The identification of dopamine in the rabbit’s carotid body

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Introduction

There is disagreement about the influence of catecholamines on the chemoreceptor discharge of the carotid body (Anitchkov & Belen’tkii 1963; Biscoe 1965; Eyzaguirre & Koyano 1965; Eyzaguirre & Zapata 1967). It is clear, however, that catecholamines are present in the carotid body. Muscholl, Rann & Watzka (1960) have shown that the carotid body of the calf contains up to 5 μg/g of noradrenaline and Niemi & Ojala (1964) have obtained cytochemical evidence for the presence of catecholamines in the human carotid body. Chiocchio, Biscardi & Tramezzani (1966) have found adrenaline, noradrenaline and dopamine in the carotid body of the cat. They suggested that the dopamine, which was present in concentrations of 0·1 to 0·2 μg/carotid body, might be stored in mast cells in the tissue.

In electron micrographs of the carotid body dense-cored vesicles have been observed (Ross 1959; Lever, Lewis & Boyd 1959; Fillenz & Woods 1966). They are similar to those in the adrenal medulla (Coupland 1965; Elfvin 1965), which have been shown to contain most of the adrenaline and noradrenaline present (Blaschko & Welch 1953; Hagen & Barnett 1960). The vesicles were seen in the glomus Type I cells (de Kock & Dunn 1964), which are generally considered to be responsible for chemoreceptor sensitivity (Ross 1959).

Fillenz & Woods (1966) have applied the Falck & Hillarp histochemical technique for catecholamines to the rabbit carotid body and have described a particularly intense fluorescence in the Type I cells. The present work reports histochemical and chemical evidence that the Type I cells of the rabbit’s carotid body contain notably high concentrations of dopamine. A preliminary account has appeared (Dearaley, Fillenz & Woods 1967).

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Carotid bodies were quickly removed from rabbits which had been killed by a blow on the head; they were then frozen in ethanol cooled with solid carbon dioxide. They were freeze-dried in a Pearse Speedivac tissue dryer for 24 to 48 hours and embedded in paraffin wax. Sections were cut 10 to 15 μm thick and mounted dry on clean slides. The wax was removed with xylol, and excess xylol was allowed to evaporate. The slides were then dried over phosphorus pentoxide for at least 2 h to remove any absorbed water. They were treated at temperatures from 22 to 75 °C with the vapour from paraformaldehyde which had been stored in a dessicator at 80% humidity (Hamberger 1967). The sections were mounted in liquid paraffin and examined with a fluorescence microscope equipped with a high-pressure mercury discharge lamp.

The parts of sections seen by their fluorescence to contain carotid body Type I cells were dissected out and assembled in a group on a glass slide, mounted in liquid paraffin and covered with a coverslip. The slide was clamped to a black masking plate and placed in the cuvette chamber of an Aminco-Bowman spectrophotofluorimeter at 45° to the light beam so that the path of reflected light was away from the photomultiplier. The group of carotid body sections was exposed to the beam through a 6 mm diameter hole in the mask and the fluorescent light which entered the photomultiplier was recorded. Activation and emission spectra were plotted.

Model systems were made by stretching rabbit mesentery on to slides. These were dried and painted with 1 μg/ml standard solutions of adrenaline, noradrenaline, dopamine and 5-hydroxytryptamine (5-HT). The slides were treated with vapour from paraformaldehyde at 75 °C for 1 h and examined in the fluorescence microscope. After wetting with liquid paraffin to reduce light scattering by the tissue the models were transferred to the spectrophotofluorimeter and their spectra were recorded. Background spectra were obtained from unpainted mesentery treated with paraformaldehyde, and from untreated model slides.

Treated slides of carotid body and the noradrenaline and dopamine models were exposed for 1 h to dry hydrogen chloride gas at 75 °C, and re-examined in the spectrophotofluorimeter. This procedure shifts the activation maximum of the noradrenaline fluorophore to shorter wavelengths (Corrodi & Jonsson 1965).

Preparation of tissue extracts for chromatography

It was not possible to dissect the carotid body entirely free of carotid artery. The tissue removed weighed 1 to 2 mg, about half of which was carotid body. Portions of carotid artery of similar weight from just below the carotid body were removed as well, and the two groups of samples were analysed and compared.

Pooled groups of eight carotid bodies adhering to pieces of carotid artery, and pooled samples of carotid artery only, were homogenized separately under 3-0 ml of 0·5 N perchloric acid containing 2 mg each of ascorbic acid and ethylene diamine tetra-acetic acid (EDTA). The homogenate was centrifuged at 0 °C for 5 min at
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500 g. The supernatant was decanted and its pH adjusted to 5.9 to 6.1 by addition of 1.5 M potassium carbonate in the presence of 0.2 ml. of 0.5 M sodium dihydrogen phosphate. The solution was centrifuged again to remove precipitated potassium perchlorate. The supernatant was decanted and the precipitate washed with 1.0 ml. of water. Dissolved carbon dioxide, which would otherwise come out of solution at the resin columns, was removed from the combined supernatant and washings by warming them to 30 °C in vacuo.

Chromatographic procedure

Sulphonated polystyrene resin (Amberlite CG-120, 200–400 mesh) was pre­reated in bulk (Håggendal 1963) and stored in the sodium form under 0.1 M phosphate buffer, pH 6.5, containing 0.1% EDTA. Chromatography columns 5 x 3.5 mm diameter were made up from this stock and washed with 5.0 ml. of the phosphate buffer solution immediately before use. The tissue extracts were applied to the columns and the columns were washed with 10 ml. of the phosphate buffer solution followed by 10 ml. of water. Absorbed catecholamines were eluted with n HCl hydrochloric acid; eight successive 2.0 ml. fractions were collected. All solutions were passed through the columns at approximately 10 ml./h under positive air pressure of 40 cm of water.

Estimation of catecholamines in tissue extracts

Fractions 1 and 2 of the column eluates were taken for adrenaline and noradrenaline assay, and fractions 3 to 8 were assayed for dopamine.

Adrenaline and noradrenaline were estimated fluorimetrically by the trihydroxy-indole method as described by Håggendal (1963). Minor modifications in the procedure, which slightly improved reproducibility, were that the ferricyanide oxidation was carried out on 1.0 ml. aliquots at pH 6.8 for 5 min after neutralization with 2 n sodium hydroxide in the presence of 0.2 ml. of 0.5 M sodium dihydrogen phosphate. Dopamine was estimated fluorimetrically after oxidation with iodine at pH 5.4 (Carlsson & Waldeck, 1958). All fluorescence measurements were carried out with an Aminco–Bowman spectrophotofluorimeter.

Control experiments showed that all the adrenaline and noradrenaline was eluted in the first two fractions, and that dopamine did not appear until the third.

Efficiency of extraction procedure

Standard solutions containing 0.1 μg of noradrenaline and 0.1 μg of dopamine in 3.0 ml. of 0.5 N perchloric acid were treated in the same way as experimental homogenates. The mean recovery of noradrenaline was 72.0% ± 3.2 (s.e. of mean), n 5, and of dopamine was 89.9% ± 5.36, n 5.

Standard compounds used for comparison

Samples of adrenaline, noradrenaline, metanephrine, normetanephrine and 5-HT were obtained from L. Light and Co. Epinine (N-methyldopamine) was kindly given by Dr H. Blaschko of the Department of Pharmacology, Oxford.
Standard solutions of 0.1 μg/ml of the catecholamines were assayed fluorimetrically and the activation and emission spectra were plotted. Metanephrine and normetanephrine were oxidized with iodine at pH 6.0 (Håggendal 1962).

The retention volumes of these materials on the resin were also determined. Quantities of 1.0 μg were applied to separate columns and the chromatographic procedure carried out as described above. For this purpose the catecholamines were assayed by their natural fluorescence at 290/325 nm (activation/emission wavelength) and the 5-HT at 295/340 nm.

Results

Fluorescence microscopy

Before treatment with paraformaldehyde, sections of carotid body showed a weak autofluorescence in the Type I glomus cells, and strong green fluorescence of the elastic fibres in the artery walls.

After treatment with paraformaldehyde there were three types of fluorescent structure: the Type I glomus cells, varicose perivascular nerve fibres, and faintly fluorescent non-varicose nerve fibres running among, and closely related to, the glomus Type I cells. The Type II cells did not fluoresce. These structures are illustrated in figure 1, plate 19.

The fluorescence of the Type I cells was particularly intense. It developed rapidly on treatment with paraformaldehyde at 75 °C and did not increase markedly after 20 min. Ten minutes' treatment at 56 °C gave an intensity comparable with that of vascular nerves treated for 1 h at 75 °C. The colour was bright yellow-green when examined with dark-field illumination with a 4 mm thick blue glass primary filter (Schott and Gen BG 12) and a yellow glass secondary filter (Zeiss ocular barrier no. 51). When a 2 mm thick black glass primary filter (Schott and Gen UG 1) was used in combination with a colourless u.v. absorbing secondary filter (Ilford 805 Q) the colour was blue-white. The fluorescence faded slowly in sections mounted in fluoromount when they were exposed to the beam of light in the microscope, but hardly decreased during 30 min exposure when they were mounted in liquid paraffin.

The rate of development of fluorescence, the colours seen with the two filter combinations and the rate of fading under u.v. light are shown in table 1. The Type I cells resembled the dopamine and noradrenaline models and were different from the DOPA, adrenaline, epinine and 5-HT models in one or more respects.

Spectroscopy of tissue sections and models

The activation and emission spectra of the untreated tissue controls were subtracted from those of the paraformaldehyde-treated carotid body Type I cells to give the spectra of the fluorophores produced by the reaction. The activation and emission maxima are listed in table 2 together with the values obtained after treatment of the slides with dry hydrogen chloride.

Because of the relatively large background of light scattered by the tissue, these
Figure 1. Fluorescence photomicrographs of a rabbit carotid body, showing groups of fluorescent Type I cells and both varicose perivascular nerve fibres and non-varicose nerve fibres. Filters: primary, 4 mm Schott and Gen BG 12; secondary, yellow filter, cut-off 460 to 470 nm. High Speed Ektachrome Daylight film, 30 s exposure. 10 μm sections; x 120.
systems gave rather poorly defined spectra. The activation maximum of the Type I cell fluorescence was not accurately reproducible, but the values obtained from four slides lay between 360 and 400 nm. The 5-HT model (emission maximum 530 nm) differed clearly from the other models and from the Type I cells, and the noradrenaline model was distinguished in that its activation maximum shifted from 400 to 335 nm after treatment with dry hydrogen chloride.

**Table 1. Fluorescence Characteristics of Type I Cells and Model Systems:**

<table>
<thead>
<tr>
<th>system</th>
<th>rate of development</th>
<th>colour with filters:</th>
<th>rate of fading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I cells</td>
<td>fast</td>
<td>yellow-green or yellow</td>
<td>blue-white</td>
</tr>
<tr>
<td>Models</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPA</td>
<td>fast</td>
<td>green</td>
<td>blue-white</td>
</tr>
<tr>
<td>dopamine</td>
<td>fast</td>
<td>yellow-green</td>
<td>blue-white</td>
</tr>
<tr>
<td>noradrenaline</td>
<td>fast</td>
<td>yellow-green</td>
<td>blue-white</td>
</tr>
<tr>
<td>adrenaline</td>
<td>slow</td>
<td>green to blue-green</td>
<td>blue-white</td>
</tr>
<tr>
<td>epinine</td>
<td>slow</td>
<td>green to blue-green</td>
<td>blue-white</td>
</tr>
<tr>
<td>5-HT</td>
<td>medium</td>
<td>yellow</td>
<td>yellow</td>
</tr>
</tbody>
</table>

Filter combinations:

A = Primary, 4 mm Schott and Gen BG 12; secondary, yellow, cut off 460-470 nm.
B = Primary, 2 mm Schott and Gen UG 1; secondary, Ilford 805 Q.

**Table 2. Activation and Emission Maxima (in nm) of Carotid Body Type I Cells and Model Systems After Treatment with Paraformaldehyde and with Paraformaldehyde Followed by Hydrogen Chloride**

Wavelengths are uncorrected instrumental values.

<table>
<thead>
<tr>
<th>system</th>
<th>paraformaldehyde</th>
<th>paraformaldehyde + HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Models</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dopamine</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>noradrenaline</td>
<td>400</td>
<td>335</td>
</tr>
<tr>
<td>adrenaline</td>
<td>410</td>
<td>400</td>
</tr>
<tr>
<td>5-HT</td>
<td>400</td>
<td>380</td>
</tr>
</tbody>
</table>

**Table 3. Fluorescence Characteristics of Standard Compounds After Oxidation in Solution**

Activation and emission maxima in nm (uncorrected instrumental values).

<table>
<thead>
<tr>
<th>compound</th>
<th>activation</th>
<th>emission</th>
<th>oxidation conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>adrenaline</td>
<td>340, 415</td>
<td>510</td>
<td>K₃Fe(CN)₆, pH 6-8</td>
</tr>
<tr>
<td>noradrenaline</td>
<td>335, 400</td>
<td>495</td>
<td>K₃Fe(CN)₆, pH 6-8</td>
</tr>
<tr>
<td>metanephrine</td>
<td>430</td>
<td>515</td>
<td>iodine, pH 6-9</td>
</tr>
<tr>
<td>normetanephrine</td>
<td>395</td>
<td>495</td>
<td>iodine, pH 6-9</td>
</tr>
<tr>
<td>dopamine</td>
<td>335</td>
<td>380</td>
<td>iodine, pH 5-4</td>
</tr>
<tr>
<td>epinine</td>
<td>335</td>
<td>390</td>
<td>iodine, pH 5-4</td>
</tr>
</tbody>
</table>
Properties of standard compounds

The activation and emission maxima of the fluorophores obtained by oxidation of catecholamines in solution are shown in table 3. The conditions of oxidation are also given.

Dopamine could not be distinguished fluorimetrically from epinine, but the presence of the other compounds, or of 5-HT, did not prejudice their individual assays. Proportions of adrenaline and noradrenaline in mixtures can be estimated by measuring the fluorescence at 400/515 and 450/515 nm and solving simultaneous equations; the presence of the other materials does not interfere.

Table 4 shows the elution patterns of the standard compounds on the resin columns. The percentage of the total recovery in each of the eight successive 2-0 ml. fractions of n hydrochloric acid is given. 5-HT was not recovered by the procedure.

Adrenaline and noradrenaline gave similar elution patterns, as did metanephrine and dopamine. These substances, however, could be differentiated fluorimetrically.

Catecholamines from tissue extracts

Figure 2 shows the elution patterns of catecholamines recovered from columns after application of the tissue extracts. The elution histogram of a mixture of 0-1 µg of noradrenaline and 0-1 µg of dopamine is also shown.

Good separation of noradrenaline and dopamine was obtained. The material eluted in fractions 1 and 2 of the tissue extracts had the characteristics of noradrenaline. Analysis of the fluorescence at 400/515 and 450/515 nm indicated that the proportion of adrenaline was not more than 10% of the total.

The level of assay in the column eluates was about 10 ng/ml. and the mean noradrenaline content of the tissue samples containing the carotid bodies, after correction for 72·0% recovery, was 1·51 µg/g ± 0·19 (s.e. of mean), n 5. The mean content of the samples of carotid artery only was 1·65 µg/g ± 0·17, n 5. These values are not significantly different (P > 0·3).

Fractions 3 to 6 of the carotid body plus artery extracts contained a material which had the characteristics of dopamine. It had activation and emission peaks at 335/380 nm, after oxidation with iodine at pH 5·4, and maximum concentrations
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were found in fraction 4, which corresponded to the elution peak of the dopamine standard. The level of assay in the different fractions ranged from 0 to 60 ng/ml. No catecholamine was detected after fraction 2 from extracts of carotid artery one. The mean dopamine content of the 40 carotid bodies (in 5 groups of 8), after correction for 89.9% recovery, was 21.1 ng/carotid body ± 3.57, n 5.

![Graph](image)

Figure 2. Catecholamines (ng) eluted from Amberlite CG-120 resin columns in successive 2.0 ml. fractions of N hydrochloric acid. (a) Standard compounds, 0.1 µg of noradrenaline + 0.1 µg of dopamine; (b) Carotid body + carotid artery extract; (c) Carotid artery extract. Fractions 1 and 2 (open), noradrenaline assay; fractions 3 to 6 (shaded), dopamine assay.

Discussion

The characteristics of the fluorescence of the model systems (table 1) are consistent with data published by Falck, Hillarp, Thieme & Torp (1962). The similarity of the fluorescence of the Type I cells to that of the dopamine model under a variety of conditions (tables 1 and 2) suggests that dopamine is the constituent of these cells which gives rise to their intense fluorescence after treatment with paraformaldehyde.

This identification is not, however, in itself absolute, since judgement of colour under the fluorescence microscope is necessarily subjective and is particularly difficult when colours of different intensities are compared. Furthermore, the high background of scattered light impairs the resolution when fluorescence spectra are plotted from tissue sections.

The substance responsible for the fluorescence of the varicose perivascular fibres of the carotid body is almost certainly noradrenaline. The fluorescence is similar in intensity and colour to that of the fibres in the carotid artery wall, where the presence of noradrenaline would be expected (von Euler & Lishajko 1958).
The nature of the fluorogenic material in the weakly fluorescent, non-vascular nerves is difficult to determine. The low intensity suggests that, if the substance is a catecholamine or hydroxytryptamine, it is present in very small quantities. A possible source of such a compound could be uptake by these nerves of an amine liberated from the Type I cells.

The use of strongly acidic cation exchange columns has allowed the preparation of reasonably pure extracts from tissues of substances which are cationic at the pH of application to the resin (pH 5.9 to 6.1). Acidic and neutral compounds are not absorbed by the resin under these conditions.

Differentiation amongst the catecholamines is possible by reference to their retention volumes on the column (Häggendal 1962), to the conditions under which they are oxidized and to the fluorescence spectra of the resulting trihydroxyindoles (Häggendal 1962, 1963; Carlsson & Waldeck 1958). Tables 3 and 4 list these properties of the amines used for comparison.

Noradrenaline was identified by its elution pattern and by the fluorescence developed after oxidation with potassium ferricyanide at pH 6.8. The presence of perivascular fibres in both carotid artery and carotid body samples would account for the extraction of similar quantities of noradrenaline from each. It is not possible to calculate, from our results, how much noradrenaline was present in the carotid body itself.

The substance extracted from samples containing carotid bodies, which was not present in samples of carotid artery alone, was identified as dopamine on the following grounds. It was absorbed on the resin along with noradrenaline. Its retention volume and fluorescence after oxidation with iodine, at pH 5.4, were identical with those of dopamine. Epinephrine, which gave a closely similar fluorescence eluted later and metanephrine, which had the same elution pattern as dopamine, had activation and emission maxima at longer wavelengths.

On the basis of the estimate that approximately half the carotid body samples were actual carotid body tissue, the weight of an individual carotid body was 0.5 to 1.0 mg. Thus the dopamine assay of 21.1 ng/carotid body is equivalent to a concentration of 20 to 40 μg/g.

Chiocchio et al. (1966) have suggested that the dopamine which they found in the cat’s carotid body may be the precursor of other catecholamines, or may be stored in mast cells. In the present work, however, no fluorescent mast cells were seen in any of the sections of the rabbit’s carotid body.

Whilst it is very likely that dopamine is the precursor of the noradrenaline in the perivascular nerves, only minute amounts can be present, as dopamine was not detected in the carotid artery extracts. The dopamine obtained in the carotid body extracts cannot therefore have arisen from the perivascular nerves seen in these samples, but must have come from the intensely fluorescent Type I cells. These cells contain numerous dense-cored granules ranging from 60 to 200 nm in diameter (Fillenz & Woods 1966; Ross 1959; Lever et al. 1959) which resemble those in the cells of the adrenal medulla in many respects. It is possible that these are the site of storage of the amine.

The dopamine concentration is greater than published estimates for other tissues.
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Javerty & Sharman (1965) have reported dopamine contents of up to 12 μg/g in the mammalian caudate nucleus. In peripheral tissues, however, Anton & Sayre (1964) found that the dopamine ranged from 0.02 to 0.12 μg/g and was always present in considerably smaller amounts than noradrenaline.

The latter finding is consistent with the view that dopamine has a rapid turn-over in peripheral tissues, and is the precursor of noradrenaline and adrenaline (review articles by Kaufman & Friedman 1965; Iverson 1967).

The carotid body is thus remarkable not only for its high dopamine content but also because there is at least ten times as much dopamine as noradrenaline. This suggests that dopamine is of significance in this organ other than as a metabolic intermediate.

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