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Histochemical studies on the depletion of noradrenaline by adrenaline in adrenergic nerves of the rat iris

C. G. HAINING, JULIA L. HEYDON AND L. R. MURRAY

From the Pharmacology Section, National Biological Standards Laboratory, Canberra, A.C.T., Australia

The intensity of fluorescence produced in adrenergic nerves by exposure to formaldehyde under controlled conditions and levels of tissue catecholamines were determined in rat irides. Incubation of irides in Krebs solution containing (−)-adrenaline (0·1–10 μg/ml) led to a temperature-dependent reduction of fluorescence intensity, a reduction in tissue noradrenaline and a gain in tissue adrenaline. The changes in fluorescence were reversed by subsequent incubation of the tissue in the presence of (−)-noradrenaline (0·1–1 μg/ml). A reduction of fluorescence intensity and tissue noradrenaline in the rat iris, followed by recovery over 16 h, was also produced by exposing the cornea to a 1 or 5% solution of (−)-adrenaline. The application of these results to studies on noradrenaline uptake in adrenergic nerves is discussed.

The uptake of exogenous adrenaline in tissues is accompanied by a release of noradrenaline (Andén & Magnusson, 1963; Andén, 1964; Angelakos, Bloomquist & King, 1965; Westfall, 1965; Nash, Wolff & Ferguson, 1968) which is thought to result from a competitive exchange reaction (Euler & Lishajko, 1963; Burgen & Iversen, 1965). In recent years it has become possible to follow changes in the level of intraneuronal noradrenaline by histochemical fluorescence methods (Falck, 1962). Such techniques have been used to demonstrate uptake of primary catecholamines at the cellular level in tissues from reserpinized animals (Malmfors, 1965) and displacement of noradrenaline from the sympathetic nerve net-work of the rat iris by adrenaline and other amines (Eränkö & Räisänen, 1966).

The present work was carried out to investigate further the noradrenaline-releasing action of adrenaline and the possibility of employing adrenaline to deplete tissues of noradrenaline in studies on catecholamine uptake into adrenergic nerves. Results of investigations into the suitability of drugs other than reserpine for this purpose have recently been reported by Malmfors & Sachs (1968).

EXPERIMENTAL

Methods

In vitro *experiments.* Wistar rats were killed with ether or carbon dioxide and their eyes removed immediately. Irides with corneas attached were removed from eyes and allotted randomly to treatments. Some irides were treated histochemically immediately after removal from the eye while others were incubated in Krebs solution in each experiment as controls for histochemical treatment and drug effects respectively. Groups consisting of 5 or 10 irides were incubated together in Krebs solution (Ringer-phosphate bicarbonate III; Long, 1961) with or without drug. Equal

numbers were included in control and test groups. Incubations were carried out in 10 ml volumes of solution and gassed with moist preheated 5% carbon dioxide in oxygen at 37° unless stated otherwise.

Dissection of the iris. The method was based largely on that of Malmfors (1965). The eye was placed in a depression in a Perspex plate. Two incisions were made at the same time on opposite sides about 2 mm posterior to the end of the rectus tendon with two razor blades and the anterior segment of the eye was then detached with fine forceps and iris scissors. The lens capsule with adherent vitreous was removed from the anterior segment and the cornea, with attached iris, placed in cold Krebs solution with other similar preparations before being distributed randomly into groups. At the end of the incubation period each preparation was rinsed in cold Krebs solution. The iris and ciliary body were removed from the cornea with fine forceps and a radial incision was made in the iris which was then stretched and allowed to dry on a microscope slide. The ciliary body was separated from the iris with Beaver Mini Blades and the preparation was then placed over phosphorus pentoxide in a desiccator for at least 1 h.

Histochemical treatment of the iris. Condensation of the noradrenaline in adrenergic nerves with formaldehyde gas was as described by Falck (1962). The prepared microscope slides in racks of thirty were placed under vacuum in a desiccator and exposed for 1 h at 40 or 80° to the vapour from paraformaldehyde (6 g/litre) which had been equilibrated at a relative humidity of 70% for one week (Hamberger, Malmfors & Sachs, 1965; Hamberger, 1967). Preparations could be kept without apparent deterioration for 3 or 4 days over phosphorus pentoxide before formaldehyde treatment but after fluorescence had been induced slides were read as soon as possible since some fading appeared to take place within 24 h.

Estimation of fluorescence intensity. The fluorescence intensity of the irides was estimated using either a Zeiss fluorescence microscope fitted with a Zeiss I (BG 12/4) filter for excitation and a combination of Zeiss 53 and Zeiss 44 as barrier filters, or a Reichert fluorescence microscope, fitted with a Reichert E 3(BG 12/6) filter for excitation and a Reichert Sp 3(GG 9/1 + OG 1/1·5) as the barrier filter. In both microscopes the source of illumination was a high pressure mercury lamp, HBO 200 W.

Slides were coded in a random manner so that the treatment received by a preparation could not be identified until all assessments had been made. Scores for fluorescence intensity were always allotted by the same observer on the following basis: 0 = negligible, 1 = indistinct and weak, 2 = distinct and weak and 3 = distinct and strong. The score for each group was expressed as a percentage of that assigned to one of the corresponding control groups of irides included in the same experiment. n = The number of irides examined.

Extraction and estimation of tissue catecholamines. The procedure followed was that of Anton & Sayre (1962).

Source of drugs. (−)-Adrenaline hydrogen tartrate (British Drug Houses Ltd., England). (−)-Noradrenaline (Fluka A.G., Switzerland).

RESULTS

Histochemical evidence for the release of noradrenaline from adrenergic nerves by adrenaline in vitro. Incubation of isolated rat irides for 1 h in Krebs solution at 37°

Table 1. *Loss of fluorescence in adrenergic nerves of the isolated rat iris by incubation with adrenaline.* Groups of rat irides were incubated for 1 h in Krebs solution containing varying concentrations of catecholamine. Fluorescence in adrenergic nerves was induced by treatment with formaldehyde under controlled conditions. The intensity of fluorescence was estimated visually on a 0 to 3 scale and is expressed as a percentage of the value obtained for corresponding control irides incubated in Krebs solution for 1 h. n = The number of irides examined.

Concentration of catecholamine μg/ml	Adrenaline		Noradrenaline	
	n	Intensity of fluorescence %	n	Intensity of fluorescence %
0·01	25	104	20	110
0·1	32	99	30	108
1	70	67	20	109
10	65	34	25	98
100	35	27	20	100

with increasing concentrations of adrenaline led to a graded reduction in the fluorescence intensity of the adrenergic nerve plexus. The threshold concentration of adrenaline necessary to cause a reduction in the intensity of fluorescence was between 0·1 and 1 μg/ml and a near maximum effect was obtained with a concentration of 10 μg/ml (Table 1).

This effect of adrenaline was temperature dependent. When groups of 20 irides were incubated for 2 h with adrenaline (10 μg/ml) at 37, 20 or 0°, little reduction of fluorescence was obtained in the nerve network at the two lower temperatures. Decreases in the fluorescence intensity of adrenaline-treated groups expressed as percentages of values obtained for corresponding control groups were 80% at 37°, 7% at 20 to 21° and 2% at 0°.

Under the conditions employed, fluorescence obtained by exposure to formaldehyde gas was specific and was not produced when stretch preparations of rat irides were heated for 1 h at 40 or 80° in a desiccator in the absence of paraformaldehyde.

No evidence was obtained in any experiments that more prolonged incubation of irides in Krebs solution for periods up to 4 h led to a reduction in the development of fluorescence. Comparison of scores allotted to groups of irides which had been mounted immediately after removal from the eye with those of control preparations incubated in Krebs solution for 1 h did not reveal any difference in the intensity of fluorescence in the two groups.

Incubation of irides with noradrenaline at concentrations from 0·01 to 100 μg/ml did not affect the intensity of fluorescence (Table 1). Irides which had been treated in solutions containing 10 μg/ml or lower concentrations of noradrenaline resembled control preparations which had been incubated in Krebs solution for the same period of time without catecholamine, but incubation with noradrenaline (100 μg/ml) resulted in an overall fluorescence of the tissue. The network of nerves enveloping blood vessels which normally exhibit fluorescence equal to or greater in intensity than other parts of the sympathetic nerve plexus were not clearly discernable, possibly because of the strong background fluorescence.

Since oxygenated Krebs solution containing adrenaline darkened during incubation,

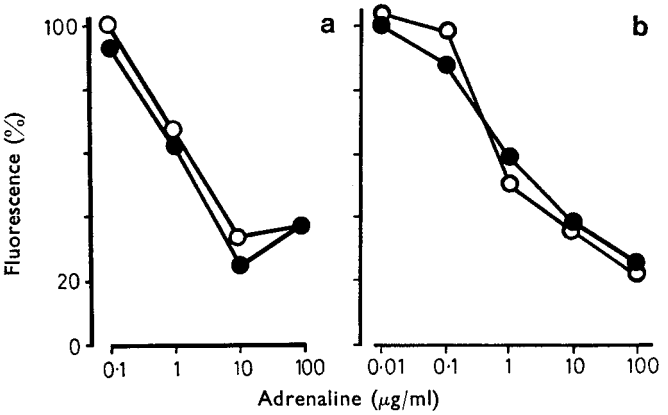

FIG. 1. Failure of ascorbic acid (a) and ascorbic acid plus sodium edetate (b) to modify the effect of incubation for 1 h with adrenaline on the fluorescence intensity of adrenergic nerves in the isolated rat iris. ○, adrenaline; ●, adrenaline + ascorbic acid (10 μg/ml), or ascorbic acid (10 μg/ml), and sodium edetate (20 μg/ml). The effect of each concentration of adrenaline was examined on 15 (a) or 20 (b) irides.

the possibility of protecting catecholamines by the addition of antioxidants was investigated. The presence of ascorbic acid (10 μg/ml) alone or with sodium edetate (EDTA) (20 μg/ml) had a negligible effect on the intensity of fluorescence in irides incubated for 1 h in the absence of catecholamine. The mean fluorescence intensity of irides which had been incubated with ascorbic acid (10 μg/ml) was 90% of maximum (n = 35) compared with 80% for controls (n = 35) whilst corresponding values for those incubated with ascorbic acid (10 μg/ml) + EDTA (20 μg/ml) were 96% (n = 40) and 98% (n = 40) for controls. The presence of ascorbic acid (10 μg/ml) for the same period of incubation alone (Fig 1a) or with EDTA (20 μg/ml) (Fig 1b) failed to modify the effect of adrenaline on the intensity of fluorescence in adrenergic nerves.

Chemical evidence for the release of noradrenaline from adrenergic nerves by adrenaline in vitro. To confirm that a reduction in the intensity of fluorescence indicated

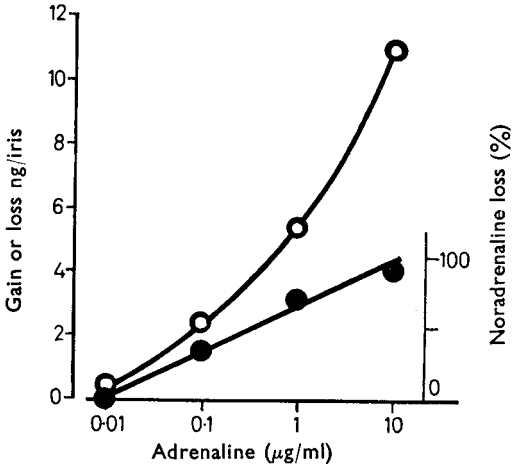

FIG. 2. Changes in noradrenaline and adrenaline content of the isolated rat iris after incubation with adrenaline for 1 h. The effect of each concentration of adrenaline was examined on 25 irides. Catecholamines were determined chemically in groups of 5 or 10 irides. ○, adrenaline gain; ●, noradrenaline loss.

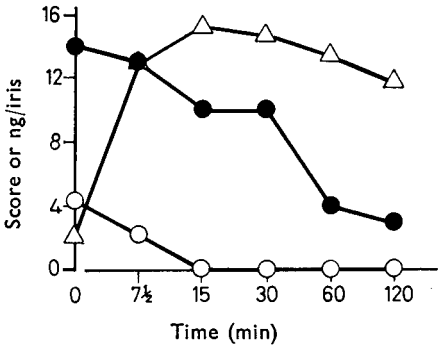

FIG. 3. Time course of noradrenaline depletion from adrenergic nerves of the isolated rat iris incubated with adrenaline (10 μg/ml). ●, Intensity of fluorescence in 5 irides; △, adrenaline and ○, noradrenaline, determined chemically in 5 pooled irides.

loss of noradrenaline, parallel experiments were made in which tissue catecholamine levels were estimated spectrofluorimetrically after extraction. Changes in the adrenaline and noradrenaline content of irides resulting from incubation with varying concentrations of adrenaline for 1 h are shown in Fig 2.

In these experiments, estimates of the quantity of adrenaline taken up by tissues exceeded those of noradrenaline displaced, the difference being most marked in irides which had been incubated with adrenaline (10 μg/ml). Ratios of adrenaline gain to noradrenaline loss obtained with increasing concentrations of adrenaline in the medium were 0·1 μg/ml, 1·6; 1 μg/ml, 1·7 and 10 μg/ml, 3·1.

Time course of noradrenaline release from adrenergic nerves by adrenaline in vitro. The time course of noradrenaline release from isolated irides incubated with adrenaline (10 μg/ml) determined by fluorescence microscopy is shown in Table 2. The

Table 2. *Time course of changes in fluorescence intensity in adrenergic nerves of the isolated rat iris incubated with adrenaline.* Groups of rat irides were incubated for varying periods of time in Krebs solution with or without adrenaline (10 μg/ml). Fluorescence in adrenergic nerves was induced by treatment with formaldehyde under controlled conditions. The intensity of fluorescence was estimated visually on a 0 to 3 scale and is expressed as a percentage of the value obtained for corresponding control irides mounted immediately after removal from the eye. *n* = The number of irides examined.

Period of incubation (min)	Krebs solution		Krebs solution + adrenaline 10 μg/ml	
	n	Intensity of fluorescence %	n	Intensity of fluorescence %
0	20	106		
7·5			25	106
15			25	90
30			27	81
60	40	103	33	51
120	35	103	33	34
240	20	121	20	33

results of a single experiment in which both the intensity of fluorescence and catecholamine levels were determined in parallel after incubation with adrenaline (10 μg/ml), for varying periods of time are shown in Fig. 3. Maximum reduction of fluorescence intensity was obtained in tissue only after exposure to adrenaline for 2 h or more (Table 2) but noradrenaline could not be detected chemically after an incubation period of 15 min (Fig. 3).

Restoration of fluorescence in adrenergic nerves after its reduction by incubation with adrenaline in vitro. The fluorescence of adrenergic nerves which had been reduced in intensity by exposure to adrenaline was restored in preparations which had been treated with noradrenaline (Table 3). Incubation of irides with adrenaline (10 μg/ml) for 1 h followed by a further period of 1 h in Krebs solution after a rinse in Krebs

Table 3. *Restoration of fluorescence in adrenergic nerves of the isolated rat iris after its partial reduction by incubation with adrenaline.* Groups of 10 rat irides were incubated for 1 h with or without adrenaline (10 μg/ml) and then for a further 1 h with varying concentrations of noradrenaline. Fluorescence in adrenergic nerves was induced by treatment with formaldehyde under controlled conditions and its intensity estimated visually on a 0 to 3 scale.

Treatment		Intensity of fluorescence		
1st h adrenaline μg/ml	2nd h noradrenaline μg/ml	Expt 1 Score	Expt. 2 Score	%
0	0	29/30	30/30	100
10	0	7/30	6/30	22
10	0·01	7/30	9/30	27
10	0·1	20/30	17/30	63
10	1	29/30	30/30	100
10	10	30/30	30/30	102

solution resulted in a 78% reduction of fluorescence intensity compared with control preparations. However, when noradrenaline at a concentration of 0·1 μg/ml or higher was present during the second incubation period, the intensity of fluorescence increased and was restored to its original level with a concentration of 1 μg/ml.

Noradrenaline release from adrenergic nerves by adrenaline in vivo. Experiments were made to determine whether the displacement of noradrenaline by adrenaline could be demonstrated *in vivo* under conditions similar to those in which adrenaline and other sympathomimetic amines might be employed clinically. Albino rats were allotted to one of two groups and one drop of a 1 or 5% w/v solution of adrenaline as the hydrogen tartrate dissolved in saline and adjusted to pH 3·5 was applied on one eye for 10 s, the other eye acting as control. Two h later the animals were killed with carbon dioxide and stretch preparations prepared from their irides were exposed to formaldehyde. A single application of adrenaline at either concentration reduced the intensity of fluorescence in the iris of the treated eye. Little difference was observed between the response to 1 and 5% solutions. With groups of 25 animals, the reduction in fluorescence intensity of the treated eyes compared with their controls was 31% when a 1% solution of adrenaline was used and 44% with a 5% solution. A more marked reduction of fluorescence was obtained when the adrenaline solution was applied repeatedly at 10 min intervals for 2 h. In groups of 5 animals, the 1% solution produced a 64% reduction of fluorescence and the 5% solution 75%.

The time course of noradrenaline depletion from the rat iris after a single application of adrenaline to the cornea and its subsequent restoration, was followed by estimating catecholamine levels chemically and determining changes in fluorescence intensity. A 5% solution of adrenaline was applied to the cornea of one eye and allowed to remain in contact with it for 10 s. The other eye was untreated and acted as control. Groups of 15 animals were killed with carbon dioxide at suitable time intervals after drug administration. Irides were removed from both eyes of 10 of the rats, rinsed in Krebs solution and dropped into 0·4N perchloric acid before determinations of their adrenaline and noradrenaline content by the trihydroxyindole method. Irides were dissected from the eyes of the remaining five animals and prepared as stretch preparations on microscope slides for histochemical treatment.

The onset of noradrenaline release was rapid. Maximum depletion within 1 h was indicated by histochemical and chemical procedures (Fig. 4). The intensity of fluorescence was reduced by 50% and the level of chemically estimated noradrenaline by 80–100%. Subsequently, levels of catecholamines gradually returned towards pretreatment levels, complete recovery being achieved about 16 h after treatment.

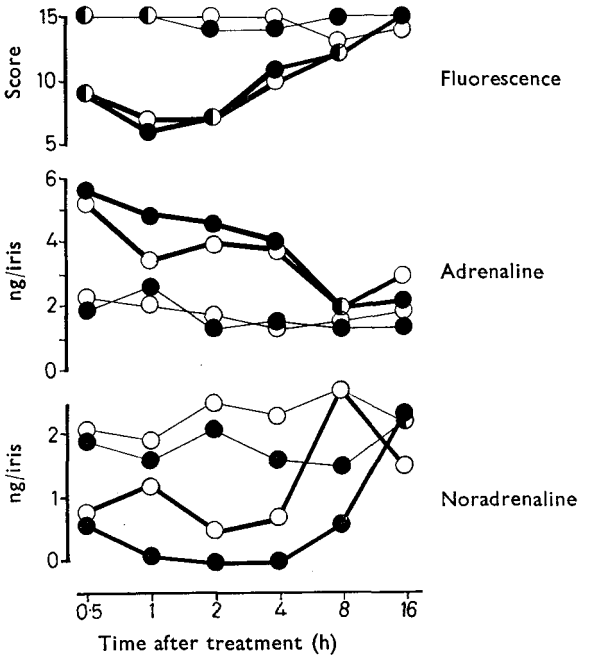

FIG. 4. Time course of noradrenaline depletion and reduction of fluorescence intensity in adrenergic nerves of the rat iris following a single application of adrenaline, 5%, to the cornea. ——, control eye; ——, treated eye. ○, 1st experiment; ●, 2nd experiment. Adrenaline and noradrenaline were determined chemically in 10 pooled irides. Fluorescence intensity was assessed in groups of 5 irides.

DISCUSSION

The histochemical demonstration of noradrenaline is based on the formation of a fluorescent compound by its condensation with formaldehyde under controlled conditions. Since secondary catecholamines require more severe reaction conditions for optimal fluorescence than primary catecholamines (Corrodi & Jonsson, 1967), the reduced intensity of fluorescence obtained in tissues exposed to adrenaline

after treatment with formaldehyde suggests that part of the endogenous noradrenaline has been replaced by adrenaline.

The loss of fluorescence in irides treated with a relatively high concentration of adrenaline and its restoration in preparations incubated with noradrenaline confirms results previously reported by Eränkö & Räisänen (1966). In the present experiments these changes have also been shown to take place with concentrations of catecholamines much lower than those so far reported. The depletion of noradrenaline stores *in vitro* and *in vivo* by adrenaline and their subsequent restoration has in some cases also been followed by chemical determinations of catecholamines. Although qualitatively results obtained by the two methods are compatible, there are disparities between them if the results are considered on a quantitative basis. This is not unexpected since the scoring system used in the histochemical studies was purely arbitrary and the relation between noradrenaline content and fluorescence intensity is not clear since quenching of fluorescence may take place with high concentrations of noradrenaline (Ritzén, 1966). Furthermore, interaction between adrenaline and formaldehyde under the conditions employed may have led to the formation of a fluorescent compound, which, even though of low intensity, could have contributed to the residual fluorescence present in neurons exposed to adrenaline.

In the noradrenaline uptake studies (Table 3) a graded increase in the intensity of fluorescence was obtained with concentrations of noradrenaline from 0·01 to 1 μg/ml (60×10^{-7}M) but no further effect could be demonstrated when the concentration was increased to 10 μg/ml. The results of chemical determinations indicate that although a reduction of fluorescence intensity is associated with depletion of noradrenaline, a large part of the adrenergic transmitter store must be lost before a reduction of fluorescence intensity is detectable. Confirmation of this is to be found in the reports of recent studies by Olson, Hamberger & others (1968) in which fluorescence histochemistry and measurements of [^{3}H]noradrenaline were made in the same tissue. Reasonable correlation was shown by these workers to exist between subjectively estimated fluorescence and the quantity of catecholamine taken up from Krebs-Ringer solution by irides from reserpine-treated rats which had been exposed to noradrenaline over a limited range of concentrations up to about 35×10^{-7}M. When the concentration of catecholamine was raised to 110×10^{-7}M there was no increase in the intensity of fluorescence of the iris although its [^{3}H]noradrenaline content was almost doubled.

The overall fluorescence observed histochemically in tissues which had been incubated with noradrenaline (100 μg/ml) indicates that at high concentrations uptake of the amine was not restricted to adrenergic nerve fibres. A similar effect was not obvious when the incubation was carried out in the presence of adrenaline instead of noradrenaline. However, there was a large increase in the ratio of adrenaline gain to noradrenaline loss determined chemically in irides which had been incubated with 10 μg/ml of adrenaline compared with those exposed to a concentration of 1 μg/ml (Fig. 2) which could be explained by the uptake of adrenaline at extraneuronal sites.

Our results suggest that provided suitable concentrations of catecholamines are employed, tissue which has been depleted of its adrenergic transmitter by previous treatment with adrenaline may be of value in studies on the uptake of noradrenaline into adrenergic neurons. Limitations which are inherent in the histochemical fluorescence technique would however prevent the application of this method in a strictly quantitative manner. These are, the presence of residual fluorescence in

neurons of adrenaline-treated preparations possibly due to an adrenaline formaldehyde condensation product and the fact that exogenous noradrenaline may accumulate intraneuronally beyond the level at which maximum fluorescence intensity is obtained.

Results of *in vivo* studies (Fig. 4) confirm the rapid onset of noradrenaline release from the iris by adrenaline observed *in vitro* (Fig. 3) and indicate that replenishment of noradrenaline by endogenous catecholamine requires only a few hours. Although adrenaline in solution, at concentrations which have been used clinically, reduced the noradrenaline content of the iris after application to the cornea, the implication of this is not clear. Andén & Magnusson (1963) failed to detect any change in adrenergic function in rats and cats after 95% depletion of peripheral noradrenaline by (+)-adrenaline, which had only about 1/10th the agonist activity of (−)-adrenaline.

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