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Histochemical fluorescence studies on noradrenaline accumulation by Uptake$_2$ in the isolated rat heart

D. E. CLARKE, C. J. JONES AND P. A. LINLEY

Postgraduate School of Studies in Pharmacology and Postgraduate School of Studies in Pharmacy, University of Bradford, Bradford 7, Yorkshire

1. The distribution of noradrenaline accumulated by Uptake$_2$ (Iversen, 1965) in the isolated perfused rat heart has been observed using histochemical fluorescence microscopy.

2. On the basis of the technique used, noradrenaline seems to be predominantly associated with the cardiac muscle cells, but sympathetic nerves and other cardiac structures also serve as retention sites.

3. Phenoxybenzamine, normetanephrine and perfusion with noradrenaline at 20° C all decreased noradrenaline accumulation.

4. Lowered temperature significantly increased the perfusion pressure and significantly decreased the [^{14}C]-sorbitol space in perfused hearts.

5. The implications of these results are discussed.

The uptake process for noradrenaline at high perfusion concentrations (1–40 μg/ml.) in the isolated rat heart was first described by Iversen (1965) and designated Uptake$_2$. Although much data has been recorded with regard to this process (Iversen, 1965, 1967), little is known about the actual location of the accumulated noradrenaline. In preliminary studies, Iversen (1965) observed a severely depressed accumulation of noradrenaline in perfused hearts obtained from immunosympathectomized rats and suggested that the locus of Uptake$_2$ might be associated with postganglionic adrenergic nerves. On the other hand, the recent histochemical fluorescence studies of Malmfors (1967, 1968) and Farnebo (1968) have revealed both intra-neuronal and extra-neuronal sites, the latter being associated with " specific cells " situated in connective tissue and an enhanced fluorescence of the cardiac muscle.

In view of the limited study and uncertain nature of the Uptake$_2$ site or sites for noradrenaline in the rat heart it was decided to further investigate this problem. Histochemical studies have been carried out on both atrial and ventricular tissue and the influence of normetanephrine, phenoxybenzamine and lowered temperature on the cardiac deposition of noradrenaline has been investigated.

Methods

Heart perfusion

Male and female albino Wistar rats (Scientific Products Farm) weighing 150–200 g received sodium pentobarbitone, 60 mg/kg, and heparin 1,000 u. both given intra-

peritoneally. Five min later the hearts were removed and perfused by the Langendorff technique at 37° C at a perfusion rate of 5 ml./min delivered from a constant output two channel pump (Watson-Marlow, Type MHRE). The perfusion fluid consisted of Krebs bicarbonate buffer containing glucose 1 g/l., $CaCl_2 \cdot 6H_2O$ 0·28 g/l., ethylene diamine tetra-acetic acid (EDTA) disodium salt 10 mg/l. and ascorbic acid 20 mg/l. (Iversen, 1963).

After a pre-perfusion for 10 min the hearts were perfused with Krebs solution containing (±)-noradrenaline 10 or 40 μg/ml. for a further 10 min.

Changes in the perfusion pressure as a result of drug treatment or modifying procedures were recorded by means of a Condon manometer.

[^{14}C]-*sorbitol space*

After the 10 min pre-perfusion period, hearts were perfused with (±)-noradrenaline 40 μg/ml. plus [^{14}C]-sorbitol, 15 mμc/ml. for a further 10 min. Unlabelled sorbitol was added to give a final concentration of 500 μg/ml. Hearts were removed from the cannula, blotted and homogenized in 6 ml of distilled water, the sorbitol being extracted as described by Morgan, Randle & Regen (1959). Aliquots of 1 ml. were counted in a Packard Tri-Carb liquid scintillation spectrometer, using the scintillator described by Bray (1960). Efficiency was determined by the use of internal standards.

Histochemical fluorescence microscopy

After removal of the heart from the perfusion cannula it was blotted between filter papers and the atria were rapidly dissected from the ventricles. Both tissues were prepared for histochemical fluorescence microscopy according to the method of Spriggs, Lever, Rees & Graham (1966). A drying time of 36–72 hr over fresh phosphorus pentoxide and the use of paraformaldehyde equilibrated over 34% v/v sulphuric acid were found to provide optimum results.

In each experiment control and test heart sections were prepared on the same slide and received identical treatment.

Microscopy

A Zeiss Ultraphot II microscope was used. Incident light was provided by an Osram HBO 200 mercury lamp and a Zeiss BG 12/3 filter was used in conjunction with the Zeiss dark field oil immersion condenser. A Zeiss barrier filter, type 50, was employed in the microscope tube.

Photography

Photographs were taken using an Exakta 35 mm camera loaded with Ilford FP4 film. Exposure times varied from 30 to 90 sec. The film was developed in Microphen (Ilford) and printed on Ilfobrom grade 5 paper.

Measurement of fluorescence intensity

A Zeiss automatic exposure control was used to give an objective estimate of fluorescence "brightness". The reciprocal of the exposure time computed was taken as an indicator of the average field brightness. The low power objective (× 10) was always used in order to cover as large a field as possible. Care was taken to estimate similar fields in both control and test sections.

Validification of the histochemical method

That specific noradrenaline fluorescence was being observed was shown from the following findings:

(1) The fluorescence exhibited the spectral characteristics of the condensation products of catecholamines (Eränkö, 1967).

(2) The specific fluorescence faded over a period of 24 hr.

(3) Pretreatment of animals with reserpine (5 mg/kg given intraperitoneally for 16 hr) or 6-hydroxydopamine (2×50 mg/kg given intravenously on day 1 followed by 2×100 mg/kg given intravenously on day 7) (Thoenen & Tranzer, 1968), caused a complete disappearance of the fluorescence attributable to adrenergic nerves.

Drugs

(±)-Noradrenaline hydrochloride (Sigma) and (±)-normetanephrine hydrochloride (Sigma) were calculated as base. Stock solutions were made up in 0·01 N hydrochloric acid. Phenoxybenzamine (Dibenyline, Smith, Kline & French) was dissolved in equal parts of propylene glycol and alcohol, and reserpine (Serpasil, Ciba) was dissolved in 20% w/v ascorbic acid solution. 6-Hydroxydopamine hydrobromide was generously donated by Dr. H. Thoenen, Hoffmann-LaRoche & Co. Ltd., Basle, Switzerland, and was dissolved in 0·01 N hydrochloric acid and injected intravenously.

Sorbitol-1-^{14}C was obtained from the Radiochemical Centre, Amersham, and non-radioactive sorbitol from British Drug Houses.

Results

Distribution of adrenergic nerves

Hearts perfused with Krebs solution without noradrenaline showed the same adrenergic nerve fluorescence characteristics with respect to intensity and definition as non-perfused hearts. In the former case, however, the non-specific background fluorescence appeared to be slightly enhanced. Both experimental conditions revealed a more dense adrenergic innervation in atrial muscle compared with ventricular muscle (Fig. 1a and b) which was characterized by the nerve fibres coursing among the cardiac muscle bundles. In both atrial and ventricular sections the coronary arteries were densely innervated just outside the media, while in the smaller vessels this innervation often ran along opposite sides of the structure.

Small orange granules in myocardial cells were also frequently observed and on some occasions mast cells were identified in the epicardium and connective tissue. Specific fluorescence attributable to " chromaffin cells " was not observed in any experiment.

Localization of noradrenaline accumulation

Hearts perfused with noradrenaline, 10 and 40 μg/ml. for 10 min, exhibited a marked increase in the specific fluorescence of the myocardium and smooth muscle of the coronary blood vessels in both atrial and ventricular sections. Figure 2

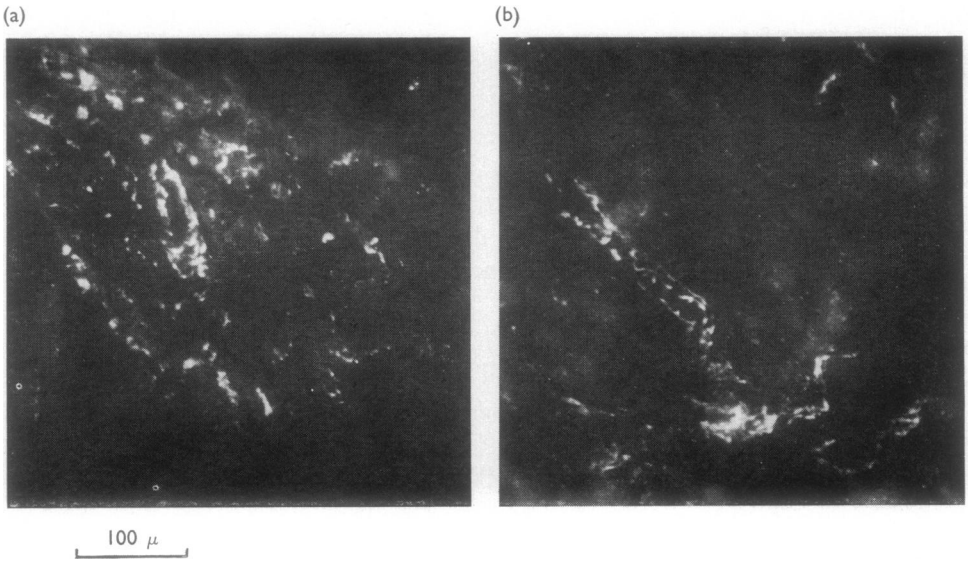

FIG. 1. Histochemical fluorescence in the rat atrium (a) and ventricle (b) after perfusion with Krebs bicarbonate solution. Fluorescent nerve fibres are more numerous in the atrium than ventricle. Non-specific background fluorescence is low.

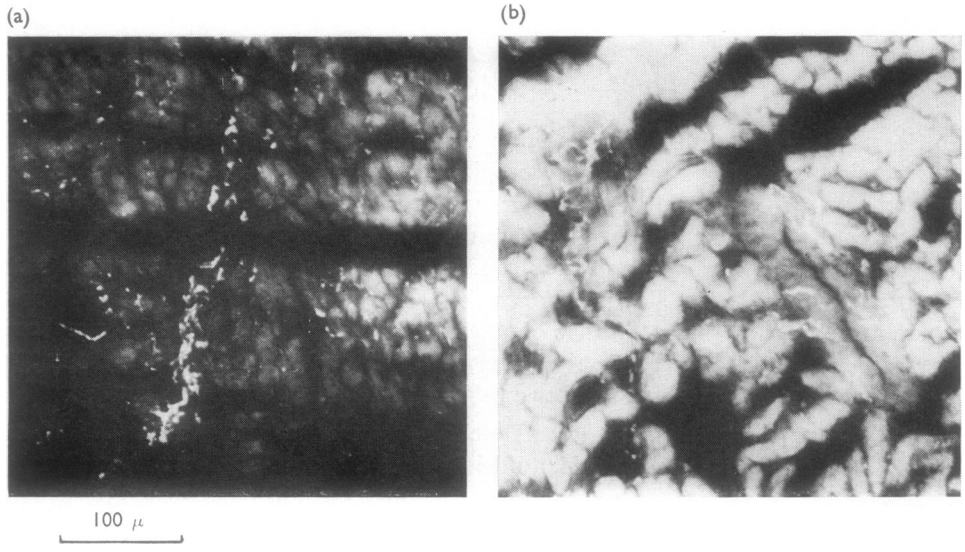

FIG. 2. Histochemical fluorescence in the rat ventricle after a 10 min perfusion with noradrenaline 10 μg/ml. (a) and 40 μg/ml. (b).

illustrates these observations and shows that there was a less marked increase in the fluorescence following perfusion with the lower concentration of noradrenaline. In addition there was an increase in the intensity of fluorescence from the adrenergic nerves, but this observation was less noticeable after perfusion with noradrenaline 40 μg/ml. because of the greatly enhanced fluorescence of the adjacent structures. One striking feature was the complete absence of any " specific fluorescent cells " in either the atria or ventricles. Except for a minority of cardiac muscle cells occasionally showing some relatively enhanced fluorescence compared with the rest of the cardiac muscle, the distribution of noradrenaline appeared uniform. Connective tissue and fat cells also exhibited an enhanced fluorescence after noradrenaline perfusion.

Over a period of 24 hr the decrease (fading) in specific fluorescence attributable to adrenergic nerves preceded that of the other fluorescent structures.

Figure 3a shows the results obtained from objective measurements of field brightness (see **Methods**). It can be seen that there is a highly significant increase

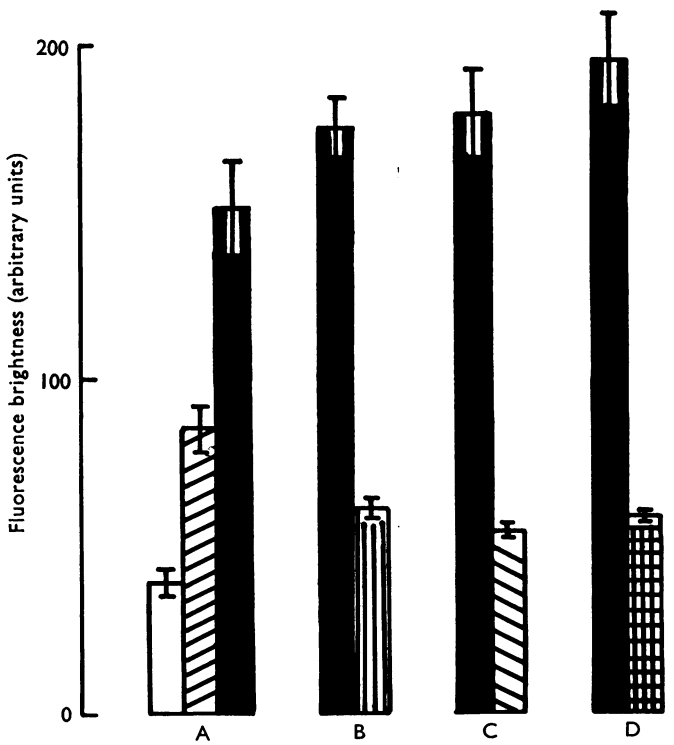

FIG. 3. Objective brightness measurements of noradrenaline fluorescence in ventricular cryostat sections from isolated perfused rat hearts. (A) Noradrenaline 40 μg/ml. (■) and 10 μg/ml. (▨) compared with Krebs alone (□). (B) Noradrenaline 40 μg/ml. and phenoxybenzamine 50 μg/ml. (|||) compared with noradrenaline 40 μg/ml. alone (■). (C) Noradrenaline 40 μg/ml. and normetanephrine 40 μg/ml. (▨) compared with noradrenaline 40 μg/ml. alone (■). (D) Noradrenaline 40 μg/ml. at 20° C (▩) compared with noradrenaline 40 μg/ml. at 37° C (■). All hearts were perfused for 10 min and each column represents the mean of at least six observations. Vertical lines indicate the standard error of the mean measurements. Within each block, all columns are significantly different from each other ($P<0{\cdot}001$).

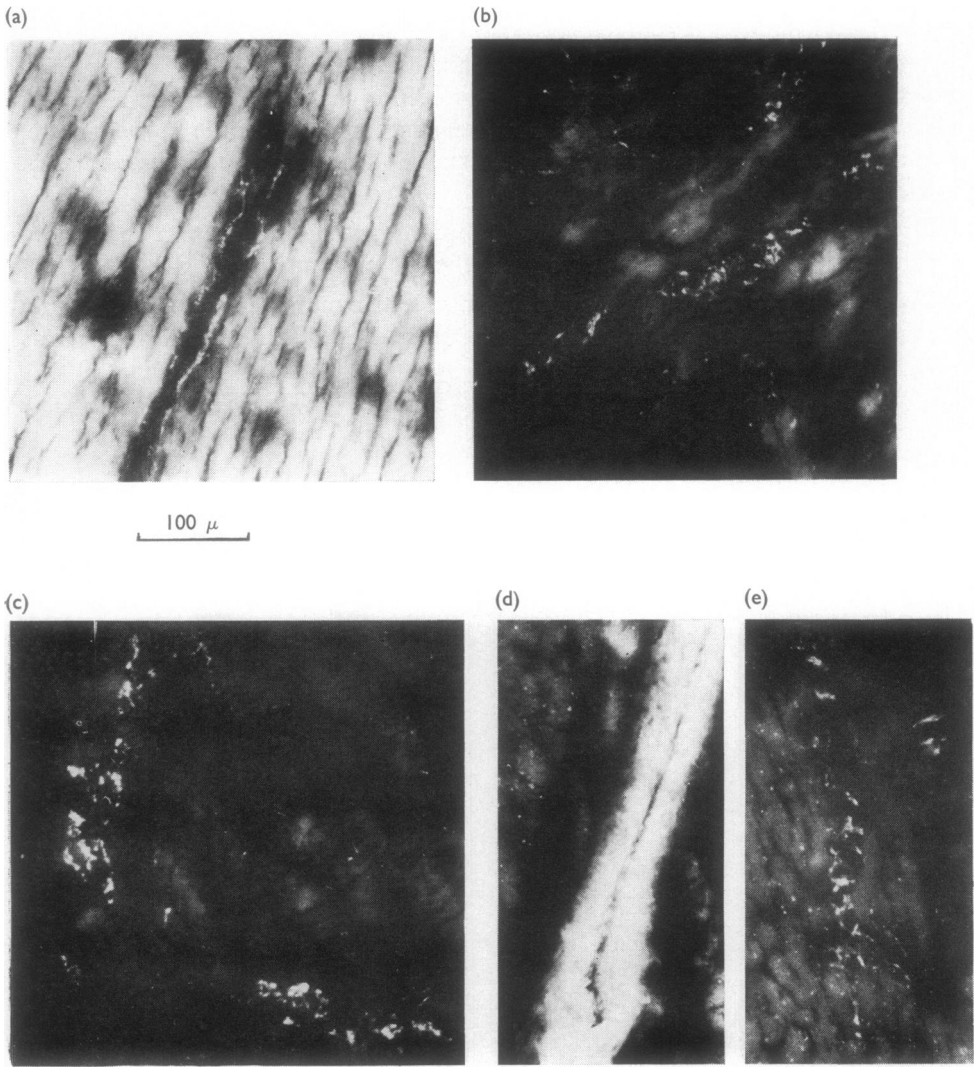

FIG. 4. Histochemical fluorescence in the rat ventricle (a) after perfusion with noradrenaline 40 μg/ml.; (b) as in (a) plus phenoxybenzamine 50 μg/ml.; (c) as in (a) plus normetanephrine 40 μg/ml.; (d) and (e) after perfusion with noradrenaline 40 μg/ml. at 20° C. Note the reduction of cardiac muscle fluorescence in (b), (c) and (e), the less intensive nerve fluorescence in (b) and (e) and the marked retention of specific fluorescence in the large coronary artery in (d). All hearts were perfused for 10 min.

($P<0{\cdot}001$) in the ventricular brightness after perfusion with noradrenaline (10 and 40 μg/ml.) compared with control hearts.

Effect of modifying agents and procedures

Hearts perfused with noradrenaline (40 μg/ml.) in combination with either phenoxybenzamine (50 μg/ml.) or normetanephrine (40 μg/ml.) for 10 min showed less increase in cardiac and smooth muscle fluorescence than hearts perfused with noradrenaline alone (Fig. 4a, b and c). This reduction was substantiated by simultaneous objective measurements (Fig. 3b and c). However, by comparing Fig. 1b with Fig. 4b and c it can be seen that some increase in myocardial fluorescence still persisted. Phenoxybenzamine partly prevented the increase in adrenergic nerve fluorescence normally observed after noradrenaline perfusion, whereas normetanephrine appeared not to affect it.

The perfusion of hearts with noradrenaline (40 μg/ml. for 10 min) at 20° C likewise produced less increase in the observed and measured fluorescence than hearts perfused with noradrenaline alone at 37° C, the reduction being similar to that observed with phenoxybenzamine and normetanephrine (Fig. 3d and Fig. 4e). However, in distinction from the effects seen with these latter two agents, at 20° C the larger coronary vessels were frequently observed to retain a high degree of specific fluorescence (Fig. 4d) which was rarely seen in the smaller vessels. Furthermore, perfusion with noradrenaline at 20° C caused a 26% rise in perfusion pressure and a 36% reduction in the [^{14}C]-sorbitol space (Table 1).

Discussion

The greater density of the adrenergic innervation to the atria compared with the ventricle is in accord with the observations of other workers (Laties, Lund & Jacobowitz, 1967; Ehinger, Falck & Sporrong, 1967; Neilsen & Owman, 1968). This finding, together with the disappearance of the specific fluorescence in control hearts after treatment with reserpine or 6-hydroxydopamine (see **Methods**), supports the validity of the fluorescence procedure adopted. However, in the absence of a freeze drying technique (Falck & Owman, 1965; Eränkö, 1967) diffusion artefacts, leading to a translocation of the accumulated or endogenous noradrenaline, cannot be excluded. It is possible that the failure to observe "chromaffin cells" (Jacobowitz, 1967; Laties *et al.*, 1967; Nielsen & Owman, 1968) in heart sections and "specific fluorescent cells" (Malmfors, 1967; Farnebo, 1968) in hearts perfused with noradrenaline might be explained on this basis, especially if the binding to such structures was weaker than that encountered with sympathetic fibres. On the other hand, these differences, particularly with respect to Uptake$_2$, might also be

TABLE 1. *Effect of perfusion at* 20° C *and* 37° C *on the perfusion pressures and* [^{14}C]*-sorbitol space in the isolated rat heart*

	Perfusion pressure (mm Hg)		[^{14}C]-sorbitol space (μl./g)
Temperature (° C)	Before noradrenaline	In presence of noradrenaline (40 μg/ml.)	in the presence of noradrenaline (40 μg/ml.)
37	39·0±1·8 (6)	45·2±2·7 (6)	334±10 (4)
20	54·8±1·3* (5)	56·8±1·5† (5)	213±3* (4)

The mean values and standard errors are shown and the numbers in brackets indicate the number of observations.
* Significantly different from 37° C ($P<0{\cdot}001$).
† Significantly different from 37° C ($P<0{\cdot}005$).

explicable in terms of a strain difference between the rats used. Biochemical estimations of noradrenaline retention after cardiac perfusion with 5 μg/ml. for 10 min (unpublished observations) have revealed a marked difference between Wistar rats and C.F.E. rats obtained from Carworth Europe (Sprague-Dawley derived strain); hearts from the former strain accumulating 51% more noradrenaline than the latter. It may be significant, therefore, that the rats used by Farnebo (1968) were of the Sprague-Dawley strain, but no information as to the strain used was provided by Malmfors (1967; 1968). Also, it may be relevant to note that a wide species variation exists with respect to $Uptake_2$ (Gillespie, 1968; Hamilton, 1968).

Under the experimental conditions used, the noradrenaline accumulated by $Uptake_2$ (Iversen, 1965) appears to be associated with all available structures in the rat heart. By virtue of its abundance it is probable that cardiac muscle accounts for most of this noradrenaline but sympathetic nerves, coronary blood vessels, fat and connective tissue can all serve as binding sites. With the exception of the presence of " specific fluorescent cells ", these observations are in agreement with the reports of Malmfors (1967; 1968) and Farnebo (1968). This pronounced lack of structural selectivity detracts from the suggestion that the $Uptake_2$ process in the rat heart is largely dependent upon the adrenergic innervation (Iversen, 1965; 1967). The fact that normetanephrine inhibited the extra-neuronal noradrenaline fluorescence without reducing the enhancement of the neuronal fluorescence also militates against this proposal. This is particularly so, because this compound is known to be a very potent inhibitor of noradrenaline accumulation by $Uptake_2$ in biochemical studies (Iversen, 1965).

Histochemically, phenoxybenzamine has been shown to reduce the accumulation of noradrenaline in the perfused isolated central artery of the rabbit ear (Avakian & Gillespie, 1968) and biochemically in the isolated perfused rat heart both in the presence (Eisenfield, Landsberg & Axelrod, 1967) and absence of cocaine (Iversen, 1965). The experiments reported in this paper demonstrate that phenoxybenzamine decreased both the extra-neuronal and neuronal accumulation of noradrenaline. From the objective extra-neuronal fluorescence brightness determinations it would appear that the inhibitory effects of phenoxybenzamine and normetanephrine on noradrenaline accumulation are approximately equal, a result which correlates well with the potency of these two agents when determined biochemically (Iversen, 1965).

Noradrenaline accumulation was markedly depressed by perfusion at 20° C. Such an effect, however, does not necessarily imply the involvement of an active transport mechanism for the $Uptake_2$ process and other considerations may be pertinent. We have observed that this decrease in perfusion temperature reduces the [^{14}C]-sorbitol space and causes a rise in perfusion pressure. Such an effect could indirectly interfere with the uptake of noradrenaline by hindering its passage from the coronary vessels and between myocardial cells to its final retention sites. Some evidence that the coronary vascular system itself may serve as a barrier in this respect could be construed from the observation that the larger coronary vessels retained a high degree of fluorescence at the reduced temperature compared with the rest of the cardiac structures. The less obvious retention of fluorescence in the smaller coronary vessels might be related to their relative lack of smooth muscle. Furthermore, there is the distinct possibility that some capillary closure occurs, because of possible myocardial swelling at 20° C, resulting in the observed rise in perfusion pressure. Such an effect would serve to limit the main access route of

noradrenaline to the cardiac muscle cells. In addition, Sutherland & Young (1966) have shown that the isolated perfused rat heart is freely permeable, through postulated " capillary pores ", to an Evans blue albumen conjugate. They attributed this effect to the anoxic conditions of the *in vitro* preparation at 37° C. It is possible that at lower temperatures, where metabolic requirements are less, this anoxic permeability may be obviated, so reducing the bulk flow of fluid and the passage of noradrenaline out of the capillary beds.

Since phenoxybenzamine and normetanephrine, in the concentration used, did not cause a rise in perfusion pressure it appears unlikely that their inhibitory effects result through a similar mechanism to that postulated for temperature reduction.

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