J. Pharm. Pharmac., 1968, **20**, 1–11 Received August 25, 1967

Mechanism of amphetamine accumulation in the isolated perfused heart of the rat

H. THOENEN, A. HÜRLIMANN AND W. HAEFELY

Rat hearts were perfused with 10 to 1,000 ng/ml of (±)-[^{3}H]amphetamine. The time course of accumulation and the maximal tissue/medium ratio (T/M) were identical for all concentrations studied. The maximal T/M varied between 5 and 6 and was reached after 5 min of perfusion. The accumulation of amphetamine was not inhibited by cocaine or noradrenaline. It was not impaired by combined inhibition of aerobic and anaerobic energy metabolism and it was not dependent on the intactness of sympathetic nerve endings, indicating that the greater amount of amphetamine was located extraneuronally. The diminished accumulation of amphetamine following reduction of temperature or sodium concentration of the perfusion fluid most probably reflects impaired tissue perfusion resulting from vascular constriction. The time course of accumulation and decay of amphetamine is compatible with a rapidly reversible phase-distribution of this amine possibly related to its relatively high lipophilic properties. The possible significance of phenolic hydroxyl groups in membrane transport and diffusion of phenethylamines is discussed.

THERE is strong evidence that amphetamine, like many other phenethylamines, exerts its sympathomimetic effect mainly by liberating noradrenaline from sympathetic nerve endings (Fleckenstein & Stöckle, 1955; Burn & Rand, 1958; Trendelenburg, Muskus & others, 1962; Haefely, Hürlimann & Thoenen, 1964). In addition to this indirect sympathomimetic effect these substances inhibit the uptake of noradrenaline into sympathetically innervated organs (Hertting, Axelrod & Whitby, 1961; Iversen, 1964a). Several of them have been shown to interfere with the membrane transfer of noradrenaline by being transported themselves and possessing kinetic uptake properties very similar to those of noradrenaline (Carlsson & Waldeck, 1965; Iversen, 1966). Thus the question arises whether amphetamine inhibits noradrenaline uptake in a similar manner or whether it interferes with noradrenaline uptake without being transported itself. The accumulation of amphetamine and its uptake kinetics were therefore studied in the isolated perfused heart of the rat.

Experimental

METHODS

Charles-River male rats weighing 180 to 210 g were injected intraperitoneally with 2,000 units of heparin. 5 to 10 min later the animals were killed by cervical dislocation. The hearts were removed and perfused by the Langendorff technique at a constant rate of 10 ml/min at 37° with the following modified Krebs-Henseleit solution: NaCl 5·54 g, KCl 0·354 g, KH_2PO_4 0·163 g, $MgSO_4.7H_2O$ 0·294 g, $CaCl_2$ 0·282 g, $NaHCO_3$ 2·1 g, Na-pyruvate 0·542 g, Na-fumarate 0·474 g, Na-l-glutamate 0·416 g, dextrose 2·08 g, ethylenediamine tetra-acetic acid disodium salt 0·01 g, ascorbic acid 0·01 g in 1,000 ml. At this perfusion

From the Department of Experimental Medicine, F. Hoffmann-La Roche & Co. Ltd., Basle, Switzerland.

rate the perfusion pressure varied between 50 and 70 mm Hg, the heart rate between 150 and 200 beats/min. Usually the perfusion pressure was checked only at the end of the equilibration period to be certain that it was within this range. In a limited number of experiments in which the sodium concentration or the temperature of the perfusion medium was reduced, the perfusion pressure was recorded throughout the experiment. The hearts could be perfused by either one of two independent perfusion systems connected to the perfusion cannula by a three-way stopcock. One of the two systems contained (±)-amphetamine in concentrations of 10 to 1,000 ng/ml; irrespective of the total concentration of amphetamine, all solutions contained 0·15 μc/ml of (±)-[^{3}H]amphetamine.

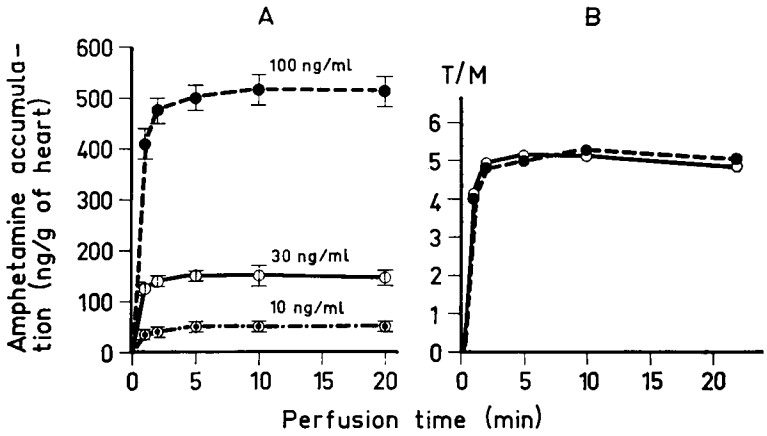

FIG. 1A and B. Time course of amphetamine accumulation in the isolated perfused rat heart. T/M = tissue/medium ratio. In Fig. 1B only the values for 30 and 100 ng/ml of amphetamine are given since those for 10 ng/ml lay between the two other concentrations. Each value in Figs 1–6 is the mean ± standard error of 4–5 experiments.

After an initial perfusion period of 2 to 3 min with amphetamine-free solution, the hearts were perfused for 1, 2, 5, 10 or 20 min with amphetamine solutions of different concentrations (10 to 1,000 ng/ml). At the end of the perfusion, the hearts were blotted, frozen in light petroleum (b.p. 90–120°) at −80°, weighed and homogenized in 5 ml of 0·4 N $HClO_4$. After centrifugation the radioactivity of the supernatant was determined in a liquid scintillation counter (Mark I, Nuclear-Chicago) using Bray's solution. The amphetamine content of the hearts was expressed in ng/g wet weight, corrected for the recovery of (±)-[^{3}H]amphetamine added to homogenates of hearts perfused without amphetamine. No corrections were made for the extracellular space.

In two series of experiments the effect of cocaine and noradrenaline on the accumulation of amphetamine was examined. The two substances were infused into a rubber tube placed immediately ahead of the perfusion cannula during the entire duration of the experiment, i.e. as early as during the initial equilibration phase with amphetamine-free solution.

For "chemical sympathectomy" rats were injected intravenously with four doses of 20 mg/kg of 6-hydroxydopamine (Tranzer & Thoenen, 1967) given over a period of 48 hr. On account of the extreme susceptibility to oxidative degradation at neutral and alkaline pH it was necessary to dilute 6-hydroxydopamine in 0·001 N hydrochloric acid.

Isolation and chromatographic identification of amphetamine and its possible metabolites formed in hearts during perfusion with (±)-[^{3}H]-amphetamine were as described previously (Thoenen, Hürlimann & others, 1966).

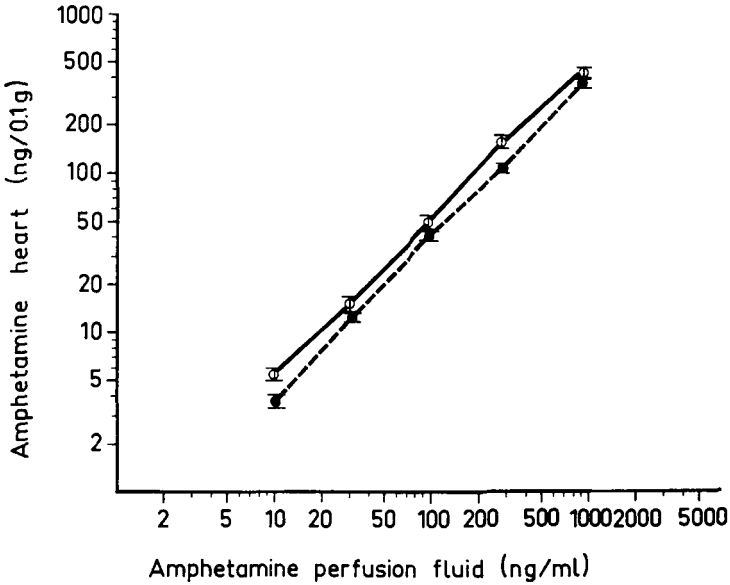

FIG. 2. Relationship between the concentration of amphetamine in the perfusion fluid (ng/ml) and its accumulation in the heart tissue (ng/0·1 g) after 1 (●——●) and 20 (○——○) min of perfusion.

Drugs used: (−)-noradrenaline (Arterenol, Hoechst), cocaine hydrochloride, 6-hydroxydopamine hydrobromide and (±)-[^{3}H]amphetamine sulphate (tritiated in *ortho*-position, activity 15 mc/mg). 6-Hydroxydopamine was synthesized by Dr. M. Scheer, tritiated amphetamine by Dr. J. Würsch and Dr. H. Bruderer of the Chemical Research Department of Hoffmann-La Roche & Co. Ltd., Basle. All doses refer to the base.

Results

METABOLISM OF AMPHETAMINE IN THE RAT ISOLATED PERFUSED HEART

The labelled compounds present in homogenates of rat hearts perfused for 20 min with 30 ng/ml of amphetamine were separated on Dowex-50 columns into acidic and basic fractions. The entire activity was found in the latter fraction and its chromatographic analysis revealed that the total activity was confined to the position of amphetamine. In particular, no

activity could be detected at the position of norephedrine (β-hydroxylated amphetamine), *p*-hydroxyamphetamine and its β-hydroxylated derivative *p*-hydroxynorephedrine, metabolites found in cat spleen and heart after pretreatment with ($\pm$)-[^{3}H]amphetamine (Thoenen & others, 1966). The activity determined in homogenates of rat hearts will therefore be expressed in terms of amphetamine.

TIME COURSE AND DOSE DEPENDENCE OF AMPHETAMINE ACCUMULATION

As shown in Fig. 1, the time course of amphetamine accumulation in isolated perfused rat hearts was virtually identical for amphetamine concentrations of 10, 30 and 100 ng/ml. After 1 min of perfusion, the amphetamine content of the hearts (expressed in ng/g wet weight) was about 4 times higher than the concentration in the perfusion fluid (ng/ml), and there was only a small further increase in the following minutes.

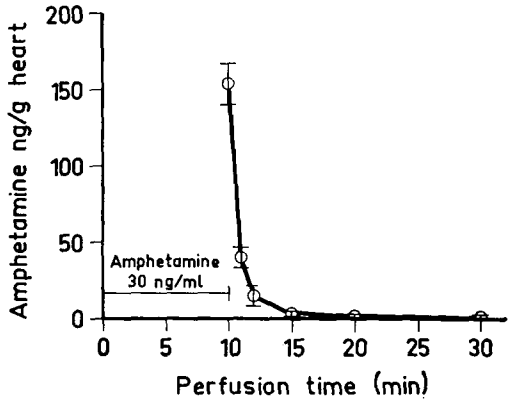

FIG. 3. Decay of the amphetamine content in rat hearts. The hearts were first perfused for 10 min with 30 ng/ml of amphetamine, followed by perfusion without amphetamine for 1, 2, 5, 10 and 20 min.

The maximal T/M was reached after 5 min and remained at this level as long as 20 min. In the dose range studied (10 to 1,000 ng/ml) the amphetamine accumulation revealed no signs of saturation which accords with observations on brain slices (Ross & Renyi, 1966b). There is a statistically significant ($P < 0{\cdot}05$) linear correlation between the amphetamine concentrations in the perfusion fluid and its accumulation in heart tissue both for 1 and 20 min of perfusion (Fig. 2). The slope of the regression line is 1·01 for the 1-min values and 1·07 for the 20-min values.

ELIMINATION OF AMPHETAMINE ACCUMULATED IN RAT HEARTS

In a further series of experiments the hearts were perfused during 10 min with 30 ng/ml of amphetamine. The amphetamine content of the hearts at the end of this perfusion period was compared with that after continuation of perfusion for 1, 2, 5, 10 or 20 min without amphetamine. The time course of the decay (Fig. 3) is virtually identical to that of accumulation during perfusion with 30 ng/ml of amphetamine (Fig. 1).

EFFECT OF COCAINE AND NORADRENALINE ON AMPHETAMINE ACCUMULATION

In a series of experiments the hearts were perfused with 10 μg/ml of cocaine, a concentration which almost completely abolishes the noradrenaline uptake into the isolated perfused rat heart (Iversen, 1964b). The effect on amphetamine accumulation, however, was very small (Fig. 4A) and there was no statistically significant ($P > 0{\cdot}05$) difference between the amount of amphetamine accumulating in hearts perfused with and without cocaine. These results agree with the observation on mouse heart slices published by Ross & Renyi (1966a) while our experiments were in progress.

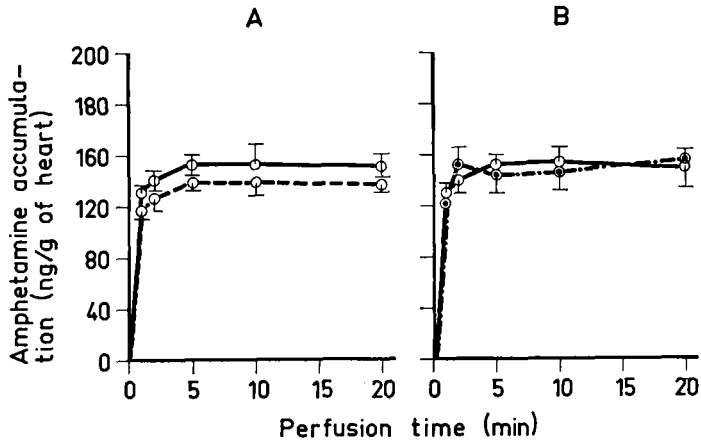

FIG. 4. The effect of (A) cocaine (10 μg/ml) and (B) noradrenaline (0·1 μg/ml) on the accumulation of amphetamine in the isolated perfused rat heart. The concentration of amphetamine was 30 ng/ml in all experiments. Control O——O; cocaine O — — O; noradrenaline O—·—O.

As amphetamine interferes with the uptake of noradrenaline into the isolated perfused rat heart, we studied the effect of noradrenaline on amphetamine accumulation to decide whether this interference is a mutual one. As shown in Fig. 4B, the presence of 100 ng/ml of (−)-noradrenaline had no statistically significant ($P > 0{\cdot}05$) effect on amphetamine accumulation, although the affinities of (±)-amphetamine and (−)-noradrenaline for noradrenaline uptake sites are very similar (Iversen, 1964a).

DEPENDENCE ON TEMPERATURE, SODIUM CONCENTRATION AND ENERGY METABOLISM

The time course of amphetamine accumulation is very similar at 37, 30 and 20° (Fig. 5A). However, the maximal T/M reached after 5 min of perfusion showed a clear-cut temperature dependence and was reduced to 69% at 20° and to 84% at 30° (37° = 100%).

The transport of noradrenaline has been found to be sodium dependent (Iversen & Kravitz, 1966), the uptake being diminished in proportion to the sodium reduction in the perfusion fluid. A reduction of the sodium

concentration to 50% (correction of osmolarity by sucrose) resulted in a reduction of the heart rate from 185 ±3 to 74 ±3, whereas the perfusion pressure remained within the normal range. The accumulation of amphetamine did not differ significantly ($P > 0.05$) from that of controls (Fig. 5B). If the sodium concentration was further reduced to 25% the hearts contracted only sporadically and the perfusion pressure regularly exceeded the control range of 50 to 70 mm Hg. The accumulation of amphetamine amounted to about 70% of that of controls after 5 min of perfusion with 30 ng/ml of amphetamine.

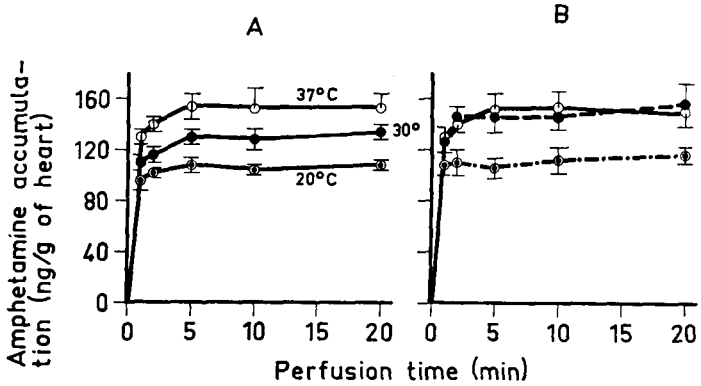

FIG. 5. Dependence of amphetamine accumulation on temperature (A) and sodium concentration (B) control O——O; 1/2 sodium ●— —●; 1/4 sodium ⊙— · —○.

Wakade & Furchgott (1966) have shown that both anoxia and glucose deprivation are necessary to block noradrenaline uptake into isolated atria of guinea-pigs, whereas alone neither was effective. If iodoacetate (2×10^{-4}M) was added to the perfusion fluid, saturated with nitrogen 95% and carbon dioxide 5%, the hearts stopped beating after 2 to 3 min. The perfusion pressure remained within the normal range. In these experiments the perfusion with amphetamine (30 ng/ml) was preceded by a perfusion period of 10 min with amphetamine-free medium. As shown in Fig. 6, impairment of the glycolytic and aerobic energy metabolism did not diminish the accumulation of amphetamine in heart tissue. On the contrary the accumulation was even larger than under control conditions. The difference, however, was statistically significant ($P < 0.05$) only for 2 min of perfusion with 30 ng/ml of amphetamine.

EFFECT OF CHEMICAL SYMPATHECTOMY

6-Hydroxydopamine has been shown to produce an efficient and extremely long-lasting noradrenaline depletion in various species (Porter, Totaro & Stone, 1963; Stone, Stavorski & others, 1963; Laverty, Sharman & Vogt, 1965). Recent electronmicroscopic studies revealed that this particular "noradrenaline depletion" was due to selective destruction of sympathetic nerve endings (Tranzer & Thoenen, 1967). 6-Hydroxydopamine therefore provides a unique tool for chemical sympathectomy.

To study whether the accumulation of amphetamine is linked to functionally intact sympathetic nerve endings, as is so for noradrenaline (Hertting, Axelrod & others, 1961; Hertting & Schiefthaler, 1964; Iversen, Glowinski & Axelrod, 1966), we examined the effect of pretreatment with 6-hydroxydopamine. The rats were injected intravenously with four doses of 20 mg/kg of 6-hydroxydopamine given over a period of 48 hr. The perfusion experiments were made 10 days after the last dose. At this time, the noradrenaline content was reduced to about 10% of that of controls and electronmicroscopic examination revealed alterations of sympathetic nerve endings (Tranzer, unpublished results) similar to those observed in various organs of the cat (Tranzer & Thoenen, 1967). The heart rate of these pretreated preparations was lower (133 $\pm$7)

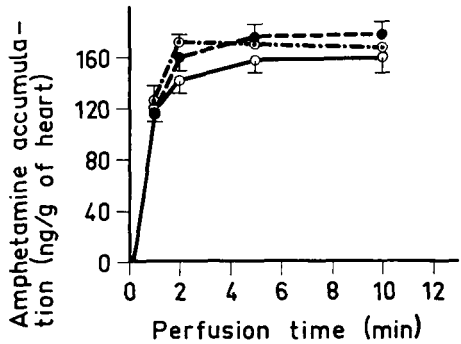

FIG. 6. Dependence of amphetamine accumulation on aerobic and anaerobic energy metabolism and intactness of sympathetic nerves. Control O——O; iodoacetate (2×10^{-4}M) and saturation of perfusion fluid by 95% N_2 and 5% CO_2 ⊙— · —⊙; hearts of rats pretreated with 4×20 mg/kg 6-hydroxydopamine (chemical sympathectomy) ●— —●.

than that of controls (185 $\pm$3), whereas the perfusion pressure was within the normal range. As evident from Fig. 6, the amphetamine accumulation was even somewhat larger than in control hearts, indicating that amphetamine is not selectively accumulated in sympathetic nerves as in the case of noradrenaline.

RELATIONSHIP BETWEEN PERFUSION PRESSURE AND AMPHETAMINE ACCUMULATION

In the preceding experiments in which the sodium concentration or the temperature of the perfusion fluid was reduced, we came to suspect the possibility of a relationship between the increase of perfusion pressure and the diminution of amphetamine accumulation in the perfused hearts.

Since the perfusion pressure was not continuously recorded but checked only during the equilibration phase, an additional series of experiments was undertaken in which the perfusion pressure was measured during the whole duration of the experiments and in which the sodium concentration was further reduced to 12·5% and the temperature to 10°. The hearts

were equilibrated for 5 min with amphetamine-free solution and then perfused for 2 min with 30 ng/ml of amphetamine.

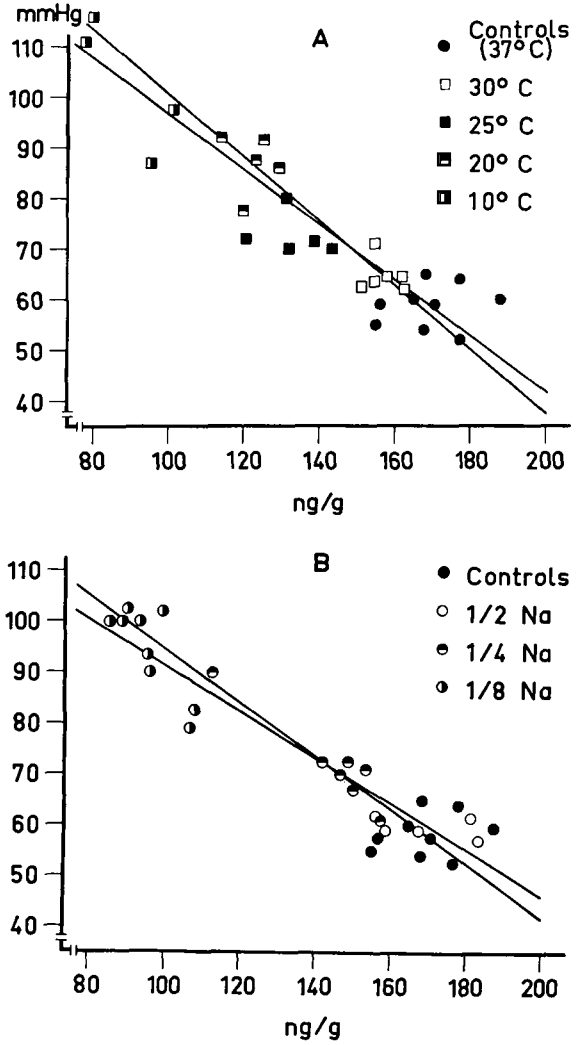

FIG. 7. Relationship between perfusion pressure and amphetamine accumulation in the isolated perfused rat heart for changes in temperature (A) and changes in sodium concentration (B). The hearts were perfused at a constant rate of 10 ml/min with 30 ng/ml of amphetamine. There is a significant correlation ($P < 0{\cdot}001$) between perfusion pressure and the amount of amphetamine accumulating in the heart both for changes in temperature (A) and sodium concentration (B). Each value is the result of a single experiment.

After 2 to 3 min of perfusion with amphetamine-free solution, the perfusion pressure equilibrated and was not changed by switching to the amphetamine solution. As evident from Fig. 7, there is a significant

($P < 0.001$) correlation between the height of perfusion pressure and the amount of amphetamine accumulating in the hearts both for changes in temperature and in sodium concentration.

Discussion

The uptake of noradrenaline into the isolated perfused rat heart has been shown to be mediated by a stereochemically specific mechanism which exhibits the properties of an active membrane transport (Iversen, 1963; Iversen & Kravitz, 1966). The complexity of the biological systems usually studied does not permit direct proof, and the assumption of an active transport is therefore based in most cases on dependence on temperature and energy metabolism, uphill transport and saturation kinetics.

The present study has shown that in the rat isolated perfused heart amphetamine, which interferes with the uptake of noradrenaline (Iversen, 1964a), is not accumulated in the same manner as noradrenaline. The accumulation of amphetamine was not inhibited by cocaine or noradrenaline. It was not impaired by combined inhibition of aerobic and anaerobic energy metabolism. It did not obey the principles of saturation kinetics. The time course of accumulation and decay after subsequent perfusion without amphetamine suggests instead a rapidly reversible phase-distribution, possibly related to the relatively high lipophilic properties of amphetamine. The distribution coefficient between benzene and Krebs-Henseleit solution (pH 7·4) is 0·212 for amphetamine and 0·004 for noradrenaline. The corresponding values for heptane and Krebs-Henseleit solution are 0·043 and <0.001 respectively (unpublished results). Both accumulation and decay curves are complex and do not fulfil the requirements of a first order reaction which might be taken as evidence that equilibration takes place between several compartments.

The dependence of amphetamine accumulation on temperature and sodium concentration could be interpreted as favouring an active membrane process. However, the fact that the quantity of amphetamine which accumulates in the rat heart is inversely correlated with the height of the perfusion pressure (Fig. 7) makes it more probable that the diminished accumulation of amphetamine reflects impaired tissue perfusion resulting from vascular constriction provoked by a reduced sodium concentration or a reduction of temperature.

Perhaps the most important difference between the accumulation of noradrenaline and amphetamine is the lack of dependence of the latter on the intactness of sympathetic nerves. Indeed no difference was found between the content of amphetamine in hearts with functionally intact sympathetic nerves and those which were chemically denervated by treatment with 6-hydroxydopamine. Thus the bulk of amphetamine accumulating in the heart must be located extraneuronally. It cannot be determined from the present results whether cardiac sympathetic nerves are capable of concentrating amphetamine to a greater degree than the extraneuronal tissue. However, an accumulation of amphetamine in

sympathetic nerves of the same order of magnitude as that found after perfusion with noradrenaline can be excluded. After 30 min of perfusion with 10 ng/ml of (±)-[^{3}H]noradrenaline the tissue/medium ratio (T/M) is about 40 (Iversen, 1963). Since noradrenaline is accumulated almost exclusively in adrenergic nerves (Hertting & others, 1961; Hertting & Schiefthaler, 1964; Iversen & others, 1966) and since the volume of nerve terminals represents only a minute part of the total heart tissue the ratio nerve terminal/perfusion medium for noradrenaline must be even much higher. If amphetamine were accumulated in adrenergic nerve terminals to a similar extent the T/M should be much higher in innervated than in chemically denervated hearts despite additional extraneuronal accumulation.

The lack of significant accumulation of amphetamine in adrenergic nerve terminals, however, does not permit the definite conclusion that it is not transported by the noradrenaline transfer mechanism. A marked uptake of amphetamine could be masked by a rapid outward diffusion along the concentration gradient. In this context it is interesting to recall that α-methyltyramine which differs from amphetamine only by a phenolic hydroxyl group in the *para*-position is very efficiently accumulated by the isolated perfused rat heart (Iversen, 1966) the T/M being 10 after 5 min perfusion with 1 ng/ml. In contrast to amphetamine this amine is concentrated selectively in sympathetic nerves (Iversen & others, 1966) and since it is not stored in the granular vesicles (Kopin, 1966), it must be present to a large extent in free diffusible form in the axoplasm of the sympathetic nerve endings.

The differences between the accumulation of amphetamine and α-methyltyramine are open to the interpretation that at least one phenolic hydroxyl group is a prerequisite for the transportation of phenethylamines by the noradrenaline transfer system and that amphetamine, though able to block this mechanism, is not itself transported. In consequence, the interference of cocaine with the sympathomimetic action of amphetamine (Fleckenstein & Stöckle, 1955; Burn & Rand, 1958) would not be due to inhibition of its active membrane transfer but possibly to interference with its effect on intraneuronal storage sites from which noradrenaline is liberated. However, it may equally well be assumed that the phenolic hydroxyl group of phenethylamines is not essential for their membrane transport into the sympathetic nerves but that this group changes their physico-chemical properties in such a way that their diffusion through the lipid membrane along the concentration gradient is impaired. Thus, in spite of an active transport an accumulation of amphetamine in sympathetic nerve endings would not occur because of its rapid passive outward diffusion.

Acknowledgement. The authors wish to thank Mr. W. J. Ziegler for the statistical evaluation.

References

Burn, J. H. & Rand, M. J. (1958). *J. Physiol., Lond.*, **144**, 314–336.
Carlsson, A. & Waldeck, B. (1965). *Acta pharmac. tox.*, **22**, 293–300.

Fleckenstein, A. & Stöckle, D. (1955). *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.*, **224**, 401–415.
Haefely, W., Hürlimann, A. & Thoenen, H. (1964). *Helv. physiol. pharmac. Acta*, **22**, C125–C127.
Hertting, G., Axelrod, J., Kopin, I. J. & Whitby, L. G. (1961). *Nature, Lond.*, **189**, 66.
Hertting, G., Axelrod, J. & Whitby, L. G. (1961). *J. Pharmac. exp. Ther.*, **134**, 146–153.
Hertting, G. & Schiefthaler, Th. (1964). *Int. J. Neuropharmac.*, **3**, 65–69.
Iversen, L. L., Glowinski, J. & Axelrod, J. (1966). *J. Pharmac. exp. Ther.*, **151**, 273–284.
Iversen, L. L. (1963). *Br. J. Pharmac. Chemother.*, **21**, 523–537.
Iversen, L. L. (1964a). *J. Pharm. Pharmac.*, **16**, 435–436.
Iversen, L. L. (1964b). *Ibid.*, **17**, 62–64.
Iversen, L. L. (1966). *Ibid.*, **18**, 481–484.
Iversen, L. L. & Kravitz, E. A. (1966). *Mol. Pharmac.*, **2**, 360–362.
Kopin, I. J. (1966). *Pharmac. Rev.*, **18**, 513–540.
Laverty, R., Sharman, D. F. & Vogt, M. (1965). *Br. J. Pharmac. Chemother.*, **24**, 549–560.
Porter, C. C., Totaro, J. A. & Stone, C. A. (1963). *J. Pharmac. exp. Ther.*, **140**, 308–316.
Ross, S. B. & Renyi, A. L. (1966a). *J. Pharm. Pharmac.*, **18**, 756–757.
Ross, S. B. & Renyi, A. L. (1966b). *Acta pharmac. tox.*, **24**, 297–309.
Stone, C. A., Stavorski, J. M., Ludden, C. T., Wenger, H. C., Ross, Ch. A., Totaro, J. A. & Porter, C. C. (1963). *J. Pharmac. exp. Ther.*, **142**, 147–156.
Thoenen, H., Hürlimann, A., Gey, K. F. & Haefely, W. (1966). *Life Sci.*, **5**, 1715–1722.
Tranzer, J. P. & Thoenen, H. (1967). *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.*, **257**, 342–343.
Trendelenburg, U., Muskus, A., Fleming, W. W. & Gomez Alonso de la Sierra (1962). *J. Pharmac. exp. Ther.*, **138**, 170–180.
Wakade, A. R. & Furchgott, R. F. (1966). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **25**, 260.