Synthesis, Binding, Release, and Metabolism of Norepinephrine in Normal and Transplanted Dog Hearts

By Lincoln T. Potter, M.D., Theodore Cooper, M.D., Ph.D., Vallee L. Willman, M.D., and David E. Wolfe, M.D.

The ability of several tissues, including the heart, to make, bind, store, release, and inactivate norepinephrine has been the subject of numerous recent investigations. Implicit in most of these studies is the idea that these processes are occurring largely, if not exclusively, within adrenergic nerves. It appeared possible to determine what part neural tissue plays in the handling of norepinephrine by comparing the synthesis, storage, and metabolism of the transmitter in normal and denervated hearts. For this purpose the autotransplanted dog heart was selected; after the operation the severed sensory, preganglionic cholinergic, and postganglionic sympathetic axons degenerate, and the heart is left with decentralized postganglionic cholinergic fibers. Our results help to describe the biochemical activity of hearts without adrenergic control, and emphasize the importance of such innervation for normal adrenergic mechanisms.

The normal heart can make norepinephrine from tyrosine or dopamine, but studies of catecholamine synthesis in denervated hearts have not previously been done. Like many other sympathetically-innervated tissues, the heart can remove norepinephrine rapidly from the circulation and store it in a bound form for long periods, about one-fifth of the endogenous catecholamine content of the rat heart has been attributed to uptake of the circulating amine. Further, binding represents the predominant physiological means of inactivation of norepinephrine, whereas enzymatic inactivation of the amine plays a lesser role. It is not known whether the denervated heart, in the absence (or near absence) of adrenergic nerves can inactivate circulating norepinephrine and epinephrine rapidly; if not, the greater responses of the transplanted tissue to amine infusions (compared to normal hearts) may be attributed in part to a higher level of the transmitter near receptor cells.

H3-norepinephrine was used for these studies because of the following evidence that it is retained with endogenous catecholamines in postganglionic sympathetic nerves: (1) Unilateral denervation of the salivary glands, iris, and eye muscles reduces the uptake of H3-norepinephrine on the denervated side by 90%. (2) Stimulation of the postganglionic nerves to several tissues causes release of the labeled amine, whereas reduction of nerve impulses by pharmacological or surgical decentralization reduces the rate of release of bound H3-norepinephrine. (3) Radioautography of H1-norepinephrine in the heart, pineal gland, choroid, ciliary body, iris, fat, and spleen of the rat has been combined with electron microscopy and demonstrates a constant association of silver grains with certain "granulated vesicles" in axons. (4) Endogenous and H2-norepinephrine have been isolated in the same small-particle fraction from homogenates of the heart, salivary

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*The term "denervated" is used for convenience in this paper to mean isolation of the heart from extrinsic neural control, and sympathetic denervation.
norepinephrine in normal and transplanted hearts

Materials
dl-7-H^3-norepinephrine (1100-7580 mc/m mole), 2-C^14-dopamine (3.1 mc/m mole), 2-
C^14-tryptamine (2.1 mc/m mole) and C^14-methyl
methylmethionine (4.0 mc/m mole) were obtained
from New England Nuclear Corporation and their purity was established by paper chromatog-
raphy. Before use as a precursor for norepineph-
rine, the dopamine was repurified by paper
chromatography in the same system used later to separate the two amines. C^14-methyl-
methylmethionine was prepared from C^14-meth-
methionine.

Methods

Experiments were performed on 26 mongrel dogs including 10 dogs with transplanted hearts.

The animals averaged 11.9 kg in body weight, and were matched as closely as possible with respect to weight for each experiment. Complete interruption of all extrinsic nerves to the heart was achieved by excision and reimplantation of the heart four days to four months before the experiments. Previous studies have shown that endogenous norepinephrine in the heart is reduced to negligible levels within three days after the operation, and that reinnervation of the heart does not occur for at least eleven months. In the present studies denervation was confirmed by demonstrating the absence of immediate response of the heart to stimulation of the sympathetic and vagal nerve trunks, and by a profound depletion of cardiac catecholamines.

The dogs were anesthetized moderately deeply by slow intravenous injection of sodium pento-
barbital and were given artificial respiration during and after thoracotomy. The activity of the heart was monitored with an electrocardiog-
graph and blood pressure was recorded from pressure transducer connected to a cannula in one femoral artery. Further preparations were as follows: (a) Uptake and retention of radio-
active dopamine were studied after infusion of the amine into a leg vein. Norepinephrine uptake was studied after its infusion into the superior vena cava of open-chest dogs. Fifteen minutes after the one-minute infusions a blood sample was taken from the left ventricle and a sample of the right atrium was fixed for electron microscopy and radioautography. The remainder of the heart was then excised, dissected in ice water, and frozen on dry ice. In one experiment with both normal and transplanted tissues, the subcellular localization of H^3-norepinephrine was also examined in continuous density gradients of sucrose. (b) Synthesis of norepinephrine from C^14-dopamine was studied in isolated perfused hearts. The azygos vein, venae cavae, right pul-
monary artery, pulmonary veins, and left sub-
clavian artery were ligated. Blood entered the coronary arteries of the heart through a cannula in the brachiocephalic trunk from a reservoir one meter above the heart. Venous blood was collect-
ed by gravity from the left pulmonary artery, and from an additional vent into the left chambers in order to return the left Thebesian drainage and any blood leaking past the aortic valve. A roller pump returned the blood via a bubble oxygenator to the reservoir which was warmed to 37°C. Each heart was perfused with the animal's own heparinized blood supple-
mented with 200 ml of Ringer's lactate solution. After 30 minutes the hearts were excised and processed as above. (c) Binding and release of H^3-norepinephrine, and its subsequent metabo-
lism, were also studied in perfused hearts. The preparation was as before except that the venous blood was collected for one-minute intervals for up to 60 minutes. The blood was then centrifuged at 2,000 x g for 15 minutes, and samples of the plasma were frozen until assayed. Because of the large fluid volumes required, perfusion was done with 20% blood in Ringer's lactate solu-
tion to which was added 2 g of glucose and 2 units of insulin per liter. The perfusion pressure was adjusted to give a flow rate of about 65 ml/min; the required reservoir elevation was 30% less for the denervated than for the innervated hearts. Each experiment was discontinued when the heart began to appear edematous or when all chambers no longer beat vigorously. At the end of the experiment these hearts were also dissected and frozen.

Assays

Samples of the atri, ventricles, and inter-
ventricular septum from all hearts were homog-
ized in isotonic potassium chloride and were separately assayed for MAO and COMT activity and for total norepinephrine content. MAO ac-
glands, and vas deferens, and this fraction is believed to contain granulated vesicles. None of these studies, however, excludes the possibility that a small amount of norepinephrine also may be made or stored outside adrenergic fibers, for example, in chromaffin cells. In vivo studies of norepinephrine inactivation in denervated tissues have not been done previously. Denervation of the heart reduces slightly the in vitro activity of catechol-
O-methyltransferase (COMT), and denervation of several tissues has been variably reported to increase, decrease, or not change the in vitro activity of monoamine oxidase (MAO). Materials

Methods

Experiments were performed on 26 mongrel dogs including 10 dogs with transplanted hearts. The animals averaged 11.9 kg in body weight, and were matched as closely as possible with respect to weight for each experiment. Complete interruption of all extrinsic nerves to the heart was achieved by excision and reimplantation of the heart four days to four months before the experiments. Previous studies have shown that endogenous norepinephrine in the heart is reduced to negligible levels within three days after the operation, and that reinnervation of the heart does not occur for at least eleven months. In the present studies denervation was confirmed by demonstrating the absence of immediate response of the heart to stimulation of the sympathetic and vagal nerve trunks, and by a profound depletion of cardiac catecholamines. The dogs were anesthetized moderately deeply by slow intravenous injection of sodium pentobarbital and were given artificial respiration during and after thoracotomy. The activity of the heart was monitored with an electrocardiograph and blood pressure was recorded from pressure transducer connected to a cannula in one femoral artery. Further preparations were as follows: (a) Uptake and retention of radioactive dopamine were studied after infusion of the amine into a leg vein. Norepinephrine uptake was studied after its infusion into the superior vena cava of open-chest dogs. Fifteen minutes after the one-minute infusions a blood sample was taken from the left ventricle and a sample of the right atrium was fixed for electron microscopy and radioautography. The remainder of the heart was then excised, dissected in ice water, and frozen on dry ice. In one experiment with both normal and transplanted tissues, the subcellular localization of H^3-norepinephrine was also examined in continuous density gradients of sucrose. (b) Synthesis of norepinephrine from C^14-dopamine was studied in isolated perfused hearts. The azygos vein, venae cavae, right pulmonary artery, pulmonary veins, and left subclavian artery were ligated. Blood entered the coronary arteries of the heart through a cannula in the brachiocephalic trunk from a reservoir one meter above the heart. Venous blood was collected by gravity from the left pulmonary artery, and from an additional vent into the left chambers in order to return the left Thebesian drainage and any blood leaking past the aortic valve. A roller pump returned the blood via a bubble oxygenator to the reservoir which was warmed to 37°C. Each heart was perfused with the animal's own heparinized blood supplemented with 200 ml of Ringer's lactate solution. After 30 minutes the hearts were excised and processed as above. (c) Binding and release of H^3-norepinephrine, and its subsequent metabolism, were also studied in perfused hearts. The preparation was as before except that the venous blood was collected for one-minute intervals for up to 60 minutes. The blood was then centrifuged at 2,000 x g for 15 minutes, and samples of the plasma were frozen until assayed. Because of the large fluid volumes required, perfusion was done with 20% blood in Ringer's lactate solution to which was added 2 g of glucose and 2 units of insulin per liter. The perfusion pressure was adjusted to give a flow rate of about 65 ml/min; the required reservoir elevation was 30% less for the denervated than for the innervated hearts. Each experiment was discontinued when the heart began to appear edematous or when all chambers no longer beat vigorously. At the end of the experiment these hearts were also dissected and frozen.

Assays

Samples of the atri, ventricles, and inter-
ventricular septum from all hearts were homog-
ized in isotonic potassium chloride and were separately assayed for MAO and COMT activity and for total norepinephrine content. MAO ac-
tivity was assayed by measuring the conversion of C\textsuperscript{14}-tryptamine to indoleacetic acid,\textsuperscript{26} and COMT activity by the transfer of C\textsuperscript{14}-methyl groups from S-adenosylmethionine to epinephrine.\textsuperscript{27} Nonradioactive norepinephrine was isolated by a butanol extraction procedure and was assayed fluorometrically by a trihydroxyindole method.\textsuperscript{16} Radioactive norepinephrine and its metabolites were isolated, and assayed by liquid scintillation spectrometry.\textsuperscript{4, 28} Briefly, the catechols norepinephrine, dihydroxymandelic acid, and dihydroxyphenylglycol were isolated on alumina and the latter two compounds separated from norepinephrine by ethyl acetate extraction. The O-methylated metabolites were not adsorbed to alumina; normetanephrine was separated from methoxymethoxyphenylglycol and methoxymethoxyphenylmandellic acid (VMA) on columns of Dowex-50 resin. All values are corrected for recoveries of standard compounds. Norepinephrine, normetanephrine and VMA accounted for 95 to 99\% of the infused radioactivity. In the studies of norepinephrine synthesis from C\textsuperscript{14}-dopamine only these two catecholamines were assayed. They were isolated on columns of alumina as above and the eluates were lyophilized, redissolved in ethanol containing 100 $\mu$g each of dopamine and norepinephrine, and were separated by ascending paper chromatography (butanol:acetic acid:water; 4:1:1). The amine spots were located by their fluorescence in ultraviolet light. They were then cut out and their radioactivity was counted after immersion in liquid scintillation phosphor.

**MORPHOLOGICAL STUDIES**

Small pieces of the right atrium (about 1 mm\textsuperscript{3}) from normal and denervated hearts were fixed by immersion in 3\% glutaraldehyde or in 1.3\% osmium tetroxide, each buffered with col- lidine to pH 7.4, and were then dehydrated in methanol and embedded in Epon 812. From atria containing H\textsuperscript{3}-norepinephrine, thick sections were prepared for light microscopy and radioautography, and thin sections for electronmicroscopic radioautography\textsuperscript{24, 25} using Gevaert 3.07 or Ilford L4 nuclear research emulsions. Specimens were examined in a RCA 3G or Philips EM 200 electron microscope.

**Results**

**CATECHOLAMINE ASSAYS**

Average norepinephrine levels in the sixteen normal dog hearts were as follows (standard errors of the means less than ±12\%), RA: 2.36 $\mu$g/g, RV: 0.75, LA: 1.42, LV: 0.66, IVS: 0.88, whole heart 0.74. The two normal hearts which had been perfused with dopamine had the same norepinephrine content as did the other fourteen normal hearts. By contrast, no part of any of the ten denervated hearts had more than 0.028 $\mu$g/g, and the average of all 50 determinations was 0.009 $\mu$g/g.

**UPTAKE OF C\textsuperscript{14}-DOPAMINE AND SYNTHESIS OF NOREPINEPHRINE**

Uptake of dopamine by the heart was studied initially in a normal dog (14.5 kg) and a dog (15 kg) with a heart denervated for two weeks; each animal received 32.6 $\mu$g C\textsuperscript{14}-dopamine (2.0 mg) intravenously, with little cardiac response. After 15 minutes the normal heart retained 240 $\mu$g of C\textsuperscript{14}-catecholamines (dopamine and norepinephrine). This radioactivity was distributed unequally among the chambers as follows: RA: 3.9 $\mu$g/g, RV: 2.3, LA: 3.6, LV: 2.5, IVS, 2.9. The denervated heart contained 4.3\% as much of the C\textsuperscript{14}-catecholamines (10 $\mu$g), nearly equally distributed per gram of tissue. Uptake of dopamine was also studied in two normal and two denervated hearts (8 and 14 days postoperative) after isolation and perfusion for 30 minutes with 16.3 $\mu$g C\textsuperscript{14}-dopamine (1.0 mg). The normal hearts showed a brief but marked increase in rate with frequent extrasystoles in response to this large dose of dopamine, whereas the denervated hearts showed only moderate rate increases. The normal hearts retained an average of 14.9\% of this dose which was unequally distributed as before. About 3.5\% (range 2.9 to 5.3\%) of the amount of C\textsuperscript{14}-catecholamines in each part of the heart was norepinephrine. Overall, about 0.52\% of the administered compound was synthesized to norepinephrine in 30 minutes, and the product accounted for 5 and 7\% of the total norepinephrine content of the two normal hearts. In the denervated hearts only 0.7\% of the perfused dopamine was retained as dopamine and norepinephrine. Although the chambers were not separately assayed, the whole hearts did show that 2.1\% of the retained C\textsuperscript{14}-catecholamines was norepinephrine. More C\textsuperscript{14}-norepinephrine may have been made but
not retained in either the normal or denervated hearts.

**UPTAKE, RELEASE, AND METABOLISM OF NOREPINEPHRINE**

Initial studies of the uptake and retention of norepinephrine were performed with six normal dogs and four dogs with cardiac denervation for 4, 6, 14, and 140 days respectively. Each animal was given 0.5 μg of H\textsuperscript{3}-dl-norepinephrine per kilogram body weight as a one-minute infusion into the superior vena cava (total dose of l-norepinephrine = 2.9 to 3.5 μg). No cardiac response was seen. After 15 minutes the hearts were removed, dissected, and frozen until assayed for norepinephrine and its metabolites (table 1). In the normal hearts the uptake of norepinephrine was greater in the ventricles than atria, as was the distribution of its metabolites. In the transplanted hearts the total uptake of norepinephrine was reduced to 6.1% of normal and the amine was evenly distributed per gram of tissue. In addition, the tissue concentration of norepinephrine was less than that in an equal weight of plasma (average value for all hearts was 27.4 μg/ml) taken from the heart just before its excision. The amounts of metabolites in these hearts were almost twice those found in the normal hearts.

The retention of H\textsuperscript{3}-norepinephrine in different parts of the heart was re-examined in normal dogs at two time intervals after the injection of the labeled amine. One unanesthetized dog (9.7 kg) was given a total dose of 0.5 μg H\textsuperscript{3}-norepinephrine intravenously and was killed 15 minutes later. The heart (67 g) retained 4.6% of the dose given, distributed as before, and the tissue amine concentration was 8.3 times that in the plasma. Two hearts were studied three hours after the same small dose. These hearts still retained 2.5% of the administered amine, in a concentration 130 times that in the plasma. However, the level of H\textsuperscript{3}-norepinephrine in the ventricles and septums had decreased nearly to that found in the atria, indicating a more rapid net turnover of the labeled transmitter in the ventricles. These results are consistent with the results of more complete studies in rats (Potter, unpublished).

Uptake, metabolism and release of H\textsuperscript{3}-norepinephrine during perfusion were also studied. In four normal and three denervated isolated hearts norepinephrine was continuously infused for 10 minutes at a rate of 0.15 μg (0.15 μg l-norepinephrine) per minute without a perceptible change in heart rate. The outflow of norepinephrine and its metabolites was studied each minute during the infusion and for as long thereafter as possible. A right atrial biopsy was taken nine minutes after the infusion was begun. The results from the most comparable pair of a normal (150 g) and a denervated heart (140 g; 28 days post-

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>H\textsuperscript{3}-norepinephrine</th>
<th>H\textsuperscript{3}-normetanephrine</th>
<th>H\textsuperscript{3}-deaminated metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal (n=6)</td>
<td>Transplanted (n=4)</td>
<td>Normal (n=6)</td>
</tr>
<tr>
<td>Right atrium</td>
<td>86.7</td>
<td>8.0</td>
<td>7.4</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>83.4</td>
<td>8.9</td>
<td>8.9</td>
</tr>
<tr>
<td>Left atrium</td>
<td>58.6</td>
<td>7.4</td>
<td>6.0</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>134.0</td>
<td>8.2</td>
<td>15.0</td>
</tr>
<tr>
<td>Interventricular septum</td>
<td>121.0</td>
<td>8.8</td>
<td>12.8</td>
</tr>
<tr>
<td>Plasma</td>
<td>27.6</td>
<td>27.1</td>
<td>11.3</td>
</tr>
</tbody>
</table>

*The numbers are average values for norepinephrine and its metabolites in six normal and four transplanted dog hearts 15 minutes after the infusion of 0.5 μg/kg of H\textsuperscript{3}-dl-norepinephrine (1100 mc/mmole) into the superior vena cava. No SEM exceeded ± 15% of the mean values given. H\textsuperscript{3}-deaminated metabolites include VMA, dihydroxymandelic acid, dihydroxyphenylglycol, and methoxyhydroxyphenylglycol. The differences in norepinephrine and normetanephrine content between the normal and transplanted hearts are statistically significant (P < 0.001).*
operative) are illustrated in figures 1 and 2 and table 2; they are typical of the other hearts studied.

During the infusion of $H^3$-norepinephrine the normal heart retained 56% of the amine delivered to it. Of the remaining norepineph-

![Figure 1](image1.png)

**FIGURE 1**
Release of $H^3$-norepinephrine and its metabolites from a normal and a transplanted dog heart. Figure shows the amounts of tritium leaving the isolated perfused hearts during and after the infusion of $H^3$-norepinephrine. The tritium represents norepinephrine and its metabolites in the proportions shown in figure 2.

![Figure 2](image2.png)

**FIGURE 2**
Metabolism of $H^3$-norepinephrine during perfusion of a normal and a transplanted dog heart. Figure shows the percentage of tritium released from the hearts, during and after the infusion of $H^3$-norepinephrine, which was present as norepinephrine, normetanephrine, and deaminated metabolites. Amounts of tritium released are shown in figure 1.
TABLE 2
Retention of Perfused H$^3$-Norepinephrine and its Metabolites by a Normal and a Transplanted Dog Heart

<table>
<thead>
<tr>
<th></th>
<th>Normal heart</th>
<th>Transplanted heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$^3$-norepinephrine perfused through the heart</td>
<td>100.0 μc</td>
<td>100.0 μc</td>
</tr>
<tr>
<td>Tritium retained in the heart*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>During 10-minute infusion of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$^3$-norepinephrine</td>
<td>55.5 μc</td>
<td>29.8 μc</td>
</tr>
<tr>
<td>2 minutes after end of infusion</td>
<td>51.4</td>
<td>17.4</td>
</tr>
<tr>
<td>15 minutes after end of infusion</td>
<td>47.0</td>
<td>9.4</td>
</tr>
<tr>
<td>30 minutes after end of infusion</td>
<td>44.8</td>
<td>3.3</td>
</tr>
<tr>
<td>40 minutes after end of infusion</td>
<td>43.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Labeled substances in an atrial biopsy during H$^3$-norepinephrine infusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>93%</td>
<td>68%</td>
</tr>
<tr>
<td>Normetanephrine</td>
<td>6</td>
<td>27</td>
</tr>
<tr>
<td>Deaminated metabolites</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Labeled substances in the heart at the end of the experiment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>93%</td>
<td>33%</td>
</tr>
<tr>
<td>Normetanephrine</td>
<td>5</td>
<td>54</td>
</tr>
<tr>
<td>Deaminated metabolites</td>
<td>2</td>
<td>13</td>
</tr>
</tbody>
</table>

*The tritium retained in the heart at a given time during the experiment was calculated by adding the amount of tritium released into the perfusion fluid after that time to the amount found in the heart at the end of the experiment. The results were comparable in studies of two other normal and two transplanted hearts.

Norepinephrine, most passed unchanged through the tissue while 3% was O-methylated to normetanephrine and about 3% was O-methylated and deaminated. After the infusion there was a rapid outflow of tritium, representing "washout" of norepinephrine and its metabolites from the water compartments of the heart. This exponential release of tritium was followed by a much slower, multiphasic appearance of the isotope (fig. 1). The tritium leaving the heart during this later period must have been derived from bound H$^3$-norepinephrine, since 93% of the isotope in the heart represented norepinephrine both in the biopsy taken during the administration of the amine and in the heart at the end of the experiment (table 2). About 43% of the released tritium represented norepinephrine, while a slowly decreasing percentage (49 to 44%) appeared as normetanephrine, and a slowly increasing percentage (8 to 11%) as deaminated metabolites (fig. 2). The actual release of H$^3$-norepinephrine from the normal heart was also multiphasic which indicates that release was occurring at different rates from more than one "pool" of bound norepinephrine. Most of the amine bound by the normal heart was still retained at the end of the experiment.

In contrast, the denervated heart retained much less of the infused norepinephrine than did the normal heart. Two minutes after the end of the infusion period, when most of the "unbound" H$^3$-norepinephrine had been washed out of the heart (fig. 1 and table 2), the transplanted heart still contained 17.4% of the infused tritium (11.0% of the infused norepinephrine) whereas the normal heart retained 51.4% of the tritium given (47.8% of the norepinephrine). Tritium continued to leave the denervated tissue at a rapid rate; only 1% of the infused norepinephrine was retained 30 minutes after the end of the infusion period when the normal tissue retained 41.7%. During the administration of norepinephrine the denervated tissue metabolized about twice as much of the norepinephrine leaving the heart (12%) as did the normal heart, almost entirely by O-methylation. After the infusion 85% of the released amine was metabolized, predominantly by COMT, indicating greater in vivo function of this enzyme. There was little change in MAO activity.
IN VITRO ACTIVITY OF COMT AND MAO

All five parts of each of the hearts studied were assayed for enzyme activity in vitro (table 3). Methyl transferase activity was distributed equally per gram of normal heart tissue and showed an insignificant decrease upon denervation. MAO activity was somewhat greater in the atria than ventricles and showed some decrease in the transplanted hearts.

INTRACELLULAR LOCALIZATION OF NOREPINEPHRINE

The subcellular localization of endogenous and H₄-norepinephrine was studied in continuous sucrose density gradients. As in the rat heart, the amine in the normal dog atrium was found almost entirely in a peak corresponding to the position in the gradient of the microsomal particles. In the denervated right atrium, however, only a very small microsomal peak of H₄-norepinephrine was apparent and most of the tritium was confined to the supernatant fluid.

MORPHOLOGICAL STUDIES

The fine structure of myocardial cells and cardiac nerves in both normal and transplanted dog hearts has been briefly described and is generally confirmed by the present studies. Further attention is given here to the possible sites of storage of norepinephrine. Three types of granular inclusions are commonly encountered in the axoplasm of autonomic nerves. Type I vesicles are membrane-limited structures 80 to 100 µm in diameter containing a dense, heterogeneous, granular material which often fills the vesicles. These vesicles are frequently seen in the terminal regions of central, peripheral, and autonomic axons, and are especially abundant in the presynaptic elements of ganglionic synapses in the autonomic nervous system. Their chemical composition is unknown. Type II vesicles are membrane-limited structures 40 to 60 µm in diameter and contain a homogeneous opaque core 20 to 30 µm in diameter which is often located eccentrically. This type of granulated vesicle is distributed selectively in postganglionic sympathetic axons, and is associated specifically with norepinephrine. Granules having the ultrastructural characteristics of glycogen are also frequently encountered in autonomic axons and should be distinguished from granulated vesicles.

In extranodal regions of the right atrium of the normal dog heart autonomic axons displayed numerous type I granulated vesicles and glycogen granules. Type II granulated vesicles were less numerous than in autonomic nerves of the rat heart. The majority of vesicles in preterminal regions of autonomic axons was nongranulated, and resembled the "synaptic vesicles" characteristic of cholinergic nerve endings (fig. 3).

Light-microscopic radioautography of the normal dog atrium, following the administration of H₄-norepinephrine, revealed specific silver grain clusters over filiform structures between myocardial cells and around small blood vessels. Myocardial cells were not labeled. An identical localization was previously observed in the rat heart; electron-micro-
Autonomic axons in the right atrium of a normal dog heart. Twelve nonmyelinated axons (A), partially encompassed by a Schwann cell (SC), course in the interstitial space (IS) near two myocardial cells. Type I granulated vesicles (1), type II granulated vesicles (2) and glycogen particles (3) are present in the axons. Myocardial cells contain the "secretory granules" (arrows) described by Palade and by Janieson. Mitochondria; F: myofilaments. The bar indicates 0.5 μ. Photomicrographs of autonomic axons in the right atrium of transplanted hearts were not distinguishable from the figure shown, except that type II granulated vesicles were not seen.

The fine structure of nerves in the transplanted dog hearts was barely distinguishable from that in the normal hearts. It was anticipated that the presence of type II granulated vesicles could be used to distinguish adrenergic from cholinergic axons. These vesicles were not seen in the transplanted tissue; however, their paucity in the normal heart precluded a statistical estimate of a change in their number upon denervation. Repeated attempts to perform light-microscopic radioautography on the denervated tissue were unsuccessful, apparently because the small amounts of tritiated substances in the tissue were not retained during preservation; therefore electron-microscopic radioautography was not performed. Type I granulated vesicles and glycogen granules were as abundant following transplantation as in the normal heart.

Myocardial cells in the dog and other mammals contain granules 100 to 400 μ in diameter and limited by a unit membrane. Because they are degranulated by reserpine it was suggested that these granules might store catecholamines. These granules were frequently observed in the normal hearts, and appeared to be unchanged in the transplanted tissues.
Discussion

The following points demonstrate that the ability of the heart to bind and store dopamine and norepinephrine, and to synthesize norepinephrine, is almost completely dependent upon its content of adrenergic nerves. Retention of administered dopamine was reduced to about 4% of normal in the autotransplanted hearts after degeneration of their sympathetic axons; the results were the same in hearts studied either in vivo or after isolation and perfusion. Uptake of norepinephrine, and retention of the amine for 15 minutes, were also comparable in hearts studied in vivo or after isolation, and were reduced to about 6% of normal in the denervated tissue. Synthesis of norepinephrine from dopamine by the enzyme dopamine β-oxidase was also greatly reduced in the denervated tissue; the amount of norepinephrine which was synthesized and retained by the transplanted hearts, after perfusion with dopamine, was only 2.8% as much as that found in normal hearts.

The ability of the perfused normal hearts to retain about 15% of the large dose of dopamine which was given demonstrates that a binding mechanism in nerves is a well-developed and major means for the removal of the substance from the circulation. The ability of the innervated heart to extract norepinephrine from the circulation is remarkable. In the present studies about 56% of the di-norepinephrine administered to perfused normal hearts was retained each minute during a 10-minute infusion of the amine. In the present studies about 56% of the dl-norepinephrine administered to perfused normal hearts was retained each minute during a 10-minute infusion of the amine. L-norepinephrine is bound in the rat heart 11 times more avidly than d-norepinephrine several minutes after such small doses of the racemic amine. The observed uptake of 56% may therefore represent an almost complete extraction of the biological L-isomer. Since the amounts of L-norepinephrine given (2.3 μg/liter) were within the physiological range of norepinephrine concentrations in the plasma, uptake of norepinephrine by neural tissue may be considered the major means of inactivation of the free amine in the heart.

It was expected that the retention of catecholamines by the heart would be related to the number of adrenergic nerves in different parts of the heart. A definite count of adrenergic and cholinergic fibers in the heart by histological or electron-microscopic techniques is not yet available. However, it is likely that the degree of sympathetic innervation of the atria is greater than that of the ventricles, as judged by the distribution of endogenous catecholamines in the dog heart, and the results of fluorescence microscopy in the rabbit heart. Unexpectedly the retention of H2-norepinephrine was considerably greater in the ventricles than in the atria (per gram of tissue), resulting in a specific activity of norepinephrine in the left ventricle 8.4 times that in the right atrium, 15 minutes after the administration of the amine. A similar but less dramatic result was noted recently in the cat heart. This finding is apparently due to a greater blood flow to the ventricles than to the atria. Preliminary results on the simultaneous delivery of Rb and H1-norepinephrine to normal and denervated hearts demonstrate that the amine is retained in exact relation to its initial delivery to various parts of the normal heart. But the distribution of C14-dopamine in the various parts of the heart did roughly parallel the distribution of endogenous catecholamines. In the case of dopamine the amounts of the amine which were given may have been sufficient to saturate the ventricular binding mechanism and disclose the greater potential capacity of the atria. Three hours after the administration of H2-norepinephrine, the distribution of this remaining bound amine also began to approach the endogenous distribution of catecholamines. Thus the net turnover of (at least) the radioactive amine was more rapid in the ventricles than in the atria. This result may be due to greater metabolic activity of the ventricles than of the atria, to a more rapid washout of released norepinephrine, to a more nearly saturated binding mechanism, or to the presence of more than one "pool" of norepinephrine (as discussed later).

The ability of cardiac tissue to retain briefly even small amounts of dopamine and norepi-
nephrine after apparently complete adrenergic denervation is of considerable interest. This binding was most evident in the isolated perfused hearts after the end of the infusions of \( \text{H}^2 \)-norepinephrine. Following the initial washout of unbound norepinephrine, 11% of the infused amine was still present in the transplanted tissue, in a form which was released with a half-life of about 10 minutes. Binding of norepinephrine in the denervated tissue may therefore be considered as an important, if temporary, means for the inactivation of the free amine. The present studies do not disclose whether or not this type of binding also occurs in the normal heart. The anatomic site of this binding process is not clear. One possibility is that a few sympathetic nerves remain in the heart. In a recent study bilateral stellate ganglion excision caused a lesser percentage fall in the catecholamine content of the cat heart, and permitted it to retain considerable amounts of \( \text{H}^2 \)-norepinephrine for several hours;\(^{88}\) these results are perhaps best interpreted as the effects of incomplete denervation. In the present studies there is no evidence for the presence of remaining sympathetic nerves. Autotransplantation effectively severs all extrinsic nerves to the heart, and the time allowed for degeneration of these nerves was sufficient, since there was no difference between the results of uptake experiments in a heart studied 140 days after autotransplantation and those examined sooner. In addition, the rapid nature of the release of \( \text{H}^2 \)-norepinephrine from binding sites in the transplanted hearts, the finding that little of the amine was bound in microsomal particles, and the lack of success in localizing it by radioautography, argue against the presence of \( \text{H}^2 \)-norepinephrine in adrenergic nerves. Another possible site of storage would be chromaffin cells, which have been reported in the dog heart,\(^{40}\) but which were not observed during these studies, and could not be located by fluorescence microscopy in the rabbit heart.\(^{37}\) Type I granulated vesicles\(^{30}\) and the large granules present in atrial muscle cells\(^{82, 83}\) are not important sites for the storage of norepinephrine since they are abundant in the transplanted tissue when endogenous norepinephrine has nearly disappeared, and because they are not associated with \( \text{H}^2 \)-norepinephrine by radioautography. It cannot now be excluded, however, that they briefly retain norepinephrine in the transplanted tissue.

Denervation produced little change in the total activity in vitro of the enzymes which metabolize norepinephrine, MAO and COMT. In contrast, the inactivation of norepinephrine by these enzymes in the intact hearts was considerably altered by denervation, apparently because of marked changes in the binding of the amine. During the administration of \( \text{H}^2 \)-norepinephrine to the perfused hearts, the transplanted tissue metabolized up to seven times as much norepinephrine as did the normal tissue. The steadily increasing inactivation of the amine by COMT in the denervated tissue (fig. 2) paralleled a decrease in binding of the amine (fig. 1), as though the binding sites in the denervated tissue were becoming saturated and more norepinephrine was exposed to the enzyme. During the post-infusion period a larger percentage and a much larger amount of norepinephrine were also metabolized by COMT in the denervated than in the normal tissue. Present views of the function of MAO and COMT indicate that MAO is most important in regulating the level of intraneuronal norepinephrine, whereas COMT metabolizes the free amine and a portion of that which has been bound for only a short time.\(^{4, 7, 11, 41}\) In normal tissues the free amine enters nerve axons and is bound in granulated vesicles;\(^{14-16}\) in the process, its metabolism shifts from O-methylation to deamination.\(^{7, 41}\) In the present studies there was a very slow increase in the activity of MAO in the normal hearts, and a slow decrease in the activity of COMT (fig. 2), in support of these ideas. In the transplanted tissues norepinephrine evidently did not enter neurones and it was exposed, after brief retention at unknown sites, to both enzymes, with the result that it was largely degraded by COMT, like circulating catecholamines. It is interesting to compare these re-
results with those observed in reserpinized tissues, in which the binding of norepinephrine is also greatly decreased. In the reserpinized rat heart, norepinephrine is taken up and concentrated, presumably in nerves, but it is not well retained in the vesicles; as a result, MAO activity overshadows that of COMT.

The overall inactivation of administered norepinephrine by binding and metabolism was considerably less in the denervated than in the normal hearts. It is reasonable to attribute part of the increased chronotropic and inotropic responsiveness of transplanted hearts to the fact that more free norepinephrine is present near receptor cells. One may speculate that the exaggerated effect of circulating norepinephrine on the transplanted tissue is of considerable value in adaptation to stresses.

These studies extend present concepts of the storage of norepinephrine in more than one “pool” or “compartment” in normal tissues. H5-norepinephrine which becomes bound in the heart appears to add to a small part of the endogenous stores of the amine, and then to exchange partially and slowly with the remainder of the stores. The first compartment has a high specific activity of norepinephrine, and turns over with a half-life of several hours. The transmitter so held is easily released by nerve stimulation or by tyramine, and the metabolic products which appear are largely the result of O-methylation. A second, larger compartment has a lower specific activity, turns over with a half-life of about 22 hours, and produces mostly deaminated metabolites. It is also released by nerve impulses and may be depleted by reserpine, but it is much less sensitive to sympathomimetic amines, including tyramine. Both of these pools are contained in small particles. The present experiments confirm previous results in several ways: the outflow of H5-norepinephrine is multiphasic as if leaving several compartments of bound norepinephrine at different rates; the amine of highest specific activity is released first (from the ventricles); and there is a gradual shift in metabolism of

the bound amine from O-methylation to deamination. In addition, another “pool” exists in the transplanted tissue which may be present also in the normal heart: a small amount of norepinephrine is held at unknown binding sites, has a half-life of about 10 minutes, and is eventually O-methylated.

The presence of these pools has been attributed to different loci of binding of exogenous norepinephrine within each nerve, to different types of binding within vesicles, and/or to different rates of release of the bound amine by drugs or by nerve impulses. Although compartmentalization of norepinephrine has been described in isolated atria, it may be noted that much of the evidence used to support concepts of several modes of storage of norepinephrine in whole hearts may only demonstrate differences between the atria and ventricles. For example, after H5-norepinephrine has been in the dog heart for a short period of time, stimulation of its sympathetic nerve supply causes the release of norepinephrine of a much lower specific activity than that leaving the heart before stimulation or present in it. This result may be explained in part by considering that nerve stimulation has a greater effect on the well-innervated atria, which contain norepinephrine of a relatively low specific activity, than on the ventricles which release amine of high specific activity before stimulation. The presence of somewhat greater MAO activity in the atria than in the ventricles might also explain in part the shift in metabolism from O-methylation to deamination, since H5-norepinephrine remains longer in the atria than in the ventricles.

The present results confirm earlier studies demonstrating that the normal heart can synthesize and store norepinephrine as well as extract it from the circulation. The rate of synthesis observed, 0.095 μg/g tissue per hour, is comparable to that previously reported; probably still more norepinephrine was made but released or metabolized during each experiment. This rate could synthesize in seven hours the amount of norepinephrine usually stored in the dog heart. It is apparent
that both the atria and ventricles have sufficient amounts of dopamine $\beta$-oxidase to make large quantities of the transmitter. The amount of norepinephrine synthesized per gram of tissue was greatest in the atria because they retained the most dopamine. The specific activity of the norepinephrine, however, was highest in the ventricles, partly because of the greater dilution of the $\text{C}^{14}$-norepinephrine by endogenous norepinephrine in the atria, and perhaps partly because of the more rapid turnover of the amine in the ventricles. The dose of dopamine administered to these hearts (1.0 mg) is known to be sufficient to release a considerable percentage of the endogenous norepinephrine of rat and guinea pig hearts, providing an explanation for the marked tachycardia which was observed in the normal but not in the denervated dog hearts. Since the total norepinephrine content of these dog hearts after perfusion was comparable to that of the other hearts studied, any norepinephrine which was released may have been replaced by synthesis. Such a balance of the effects of dopamine as a norepinephrine-releasing agent and as a norepinephrine precursor has been observed previously.

**Summary**

The ability of normal and transplanted dog hearts to make, bind, store, and metabolize norepinephrine was studied. Transplanted hearts were used in order to assess the effects of adrenergic denervation. Normal hearts bound large quantities of administered $\text{C}^{14}$-dopamine and synthesized considerable quantities of norepinephrine in both the atria and ventricles. Isolated perfused normal hearts steadily removed about 56% of infused $\text{dl}$-$\text{H}^{3}$-norepinephrine; a binding mechanism was the major means of inactivation of the amine. Uptake of radioactive norepinephrine was greater in the ventricles than in the atria because they received more of the amine, and the turnover rate of the labeled amine was also highest in the ventricles. Subcellular fractionation of the bound amine demonstrated that it was localized in particles of microsomal size; radioautographic studies demonstrated the presence of $\text{H}^{3}$-norepinephrine only in association with nerves. About 57% of the $\text{H}^{3}$-norepinephrine released from normal hearts was metabolized, primarily by $\text{O}$-methylation.

After autotransplantation, and degeneration of postganglionic sympathetic nerves, the average catecholamine content of the hearts fell to 1.2% of normal, $\text{C}^{14}$-dopamine uptake and formation of norepinephrine were reduced to a few per cent of normal, and the uptake and retention of $\text{H}^{3}$-norepinephrine was 6.1% of normal under the conditions used. Binding of norepinephrine to unknown sites in the transplanted tissue served temporarily as an important mechanism of inactivation of the amine, but this norepinephrine was very rapidly released and nearly 85% metabolized, again predominantly by $\text{O}$-methylation. The fine structure of the transplanted tissues was barely distinguishable from that of normal hearts by electron microscopy. In vitro assays of catechol-$\text{O}$-methyltransferase and monoamine oxidase showed little change in activity upon adrenergic denervation. These results demonstrate the importance of the adrenergic innervation of the heart for the normal synthesis, storage and inactivation of norepinephrine.

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**References**


Synthesis, Binding, Release, and Metabolism of Norepinephrine in Normal and Transplanted Dog Hearts
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