaltered fluorescence of the cortex.

When sections of the sheep superior cervical ganglion (Pl. 2) were treated with immune sera (both AsDH and AsCB), followed by the fluorescein labelled goat anti-rabbit globulin serum, the cytoplasm of the sympathetic cell bodies reacted strongly and became bright green, but their nuclei remained dark. There was little change in the bluish native fluorescence when preimmune serum was used instead. Similarly, when the immune serum was incubated with its corresponding antigen, before being layered on, little specific fluorescence developed after treatment with the goat globulin.

Certain differences emerged when comparing the distribution of the immuno-fluorescence with that of the catecholamine fluorescence in different parts of the neurone (Pl. 3). Whereas catecholamine fluorescence



Text-fig. 3. (*a*) Immunodiffusion in agarose of AgDH (in centre well) against AsDH produced in three different rabbits (wells, 2, 4 and 6), and against their respective pre-immune sera (wells, 1, 3, 5). (*b*) Micro-immuno-electrophoresis in agarose of AgCB, run from the well in a field of 6 V/cm against AsCB placed in the troughs on either side. Photograph retouched.

was more concentrated in the axon terminals than in the ganglia, the converse applied to immunofluorescence. The peripheral ground plexus of axon terminals proved more difficult to stain than their cell bodies. There was more non-specific staining and the axon terminals on the outer border of the media of blood vessels reacted less strongly and more diffusely. The sympathetic nerve fibres emanating from the ganglia showed only weak specific fluorescence but when sympathetic nerves were sectioned longitudinally through a constriction which had been in place for 1–5 days, an increase in specific fluorescence was seen proximal to the constriction (Pl. 4).

TABLE 1. Inhibition of dopamine-β-hydroxylase activity by its antiserum (AsDH). Enzyme samples were pre-incubated at 4° C for 60 min with either pre-immune or immune serum and then centrifuged at 10,000 *g*. The supernatant was added to an incubation mixture containing [^{14}C]dopamine as the substrate, and the amount of [^{14}C]noradrenaline formed after 30 min determined

Enzyme source	Addition	dpm	Inhibition (%)
Bovine	Pre-immune serum	1473	—
	AsDH	143	97
Sheep	Pre-immune serum	971	—
	AsDH	108	87
No enzyme	Nil	97	—

This was localized mainly proximal to the constriction in the region where we have also observed specific catecholamine fluorescence, but there was also a slight increase in fluorescence distal to the constriction. By contrast the supporting connective tissue and the tissue at the constriction showed only the dark-blue native fluorescence characteristic of the untreated tissue and that treated with pre-immune sera.

DISCUSSION

Antibodies to the soluble proteins of the chromaffin granules of the adrenal medulla have recently been prepared to study the mechanism of secretion of catecholamine by the adrenal gland (Blaschko *et al.* 1967; Sage, Smith & Kirshner, 1967; Schneider *et al.* 1967; Gibb *et al.* 1967). We have used them to provide immunohistochemical evidence that some of the proteins involved in the synthesis and storage of noradrenaline in the adrenal medulla are present in sympathetic neurones.

The term 'chromogranin(s)' has been given to the total soluble proteins from bovine chromaffin granules (Blaschko *et al.* 1967). It has since been shown that about 50% of the dopamine-β-hydroxylase content of chromaffin granules is in a soluble form (Belpaire & Laduron, 1968; Viveros, Arqueros & Kirshner, 1968) and so this enzyme is one of the chromo-

granins. We made antibodies to both dopamine-β-hydroxylase and to the fraction from sheep adrenals corresponding to chromogranin A or S_1 from bovine adrenals. Since the exact equivalence of chromogranin A and S_1 has yet to be established, a problem of nomenclature arises for one of the sheep fractions we have used. The term 'a catecholamine binding protein' has been employed (Livett *et al.* 1969), while recognizing that its binding properties alone were inadequate to account for the storage capacity of the intact vesicles.

The specificity of the immunohistochemical technique depends in the first instance on the purity of the antigens used for antibody production. The dopamine-β-hydroxylase fraction obtained by DEAE-cellulose chromatography was shown to have a high specific activity by enzymic radioassay, whereas the major protein fraction which showed some ability to bind noradrenaline had negligible enzymic activity. Electrophoresis demonstrated a satisfactory degree of purity of the two antigens whose mobilities differed markedly. Dopamine-β-hydroxylase migrated very slowly as has also been demonstrated by Gibb *et al.* (1967). As a final check, the specific reactivities of the antisera were demonstrated by immunodiffusion and immunoelectrophoresis.

Antibodies against dopamine-β-hydroxylase and a catecholamine binding protein fraction produced a localized specific fluorescence in both the sheep adrenal medulla and sympathetic nerves, each of them with a distribution essentially identical to the catecholamine fluorescence induced by formaldehyde treatment (Falck & Owman, 1965). However, the adrenal medulla and cell bodies in sympathetic ganglia reacted more strongly with the antisera than the nerve trunks and their axon terminals, whereas catecholamine fluorescence was more concentrated in the nerve terminals than in the perikaryon. Laduron & Belpaire (1968) have reported a decreasing gradient of dopamine-β-hydroxylase activity from sympathetic cell bodies to axon terminals and the same might be true for other proteins.

The observation that dopamine-β-hydroxylase and a catecholamine binding protein accumulated proximal to an axonal constriction, in a similar fashion to noradrenaline (Dahlström, 1965), and to granular vesicles (Kapeller & Mayor, 1967; Geffen & Ostberg, 1969) is consistent with the concept that the site of synthesis of these proteins is in the soma of the neurone. Evidence for axoplasmic transport of proteins in sympathetic nerves has come from the studies of Livett *et al.* (1968), who showed that [^{14}C]proteins were transported somatofugally at the same rapid rate as [^{14}C]noradrenaline, and from Laduron & Belpaire (1968), who demonstrated an increase in the dopamine-β-hydroxylase activity proximal to nerve ligations. Mueller & Shidemann (1968) have provided indirect evidence for the site of synthesis of the granular vesicles being the soma of the neurone,

in experiments where they delayed the formation of new peripheral amine binding sites after reserpine by inhibiting protein synthesis in the cell bodies. Once formed, the proteins could be transported to the axon terminals either as components of the granular vesicles (Dahlström, 1967) or else separately, perhaps by the neurotubules, and incorporated into vesicles within the axon (Pellegrino de Iraldi & de Robertis, 1968).

When catecholamines are secreted from the adrenal medulla, chromogranins are also released (Banks & Helle, 1965; Kirshner, Sage, Smith & Kirshner, 1966; Blaschko *et al.* 1967; Sage *et al.* 1967; Schneider *et al.* 1967), together with adenine nucleotides (see Douglas, 1966), but the lipids of the chromaffin granule membranes are not discharged (Schneider *et al.* 1967; Trifaro, Poisner & Douglas, 1967). This has been interpreted as evidence for the release of the medullary vesicle contents by exocytosis. It is not yet known whether a similar release of proteins and nucleotides accompanies noradrenaline release in sympathetic transmission. The rates at which the two tissues secrete catecholamines are very different, and in addition the nerve possesses an efficient uptake mechanism for conserving noradrenaline during transmission (Brown, 1965). If the release of the sympathetic transmitter involves exocytosis of the granular vesicles, the nerve terminals may also possess a mechanism for protein conservation. The use of antibodies to protein components of the vesicles could provide the means to study this problem.

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REFERENCES

Banks, P. & Helle, K. (1965). The release of protein from the stimulated adrenal medulla. *Biochem. J.* **97**, 40–41*C*.

Belpaire, F. & Laduron, P. (1968). Tissue fractionation and catecholamines. I. Latency and activation properties of dopamine-β-hydroxylase in adrenal medulla. *Biochem. Pharmac.* **17**, 411–421.

Blaschko, H., Comline, R. L., Schneider, F. H., Silver, M. & Smith, A. D. (1967). Secretion of a chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. *Nature, Lond.* **215**, 58–59.

Brown, G. L. (1965). The release and fate of the transmitter liberated by adrenergic nerves. The Croonian Lecture 1964. *Proc. R. Soc.* B **162**, 1–19.

Crout, J. R. (1961). Catecholamines in urine. In *Standard Methods of Clinical Chemistry*, vol. 3, ed. Seligson, D., p. 62. New York: Academic Press.

Dahlström, A. (1965). Observations on the accumulation of noradrenaline in the proximal and distal parts of peripheral adrenergic nerves after compression. *J. Anat.* **99**, 677–689.

DAHLSTRÖM, A. (1967). The intraneuronal distribution of noradrenaline and the transport and lifespan of amine storage granules in the sympathetic adrenergic neuron. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **257**, 93–115.

DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121**, 404–427.

DAVIS, G. A., SANTEN, R. J. & AGRANOFF, B. W. (1965). The production of a linear density gradient by means of a proportioning pump. *Analyt. Biochem.* **11**, 153–157.

DOUGLAS, W. W. (1966). The mechanism of release of catecholamines from the adrenal medulla. *Pharmac. Rev.* **18**, 471–480.

FALCK, B. & OWMAN, C. H. (1965). A detailed methodological description of the fluorescence method for cellular localization of biogenic monoamines. *Acta Univ. Lund.* II, **7**, 1–24.

FRIEDMAN, S. & KAUFMAN, S. (1965). 3-4 Dihydroxyphenylethylamine-β-hydroxylase: Physical properties, copper content, and role of copper in the catalytic activity. *J. biol. Chem.* **240**, 4763–4773.

GEFFEN, L. B. & OSTBERG, A. (1969). Distribution of granular vesicles in normal and constricted sympathetic neurones. *J. Physiol.* **204**, 583–592.

GEFFEN, L. B. & RUSH, R. A. (1968). Transport of noradrenaline in sympathetic nerves and the effect of nerve impulses on its contribution to transmitter stores. *J. Neurochem.* **15**, 925–930.

GIBB, J. W., SPECTOR, S. & UDENFRIEND, S. (1967). Production of antibodies to dopamine-β-hydroxylase of bovine adrenal medulla. *Molec. Pharmacol.* **3**, 473–478.

HILLARP, N. A. (1960). Some problems concerning the storage of catecholamines in the adrenal medulla. In *Ciba Foundation Symposium on Adrenergic Mechanisms*, ed. VANE, J. R., WOLSTENHOLME, G. E. W. & O'CONNOR, M., pp. 481–486. London: Churchill.

HOLBOROW, E. J. & JOHNSON, G. D. (1967). Immunofluorescence. In *Handbook of Experimental Immunology*, ed. WEIR, D. M. Oxford: Blackwell.

KAPELLER, K. & MAYOR, D. (1967). The accumulation of noradrenaline in constricted sympathetic nerves as studied by fluorescence and electron microscopy. *Proc. R. Soc.* B **167**, 282–292.

KIRSHNER, N., SAGE, H. J., SMITH, W. J. & KIRSHNER, A. G. (1966). Release of catecholamines and specific protein from adrenal glands. *Science, N.Y.* **154**, 529–531.

LADURON, P. & BELPAIRE, F. (1968). Transport of noradrenaline and dopamine-β-hydroxylase in sympathetic nerves. *Life Sci. Oxford* **7**, 1–7.

LEVIN, E. Y., LEVENBERG, B. & KAUFMAN, S. (1960). The enzymic conversion of 3-4 dihydroxyphenylethylamime to norepinephrine. *J. biol. Chem.* **235**, 2080–2086.

LIVETT, B. G., GEFFEN, L. B. & AUSTIN, L. (1968). Proximo distal transport of [^{14}C] noradrenaline and protein in sympathetic nerves. *J. Neurochem.* **15**, 931–939.

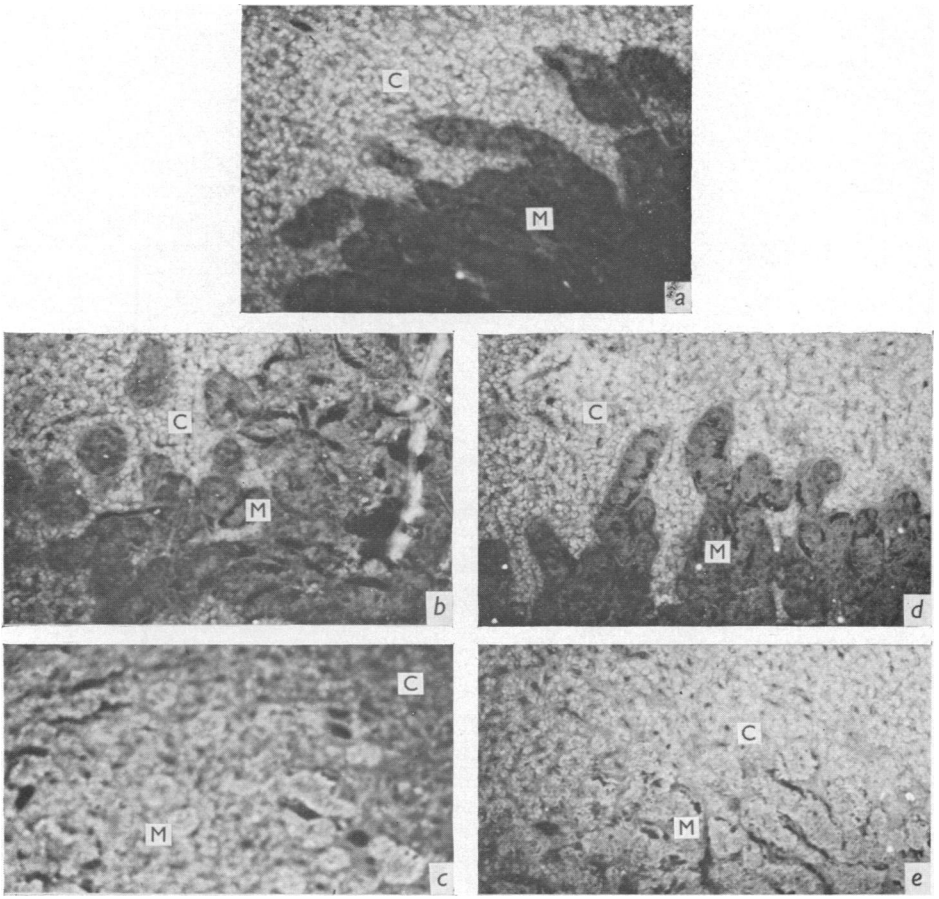
LIVETT, B. G., GEFFEN, L. B. & RUSH, R. A. (1969). Immunohistochemical evidence for the transport of dopamine-β-hydroxylase and a catecholamine binding protein in sympathetic nerves. *Biochem. Pharmac.* **18**, 923–924.

MUELLER, R. A. & SHIDEMAN, F. E. (1968). The prolongation of reserpine-induced cardiac norepinephrine depletion by metabolic inhibitors. *Biochem. Pharmac.* **17**, 451–467.

NAIRN, R. C. (1968). Standardization in immunofluorescence. *Clin. exp. Immunol.* **3**, 465–476.

NAIRN, R. C. (1969). *Fluorescent Protein Tracing*, 3rd edn. London: Livingstone.

PELLEGRINO DE IRALDI, A. & DE ROBERTIS, E. (1968). The neurotubular system of the axon and the origin of granulated and non-granulated vesicles in regenerating nerves. *Z. Zellforsch. mikrosk. Anat.* **87**, 330–344.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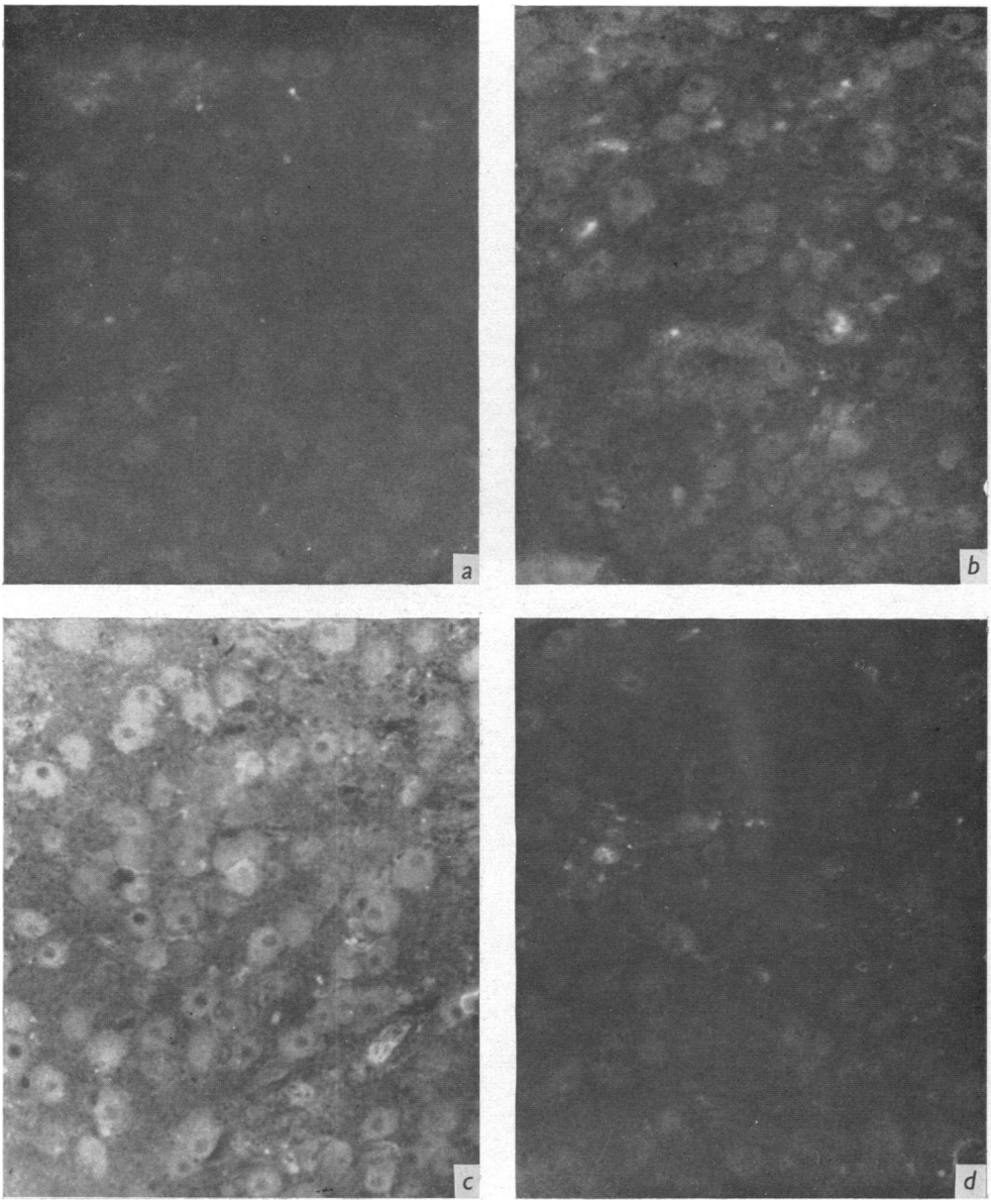
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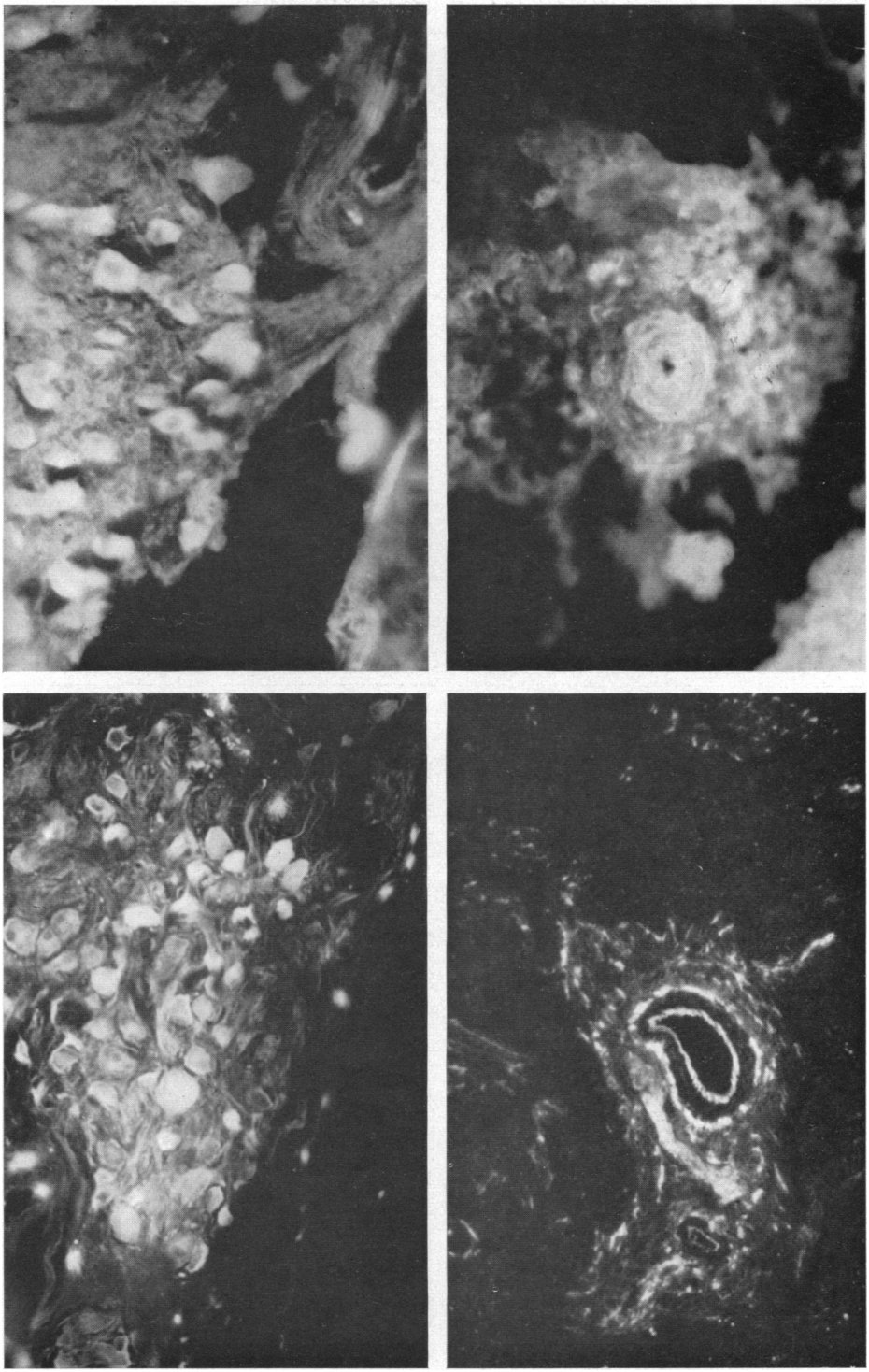

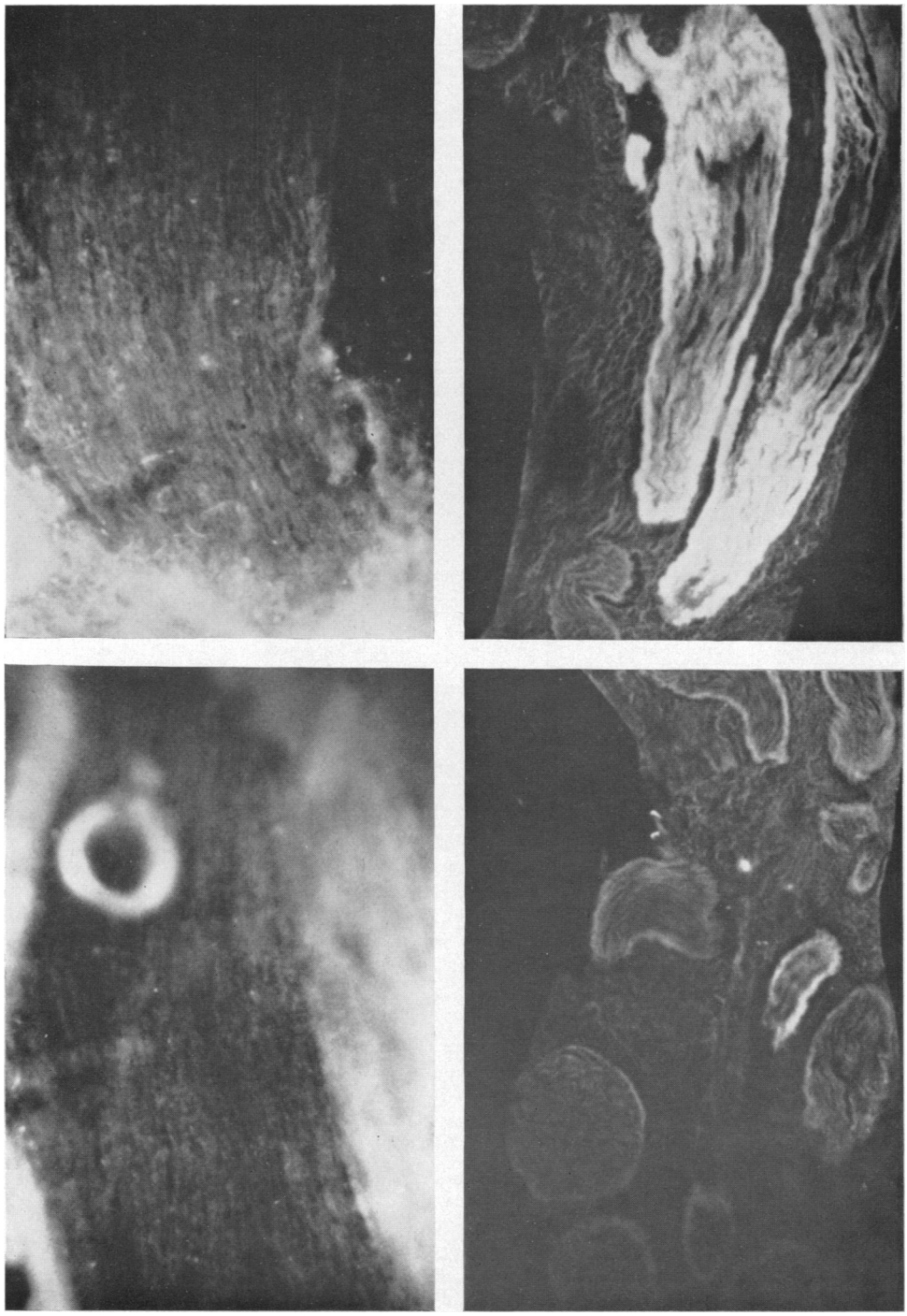

POTTER, L. T. (1967). Role of intraneuronal vesicles in the synthesis, storage and release of noradrenaline. *Circulation Res.* suppl. 3, **21**, 13–24.

SAGE, H. J., SMITH, W. J. & KIRSHNER, N. (1967). Mechanism of secretion from the adrenal medulla. I. A microquantitative immunologic assay for bovine adrenal catecholamine storage vesicle protein and its application to studies of the secretory process. *Molec. Pharmacol.* **3**, 81–89.

SCHNEIDER, F. H., SMITH, A. D. & WINKLER, H. (1967). Secretion from the adrenal medulla: biochemical evidence for exocytosis. *Br. J. Pharmac. Chemother.* **31**, 94–104.

SCHUMANN, H. J., SCHNELL, K. & PHILIPPU, A. (1964). Subcellulare Verteilung von Noradrenalin und Adrenalin im Meerschweinchenherzen. *Arch. exp. Path. pharmak.* **249**, 251–266.

SMITH, W. J. & KIRSHNER, N. (1967). A specific soluble protein from the catecholamine storage vesicles of bovine adrenal medulla. *Molec. Pharmacol.* **3**, 52–62.

SMITH, A. D. & WINKLER, H. (1967*a*). A simple method for the isolation of adrenal chromaffin granules on a large scale. *Biochem. J.* **103**, 480–482.

SMITH, A. D. & WINKLER, H. (1967*b*). Purification and properties of an acidic protein from chromaffin granules of bovine adrenal medulla. *Biochem. J.* **103**, 483–492.

TRIFARO, J. M., POISNER, A. M. & DOUGLAS, W. W. (1967). The fate of the chromaffin granule during catecholamine release from the adrenal medulla. I. Unchanged efflux of phospholipid and cholesterol. *Biochem. Pharmac.* **16**, 2095–2100.

VIVEROS, O. H., ARQUEROS, L. & KIRSHNER, N. (1968). Release of catecholamines and dopamine-β-oxidase from the adrenal medulla. *Life Sci. Oxford* **7**, 609–618.

WEIR, D. M. (1967). *Handbook of Experimental Immunology.* Oxford: Blackwell.

EXPLANATION OF PLATES

PLATE 1

Indirect immunofluorescent staining of sheep adrenal glands. Sections of the cortico-medullary junction were stained with goat anti-rabbit globulin serum, conjugated with fluorescein isothiocyanate, after treatment with (*a*) phosphate buffered saline, (*b*) pre-immune rabbit serum, (*c*) AsCB, (*d*) pre-immune serum, (*e*) AsDH. The medullary cells (M) are larger with little non-specific and strong specific staining compared to the cortical cells (C).

PLATE 2

Indirect immunofluorescent staining of sympathetic neurones in sheep superior cervical ganglion. Sections were stained with fluorescein conjugated goat serum after treatment with (*a*) phosphate buffered saline, (*b*) pre-immune rabbit serum, (*c*) AsCB (*d*) AsCB pre-incubated with AgCB. The specific fluorescence of the perikaryon of the neurones develops only with the immune serum and is abolished by titration against the antigen.

PLATE 3

A comparison of the distribution of fluorescence due to antibodies to dopamine-β-hydroxylase (above) and to catecholamines (below) in the sheep coeliac ganglion (left) and splenic arterioles (right).

PLATE 4

The effect of ligating the hepatic nerves in the sheep for 24 hr on immune dopamine-β-hydroxylase fluorescence (left) and catecholamine fluorescence (right). The fluorescence of the nerves is increased proximal (above) compared to distal to the constriction (below) and this is more pronounced for catecholamines than for the enzyme. The specific immunofluorescence in the nerve was green in contrast to the blue autofluorescence of the connective tissue on either side of the bubble artifact just below the constriction.