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Immunochemical Methods for Demonstrating Macromolecules in Sympathetic Neurons

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Immunochemical methods for demonstrating macromolecules in sympathetic neurons

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[Plate 16]

Sensitive and specific immunological techniques have been used to localize two macromolecular components of noradrenergic vesicles (chromogranin A and dopamine β-hydroxylase) in sympathetic neurons. Evidence is provided that they are transported from their site of synthesis in the cell bodies to their site of storage in the terminals from which they are released, probably by exocytosis.

Introduction

The adrenal medulla as a model for adrenergic neurons

Progress in the field of adrenergic transmission in sympathetic neurones has benefited greatly from studies on catecholamine storage, synthesis and secretion in the adrenal medulla (see Smith 1968; Kirshner 1969). The catecholamine storage vesicles in the adrenal medulla contain up to 35 % of their dry mass as protein (Hillarp 1959) and a characteristic feature of the vesicles is their high content of water soluble proteins, termed chromogranins (Blaschko *et al.* 1967). Chromogranins, catecholamines and adenine nucleotides are released in stoichiometric proportions from actively secreting adrenal glands (for review see Kirshner & Kirshner, p. 279) and on the basis of recent calculations, it has been proposed that the secretion is 'quantal' in nature and involves release of the entire soluble contents of any one vesicle by a calcium dependent mechanism (Viveros, Arqueros & Kirshner 1969).

One way to gain further insight into the mechanisms underlying synaptic transmission in adrenergic neurons has been to examine the macromolecular components of the vesicles associated with catecholamines.

Methods

Immunological methods lend themselves readily to the identification of macromolecules because of their high specificity and sensitivity, and antibodies to one of the chromogranins (Banks & Helle 1965; Helle 1966), and to two purified components of the chromogranins, dopamine β-hydroxylase (Gibb, Spector & Udenfriend 1967) and chromogranin A (Sage, Smith & Kirshner 1967; Schneider, Smith & Winkler 1967) have been used to study the mechanism of secretion from the adrenal gland (for review see Geffen & Livett 1971). The adrenal medulla

Results

Immunodiffusion and complement fixation

Immunodiffusion analysis of subcellular fractions from sheep medullae, sympathetic ganglia and nerves, and sympathetically innervated tissue, has shown that dopamine β-hydroxylase and chromogranin A are present in microsomal and supernatant fractions in approximately the same proportions as noradrenaline (Geffen, Livett & Rush 1970*a*). Similarly, Banks, Helle & Mayor (1969) have found that antiserum to bovine adrenal chromogranin A cross-reacts with a noradrenaline-containing vesicle fraction from bovine splenic nerves. By complement fixation we have shown that release of chromogranin A and dopamine β-hydroxylase accompanies the release of noradrenaline from perfused sheep spleen during nerve stimulation (Geffen *et al.* 1969*b*); these results agree with those of De Potter, de Schaepdryver, Moerman & Smith (1969) and suggests that release of the vesicle contents occurs by a process of exocytosis.

Immunohistochemistry

Immunofluorescence histochemical techniques have been used to localize these proteins in the adrenal medulla and adrenergic neurones (Hopwood 1968; Geffen *et al.* 1969*a*) and to provide evidence that these protein components of the noradrenergic vesicles are synthesized in the cell body of the neurons and transported to the nerve terminals by axoplasmic flow (Livett, Geffen & Rush 1969; Geffen *et al.* 1969*a*). The results of these experiments have been reviewed recently (Geffen, Livett & Rush 1970*a*, *b*; Livett 1970) and so only a brief account will be given here.

All immunofluorescence photographs in figure 1, plate 62, are from cryostat sections of sheep adrenergic tissues which have been incubated first with rabbit anti-sheep chromogranin A or dopamine β-hydroxylase (as indicated), and then with fluorescein-labelled goat anti-rabbit-γ-globulin by the indirect or 'sandwich' technique of immunofluorescence (see Nairn 1969). As controls, pre-immune serum, fluorescein-conjugate alone, and immune serum adsorbed with its specific antigen, were used to establish the specificity of the staining. The distribution of the specific green immunofluorescence attributable to the chromogranins was always compared with that of the catecholamines, as demonstrated by the formaldehyde technique of Hillarp & Falck (for review of methodology see Corrodi & Jonsson 1967). In all sections examined chromogranin A and dopamine β-hydroxylase exhibited a similar distribution. In sections of the adrenal gland (figure 1*a*, *b*) both were present exclusively in the medulla. Catecholamines and chromogranins shared a similar distribution in sympathetic ganglia (figure 1*c*, *d*) and all three accumulated proximal to a ligature on sympathetic nerves (figure 1*e*) providing evidence for axoplasmic flow of the noradrenergic vesicles. Owing to differences in the methodology of the two techniques it has proved more difficult to obtain comparable results with immunofluorescence in peripheral axon terminals (for discussion see Geffen *et al.* 1970*a*), but when preterminal axons were cut in longitudinal section so the chromogranins could be seen inside them (figure 1*g*), the axons could be traced to the innervated tissue.

Conclusion

The immunohistochemical technique provides a powerful means of localizing macromolecular components of noradrenergic vesicles in peripheral adrenergic neurones, and preliminary

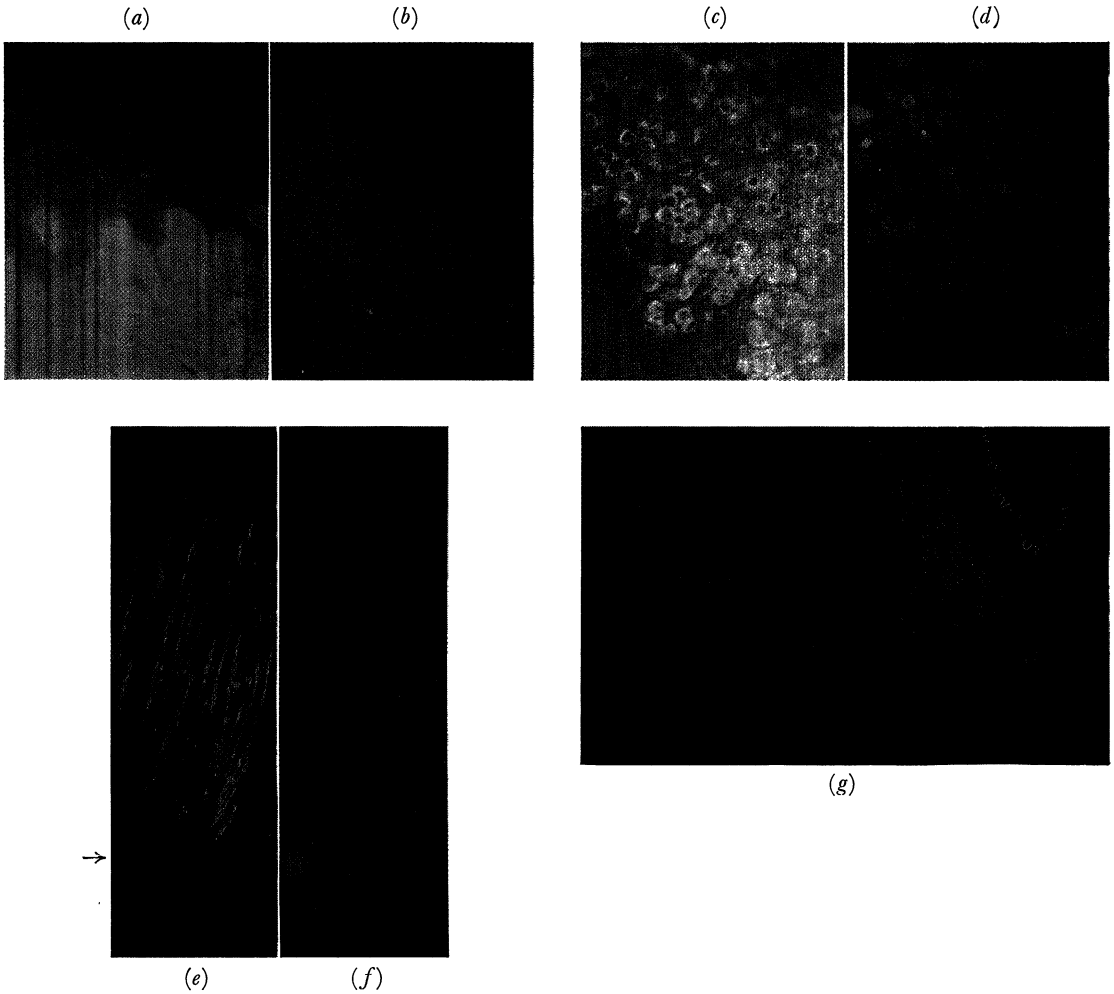

FIGURE 1. Catecholamine fluorescence and chromogranin immunofluorescence of sheep adrenergic tissues.

(*a*) Sheep adrenal gland. Formaldehyde-induced fluorescence of catecholamines in the medulla is shown by the specific yellow fluorescence (below); the cortex exhibits only the native background fluorescence.

(*b*) Indirect immunofluorescent staining of sheep adrenal glands. Sections of cortico-medullary junction were incubated with rabbit anti-sheep dopamine β-hydroxylase, followed by goat anti-rabbit-γ-globulin conjugated with fluorescein isothiocyanate. The medullary cells (below) are larger and exhibit strong specific fluorescence (green) compared to the cortical cells which show only the blue native fluorescence.

(*c*) and (*d*) A comparison of the distribution of fluorescence due to catecholamines (*c*) and to chromogranin A (*d*) in the sheep coeliac ganglion.

(*e*) and (*f*). The effect of ligating the hepatic nerves in the sheep for 24 h on the catecholamine fluorescence (*e*) and immune dopamine β-hydroxylase fluorescence (*f*). The fluorescence of the nerves is increased proximal to the constriction (arrow) while very little fluorescence is observed distal to the constriction (see Livett *et al.* 1969; Geffen, Livett & Rush 1969*a*).

(*g*) Pre-terminal axons containing dopamine β-hydroxylase are seen innervating the splenic artery of the sheep.

All photographs were taken on Agfa CK colour film under the conditions described in Geffen *et al.* (1969*a*).

(*Facing p.* 360)

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evidence (Cheah & Geffen 1970; Hartman & Udenfriend 1970) indicates that it may be a useful tool for localizing enzymes involved in the metabolism of transmitters in the central nervous system. The chemical heterogeneity of the brain complicates the interpretation of enzyme localization in subcellular fractions of homogenates and therefore, a specific histochemical method such as this is invaluable in establishing a substance as a transmitter in a specific neuronal pathway.

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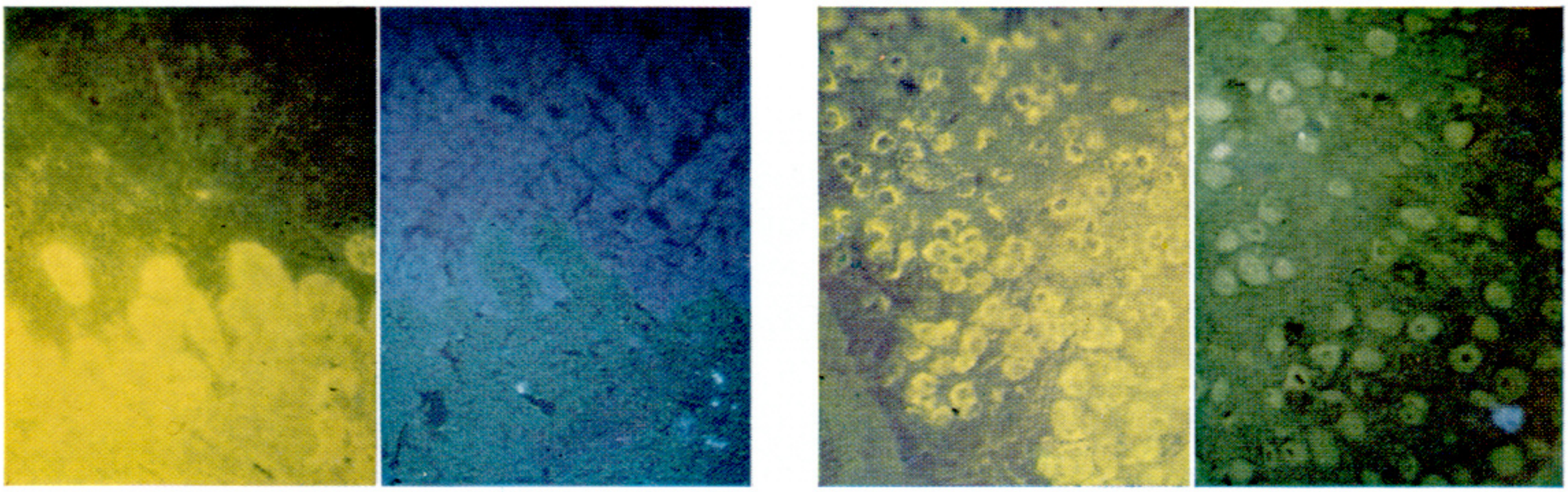

(a) (b) (c) (d)

(e) (f) (g)

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FIGURE 1. Catecholamine fluorescence and chromogranin immunofluorescence of sheep adrenergic tissues.

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