THE UPTAKE AND LOCALIZATION OF CATECHOLAMINES IN CHICK EMBRYO SYMPATHETIC NEURONS IN TISSUE CULTURE

J. M. ENGLAND AND M. N. GOLDSTEIN
Department of Anatomy, Washington University School of Medicine, St Louis, Missouri, U.S.A.

SUMMARY

The uptake of exogenous \[^3H\]dopamine, \[^3H\]norepinephrine, and \[^3H\]epinephrine by dissociated chick embryo sympathetic neurons growing in tissue culture was studied by autoradiography. The neurons, growing in a medium containing nerve growth factor, rapidly and specifically took up all three catecholamines for at least 60 days, while no uptake was observed in several other cell types, including satellite cells and chick dorsal-root ganglion cells. The uptake was dependent on the concentration of the catecholamine and the duration of the pulse and was inhibited by cocaine and several sympathomimetic amines.

Labelling was visualized only with fixatives which react with catecholamines to form water-insoluble compounds.

Autoradiographs showed that the label was much denser over the axons than the cell bodies. The label was distributed uniformly along the axons and did not seem to be preferentially localized at the axon terminals or varicosities which contain aggregates of dense core granules. These observations indicate that a large portion of the exogenous \[^3H\]catecholamine is localized in an extragranular compartment and suggest that the differentiated function of the sympathetic neuronal cell membrane, which plays an important role in uptake, is retained after prolonged tissue culture.

INTRODUCTION

Postganglionic sympathetic neurons take up exogenous norepinephrine and structurally related amines (for review see Iversen, 1967). The kinetics of this uptake process and the effect of a variety of drugs which alter this process have been studied in intact animals (Whitby, Hertting & Axelrod, 1960; Whitby, Axelrod & Weil-Malherbe, 1961), perfused organs with heavy adrenergic innervation (Kopin, Hertting & Gordon, 1962; Iversen, 1963), and sympathetic ganglia (Fischer & Snyder, 1965). \[^3H\]norepinephrine has been used with light- and electron-microscope autoradiography to localize uptake in the pineal body (Wolfe, Potter, Richardson & Axelrod, 1962), atrial myocardium (Wolfe & Potter, 1963), hypothalamus (Aghajanian & Bloom, 1966, 1967), spleen (Gillespie & Kirpekar, 1966), brain (Lenn, 1967), and vascular sympathetic axons (Devine & Simpson, 1968). In these organs the label was predominantly found in relation to small unmyelinated axons and nerve endings, many of which contained dense core granules.

Levi-Montalcini & Angeletti (1963) reported that dissociated embryonic chick sympathetic neurons survive in tissue culture and undergo axonal regeneration when grown
in a medium containing nerve growth factor (Levi-Montalcini & Booker, 1960). The purpose of this study is to examine the uptake of [3H]catecholamines by chick sympathetic neurons in tissue culture, in order: (1) to see if these cells retain this differentiated function after prolonged growth in vitro, and (2) to determine the disposition of exogenous catecholamines in the entire neuron, rather than in only its terminal axons.

METHODS

Sympathetic ganglia were dissected from 10-day-old chick embryos and incubated for 90 min in 0.3% crude trypsin (Difco 1–300) in Hanks’s solution. The cells were dissociated by gentle pipetting in a small volume of trypsin solution, then washed 3 times in the culture medium consisting of 70% medium 199, 30% calf serum, 100 units/ml purified nerve growth factor (Levi-Montalcini & Booker, 1960), and 100 units/ml penicillin. The washed cells were suspended in a small volume of medium and about 0.2 ml of suspension was placed on a 22 x 22 mm collagen-coated coverslip in a 35-mm plastic Petri dish. Eight to 10 cultures can be made from the pooled ganglia of 3 dozen embryos. Each culture was fed with 2 ml of medium and gently swirled to return the small cellular aggregates to the centre of the coverslip. Cultures were incubated at 36.5 °C in a humidified atmosphere of 95% air and 5% CO2 and re-fed at 48-h intervals.

After 3–68 days in vitro the uptake of catecholamines was studied by incubating cultures for 1–60 min in medium containing 1–10 μc/ml of the following radioactive catecholamines: DL-norepinephrine-7-H3.HCl (specific activity 7.27 c/mM), 3,4-dihydroxyphenylethyl-2-H3 amine.HCl ([3H]dopamine) (specific activity 1.84 c/mM), and DL-epinephrine-7-H3 (specific activity 10–1 c/mM). After the pulse, the cultures were rinsed 4 times in warm Hanks’s solution and fixed or chased in normal medium or in medium containing 10–5 to 10–4 m unlabelled catecholamines for periods ranging up to 24 h.

Several fixatives were tested, including: 10% formalin in Hanks’s; acetic acid: ethanol (2:6); acetic acid:ethanol:formalin (10:85:5); Bouin’s solution; 3% glutaraldehyde in Hanks’s (pH 7.2–7.4); and buffered formol-dichromate (Wood, 1963). After fixation, autoradiographs were prepared using AR-10 stripping film as described previously (Goldstein, 1967). These preparations were exposed for 7 days at room temperature and stained with either 0.02% toluidine blue in 0.15 M (pH 6) phosphate buffer or 0.04% aqueous crystal violet.

Inhibition of [3H]norepinephrine uptake was studied by incubating cultures in medium containing dopamine HCl, L-norepinephrine bitartrate, L-epinephrine bitartrate, or cocaine for 5–30 min and then adding [3H]norepinephrine to this medium for the desired pulse period.

For histological study, cultures were also fixed in 3% glutaraldehyde in Hanks’s and stained by the Bodian method, or mounted in the fixative for study with positive phase-contrast optics.
RESULTS

Dissociated sympathetic neurons in vitro

Within 1 h after explantation the dissociated sympathetic ganglion cells reaggregated into small clumps on the surface of the collagen and by 24 h axons began to grow from the cellular aggregates. These early axonal outgrowths were typically very fine and had a complex pattern of terminal arborizations. With time the axons elongated greatly and by 15 days in vitro a dense network of axons was observed (Fig. 1).

With a clump of ganglion cells as a centre of orientation, the axons were arranged differently in various regions of the culture. Immediately adjacent to the aggregate of cell bodies, the axons coursed randomly to form a dense mat of nerve fibres. As these fibres proceeded more peripherally, they established a parallel orientation with each other and formed bundles of axons which characteristically branched and rejoined, forming a complex network. Many axons terminated as free endings at the periphery, while others became entangled in the axonal network or within the clumps of cell bodies.

After approximately 15 days in culture, small groups of neurons formed epithelial-like arrangements. These neurons had large nuclei and prominent nucleoli and were surrounded by small fusiform satellite cells with ovoid nuclei. Most of the satellite cells were in the aggregates of ganglion cell bodies, while only a few were associated with the bundles of axons. Fine neurites coursed over and around the neurons and their satellites (Figs. 2, 3). Long axons and shorter dendrites were observed arising from the neuron cell bodies (Fig. 4). The terminal axons and the axons which projected into the peripheral network of nerve fibres had many varicosities.

A small number of fibroblasts were also observed in these cultures, but they did not survive under these culture conditions and often appeared vacuolated by the second week and degenerated shortly thereafter. The nerve cells on the other hand survived and appeared healthy even after 68 days in culture.

Uptake of [3H]catecholamines

Autoradiographs of sympathetic ganglion cultures pulsed with [3H]dopamine, [3H]norepinephrine, or [3H]epinephrine showed graining specifically concentrated over the axons. The distribution of the label was identical for the three catecholamines and was uniform along the entire length of the axons with no preferential accumulation at the axon terminals (Fig. 5). The visualization of this radioactive label was dependent on the method of fixation; graining resulting from [3H]dopamine or [3H]norepinephrine was observed after fixation with 3% glutaraldehyde in Hanks's. However, [3H]epinephrine could not be visualized with glutaraldehyde, although it, as well as other catecholamines, could be demonstrated after formol-dichromate fixation. The other fixatives which were used failed to show label over axons in cultures pulsed with any of the [3H]catecholamines.

The intensity of the graining, indicative of catecholamine uptake, was dependent on the concentration of the [3H]catecholamine and the duration of the pulse. Because of the limits of resolution and the variability in axonal volume, it is difficult to quanti-
tate these data precisely. Therefore, only a simple comparison can be made. The axons of cultures pulsed for 60 min with 5 μc/ml [3H]norepinephrine were more densely labelled than those pulsed with 1 μc/ml for the same time. Similarly those pulsed with 5 μc/ml [3H]norepinephrine for 60 min were more densely labelled than those pulsed for 1 min.

There was a marked disparity in the density of graining over the cell bodies and the axons. In contrast to the densely labelled axons, the large neuronal cell bodies were much less densely labelled (Fig. 6). A portion of the graining over the aggregate of cell bodies in Fig. 6 appears to be originating from axons coursing around and over the cell bodies. This close relationship between cell bodies and neuron fibres can be easily seen in the Bodian preparations in Figs. 2 and 3. The relatively lighter labelling of cell bodies as compared to axons does not appear to be an artifact of this autoradiographic technique, because the cell bodies in cultures pulsed with [3H]leucine or [3H]uridine were very densely labelled (unpublished observations). An apparent paradox was encountered when cell bodies lying on dense bundles of axons appeared densely labelled. However, close examination revealed that the graining pattern over these cell bodies was identical to and continuous with that over the adjacent bundles of axons. This dense graining may be an artifact caused by radiation from the [3H]-catecholamines taken up by the axons which lie under these cell bodies.

[3H]norepinephrine uptake, a differentiated function of sympathetic neurons, was maintained even after prolonged culture. Neurons growing in culture for more than 60 days showed moderate to heavy graining over their axons when pulsed for 60 min with 5 μc/ml [3H]norepinephrine. In this system the labelling resulting from [3H]-catecholamine uptake was specific for sympathetic neurons. No labelling was observed in HeLa cells, Chinese hamster fibroblasts or in chick fibroblasts, satellite cells, or the cell bodies and axons of dorsal root ganglion cells.

**Turnover of [3H]catecholamines**

After a pulse of 5 min with 5 μc/ml [3H]dopamine or [3H]norepinephrine, there was a gradual decrease in the density of graining over the axons as cultures were incubated for increasing periods of time in a medium free from catecholamines. After a 24-h chase, the graining was still significant over the axons. However, when 5 x 10^{-4} M unlabelled dopamine or norepinephrine was added to the chase medium of cultures previously pulsed with 5 μc/ml [3H]dopamine or [3H]norepinephrine for 5 min, there was a more rapid disappearance of label from the axons than in cultures chased with catecholamine-free medium. This decrease was very striking by the 5th hour of incubation in the chase medium.

**Inhibition of [3H]norepinephrine uptake**

The uptake of [3H]norepinephrine was partially or totally inhibited by cocaine and several unlabelled catecholamines. Cultures which were incubated with 10^{-6} M cocaine for 30 min and then pulsed with 5 μc/ml [3H]norepinephrine for 20 min in the presence of this concentration of cocaine, showed a partial inhibition of uptake (Fig. 8) as compared to cultures pulsed without cocaine in the medium (Fig. 7). Total inhibi-
Catecholamine uptake by chick neurons

681

The uptake of $2 \mu g/ml \left[ ^3H \right]$norepinephrine during a 5-min pulse was completely inhibited by incubating cultures for 5 min with either $6 \times 10^{-5} M$ L-epinephrine bitartrate or $6 \times 10^{-5} M$ dopamine. HC1 and pulsing in the presence of the unlabelled catecholamines. Inhibition of uptake by dopamine is shown in Fig. 9, where axons are completely unlabelled. Identical results were obtained when uptake was inhibited with epinephrine. In all cases graining in the control cultures was uniformly dense along the entire length of the axon.

DISCUSSION

Dissociated chick sympathetic neurons actively grow in tissue culture by axonal proliferation and elongation. The retention of differentiated function by these cells after prolonged growth in vitro provides a system for analysing catecholamine uptake and turnover in both the cell bodies and axons of growing sympathetic neurons.

At all stages of axonal outgrowth the cultured neurons rapidly took up $\left[ ^3H \right]$dopamine, $\left[ ^3H \right]$norepinephrine, and $\left[ ^3H \right]$epinephrine from the culture medium so that there was intense axonal labelling after pulse periods as short as 1 min. Examples of this phenomenon have been reported after the in vivo administration or in vitro organ perfusion of $\left[ ^3H \right]$catecholamines (Iversen, 1967). The present work demonstrates that sympathetic neurons isolated from presynaptic input and end-organ connexions are able to take up exogenous catecholamines.

The label resulting from the exogenous $\left[ ^3H \right]$catecholamines was uniformly distributed along the entire axon. It did not appear to be preferentially localized at the varicosities or in the terminal portion of the axons. Electron microscopy demonstrated concentrations of dense core granules in these dilatations and relatively few granules in other portions of the axon (unpublished observations). This supports the observations of Malmfors (1965), who found that, when catecholamine-specific fluorescence was restored to reserpine-depleted axons by exogenous norepinephrine with a monoamino oxidase inhibitor, the terminals appeared more uniformly fluorescent than the controls. In addition, Van Orden, Bensch & Giorman (1967) found this restoration of fluorescence did not result in a concomitant increase in the number of dense-core granules. This evidence suggests that in addition to the $\left[ ^3H \right]$catecholamine taken up and localized at nerve endings in association with dense-core granules (Wolfe et al. 1962; Wolfe & Potter, 1963; Aghajanian & Bloom, 1966, 1967; Lenn, 1967; Devine & Simpson, 1968) some is stored, at least temporarily, in the axoplasm independent of the dense-core granules. The present data support the work of Van Orden et al. (1967), who demonstrated an extragranular catecholamine storage compartment. Both studies emphasize the importance of a functionally differentiated axonal membrane in the uptake process.

Glutaraldehyde preserved $\left[ ^3H \right]$dopamine and $\left[ ^3H \right]$norepinephrine label best of the fixatives tested, although label could be visualized also with formol-dichromate.
ever, \([\text{H}]\)epinephrine was preserved only by formol-dichromate. Hopwood & Coupland (1965) demonstrated that several biogenic amines, including norepinephrine and dopamine, react with glutaraldehyde to form an insoluble complex while epinephrine fails to react. On the other hand, epinephrine, as well as other catecholamines, after exposure to an oxidizing agent such as potassium dichromate, is transformed by the chromaffine reaction to a dark, water-insoluble precipitate (Hillarp & Hökfelt, 1955). This supports the hypothesis that at least a portion of the \([\text{H}]\)catecholamines are not firmly bound in the axon, but free in the axoplasm. Catecholamines appear to be washed out of the axons when fixation does not convert them into water-insoluble compounds. Thus, they appear to be visualized only when precipitated \textit{in situ} by the fixative.

There appears to be a striking non-uniformity in the distribution of \([\text{H}]\)catecholamines in the neurons. Most of the label appears to be localized within the axons, with much less in the cell bodies. In fact, a portion of the sparse label seen over the cell bodies may actually be due to the \([\text{H}]\)catecholamine in the small nerve fibres in this region. It is impossible to determine the precise site of the radioactivity in the cellular aggregates by using whole-mount preparations. It is hoped that thin-section autoradiography, now in progress, will provide conclusive results.

Fischer & Snyder (1965) reported rapid \([\text{H}]\)norepinephrine uptake by the superior cervical ganglion; however, it is difficult in their study to assess the relative contribution of the cell bodies and intraganglionic neurites to this uptake. Three possible explanations for this unequal distribution of label are: (1) the exogenous catecholamine may be degraded more rapidly in the cell body than in the axon to compounds which possibly do not undergo the glutaraldehyde or chromaffine reactions; (2) there may be a very rapid flow of catecholamines from the cell body into the axons; or (3) there may be a selective difference in the uptake mechanism in the membrane of the cell body and axon. Studies are in progress to determine which of these hypothesized mechanisms is mainly responsible for this unequal distribution.

The uptake of \([\text{H}]\)norepinephrine by cultured neurons is inhibited by cocaine and several sympathomimetic amines, a finding similar to the results described for other systems, including the isolated rat heart (Burgen & Iversen, 1965; Iversen, 1965). This functional similarity to other more conventional systems and the relative stability of this differentiated function in prolonged tissue culture suggests that sympathetic neurons and perhaps selected regions of the central nervous system \textit{in vitro} may provide useful models for studying the uptake, storage, and possibly release of catecholamines in an accessible and isolated environment. Investigations with human foetal sympathetic and human sympathicoblastomas in tissue culture have given similar results (Goldstein, 1967).

This investigation was supported by U.S.P.H.S. grant number GM 240-09.
Catecholamine uptake by chick neurons

REFERENCES


(Received 12 August 1968)
The photomicrographs are of dissociated 10-day-old chick embryo sympathetic ganglia grown for various periods in tissue culture.

Fig. 1. Note the dense fascicles of axons which extend from the aggregates of neuron cell bodies and satellite cells. Fifteen days in vitro. × 40.
Catecholamine uptake by chick neurons
Figs. 2, 3. Large flattened ganglion cell bodies with associated neurites are surrounded by numerous small satellite cells with dense ovoid nuclei. Note the axons and dendrites which course below and above the ganglion cells. Bodian stain. Thirty days in vitro. Fig. 2, ×1250; Fig. 3, ×750.

Fig. 4. Numerous neurites arise from the large ganglion cells to form a dense network. Satellite cells adjacent to neurons are out of the plane of focus. Fixed in 3% glutaraldehyde; phase-contrast. Fifteen days in vitro. ×900.
Fig. 5. Neurons pulsed with 2.5 µg/ml [3H]norepinephrine for 10 min; washed and chased for 1 h. Note the even distribution of grainning along the axon bundles and terminals. This distribution is seen in experiments fixed immediately after pulsing. Eight days in vitro. × 475.

Fig. 6. Neurons pulsed with 5 µg/ml [3H]norepinephrine for 60 min; washed and chased for 10 min. There is much less grainning over the cell bodies than over the adjacent axons. A portion of the grainning over the aggregate of cell bodies appears to be derived from axons which course over and around these cell bodies. Because of the thickness of the preparation, two photographs taken at different focal planes were superimposed to show grainning accurately over both cell bodies and axons. Ten days in vitro. × 950.
Catecholamine uptake by chick neurons
Fig. 7. Neurons pulsed with 5 μc/ml [3H]norepinephrine for 20 min; washed and chased for 10 min. Note the very dense graining over the bundles of axons. Five days in vitro. × 900.

Fig. 8. Neurons pretreated with 10⁻⁴ M cocaine for 30 min, then pulsed in the presence of cocaine with 5 μc/ml [3H]norepinephrine for 20 min; washed and chased for 10 min. Note the marked decrease in graining over axons as compared with control in Fig. 7. Five days in vitro. × 900.

Fig. 9. Neurons pretreated with 6 × 10⁻⁴ M dopamine.HCl for 5 min, then pulsed in the presence of dopamine with 2 μc/ml [3H]norepinephrine; washed and chased for 10 min. Note complete absence of graining over axons; comparable controls were densely labelled. Eight days in vitro. × 900.