Uptake and Metabolism of Tritiated Norepinephrine in the Isolated Canine Heart

By Charles A. Chidsey, M.D., Richard L. Kahler, M.D., Leslie L. Kelminson, M.D., and Eugene Braunwald, M.D.

With the technical assistance of Beverlee Lehr.

The adrenergic neurotransmitter, norepinephrine, is known to be metabolized almost completely prior to its excretion in the urine.1 The demonstration that 3-methoxy-4-hydroxy-mandelic acid is a major metabolic product of norepinephrine2 indicated that two steps were involved in the metabolism of this amine, O-methylation and deamination. A number of studies using isotopically labeled catecholamines have been carried out to establish which of these two is of greater importance in the inactivation of norepinephrine.3-6 These studies have indicated that O-methylation appears to be the initial step in the metabolism of injected catecholamine and that the O-methylated amine is then a substrate for deamination.

Since the enzymes responsible for both O-methylation and deamination, catechol-O-methyl transferase and monoamine oxidase, are present in tissues innervated by sympathetic nerves,7-8 a significant amount of the norepinephrine released from the nerve may undergo metabolism before entering the circulation. The metabolic fate of norepinephrine released from the nerve endings may differ from that of injected and circulating amine, since in the latter instance the role of hepatic metabolism will predominate, and it may be questioned whether the relative activities of the two enzymes are the same in the sympathetic nerves and in the liver. In fact, it has been indicated that monoamine oxidase may be the more important enzyme in the degradation of norepinephrine locally in the heart and brain of rats.9

In order to assess this problem more directly the metabolism of norepinephrine was studied in an isolated perfused canine heart preparation. With this technic it was possible to examine the metabolism of norepinephrine which occurs locally in the heart. After labeling the cardiac norepinephrine store with tritiated norepinephrine, isotopic activity was determined in the coronary venous blood. The appearance of activity in norepinephrine and its O-methylated and deaminated metabolites was examined both during spontaneous release of norepinephrine from the heart and during augmented release induced by tyramine. In addition to demonstrating the predominant role of O-methylation in the canine heart, these studies relate the amount of norepinephrine appearing in coronary venous blood to the total amount released from the nerve ending, during both spontaneous release and release augmented by tyramine.

Methods

The experiments were performed in five isolated canine heart preparations. The experimental and donor dogs underwent bilateral adrenalectomy several days prior to study and were then maintained on daily parenteral cortisone. Adrenalectomy was performed in order to reduce the excessively elevated levels of norepinephrine and epinephrine found in blood (2-4 μg/L norepinephrine; 4-6 μg/L epinephrine) obtained by exsanguination of donor dogs. A schematic diagram of the preparation is shown in figure 1. Oxygenated blood was pumped into the coronary arterial bed by means of an oscillating Sigma motor pump through the cannulated left subclavian artery. The brachiocephalic artery was ligated and the aorta was clamped just distal to the origin of the left subclavian artery. Both venae cavae were ligated near their entry into the right atrium, and a large bore cannula with multiple side holes was placed through the aygus vein and right atrium into the right ventricle; the main pulmonary artery was ligated. Thus, the total coronary...
venous drainage to the right side of the heart could be collected. A wide bore cannula was placed into the left ventricle through its apex and left ventricular pressure was maintained at 5 mm Hg. A thermistor in the right ventricle, placed into the left ventricle through its apex, was used to monitor the blood temperature constant at 36.0 ± 1.0°C. Coronary blood flow was kept constant at approximately 38 ml/min in all experiments, and although coronary artery flow was monitored by a recording rotameter in the arterial line, coronary blood flow measurements were made by timed collecting of the coronary venous drainage into a graduated cylinder.

The DL norepinephrine-7-H3 used in these studies (New England Nuclear Corp.) was purified by adsorption on Dowex 50 × 4 (H+) and elution with 0.4N HC1 and the purity was demonstrated by paper chromatography. Four μg of tritiated norepinephrine (specific activity 13.0 μc/μg) was mixed with T-1824 dye (0.25 to 0.5 ml) and diluted to 5 ml with 0.2M sodium acetate buffer (pH 6.5) immediately before it was injected, during a 15-second interval, into the cannula supplying the coronary arterial bed. Following the injection of norepinephrine and dye, none of the coronary venous blood was allowed to return to the oxygenator, the blood required for continuous perfusion being supplied from a separate reservoir. Coronary venous samples were taken at varying time intervals for one hour.

The T-1824 dye was injected with the norepinephrine in order to estimate the extraction of norepinephrine by the heart. The concentrations of the two substances and the plasma flow were determined at 10-second intervals for two minutes after the injection. From these measurements the fraction of the injected dose leaving the heart at each sampling interval was calculated and the sum of these values during the time interval required for the dye to pass through the heart permitted an estimation of the extraction of the administered norepinephrine (NE):

Extraction (%) = \[ 1 - \frac{\text{Total NE recovered/NE administered}}{\text{Total dye recovered/dye administered}} \] \times 100

In these calculations it has been assumed that essentially all the norepinephrine remained in the plasma and only insignificant amounts were present in the red cell. This assumption is based on in vitro studies which demonstrated that red blood cells failed to take up norepinephrine.11

Two samples of blood were taken for hematocrit determinations, permitting calculation of plasma flow through the coronary vascular bed. The blood samples were refrigerated upon withdrawal and within 60 minutes were centrifuged at 12000 × g at 4°C for 30 minutes. T-1824 dye concentrations in plasma were determined spectrophotometrically. Radioactivity measurements were made in a liquid scintillation spectrometer using a counting solution12 which accommodated one ml of aqueous phase with a 10.3% efficiency for tritium. Internal standards were added to each sample and all values reported were corrected for quenching.

The total activity in plasma was determined from an aliquot of plasma deproteinized with 5% trichloroacetic acid. During the first two minutes total activity was assumed to be all norepinephrine and the norepinephrine extraction was calculated from this activity. This was a valid assumption since it was determined that at three minutes more than 80% of the total activity was in the NE fraction. Fifteen ml of plasma were diluted with an equal volume of cold 0.2M sodium acetate and 0.2 ml 0.2M disodium ethylenediamine tetraacetate. This mixture was adsorbed on aluminum oxide (alumina) at pH 8.4 and eluted first with 4.0 ml 0.2N acetic acid10 which removed catecholamines and then with 0.2N sulfuric acid which removed catechol acids.13 The effluent of the alumina oxide column, which contained non-catechol metabolites, was collected and deproteinized with perchloric acid. The effluent fractions were centrifuged for 20 minutes at room temperature and the supernatant obtained was adjusted to pH = 5.6 with 5N potassium hydroxide. After chilling, the potassium perchlorate precipitate was removed by centrifugation for five minutes at 3000 × g and the clear supernatant passed over Dowex 50 × 4 (H+) columns (28 mm × 11.5 mm2). The Dowex column was washed with 15 ml of water and eluted with 2 ml of 1N ammonium hydroxide which removed normetanephrine adsorbed on the Dowex 50. The effluent of the Dowex column represented the residual activity, which was not catechol and not amine. Carrier normetanephrine was added to plasma and the normetanephrine activity of plasma was corrected for recovery of carrier which averaged 75% (61% — 90%). Carrier norepinephrine was also added to plasma and the activity of plasma norepinephrine was corrected for the recovery of
FIGURE 1
Schematic diagram of the canine heart preparation utilized to study the metabolism of tritiated norepinephrine. Arterial blood is pumped by the "coronary pump" through a rotameter into the left subclavian artery (L.S.A.). The aorta is clamped just below the L.S.A. Aortic pressure is measured by means of a catheter inserted via the brachiocephalic artery (B.C.A.). The left ventricle (L.V.) is drained. Temperature is monitored by means of a thermistor inserted into the right ventricle (R.V.) through the right atrial appendage. Temperature is held constant by means of the heat exchanger. The pulmonary artery (P.A.) is ligated around a catheter inserted into the right ventricle (R.V.) for the measurement of pressures. A large multi-holed cannula is inserted through the azygous vein and the right atrium (J.A.) into the right ventricle. The coronary venous return is drained through this cannula into a graduated cylinder. The superior vena cava (S.V.C.) and inferior vena cava (I.V.C.) are ligated. Venous blood is sampled at the points designated.

carrier which averaged 80% (58% - 89%). The chemical identification of the activity of the aluminum oxide and Dowex eluates was carried out using paper chromatography. Aliquots of these eluates with carrier material in each, containing approximately 25 mμg of tritium were lyophilized, dissolved in ethanol, and chromatographed on Whatman #1 for 17 hours using an ascending method (butanol:acetic acid:water = 12:3:5). The radioactivity scan of two such chromatograms is shown in figure 2. The activity of the sulfuric acid eluate was not identified because of the small quantities present. However, it is known that catechol acids require stronger acid for elution from aluminum oxide than the amines and it was demonstrated that dihydroxymandelic acid is adsorbed on aluminum oxide at pH = 8.4 and is removed only by elution with sulfuric acid. By adding tritiated norepinephrine to nonradioactive plasma and fractionating the mixture by this method, the sulfuric acid eluate was shown to contain less than 4% of the norepinephrine added to plasma. Therefore, this fraction was presumed to represent dihydroxymandelic acid, the catechol acid metabolite which has been identified in urine.

Results

EXTRACTION OF TRITIATED NOREPINEPHRINE FROM THE CORONARY CIRCULATION

In three experiments in which coronary venous blood was sampled immediately following the injection of tritiated norepinephrine and T-1824 dye, indicator dilution curves were constructed for the two substances and from these the extraction of the diffusible norepinephrine was calculated. The results of a typical experiment are shown in figure 3 where dye and norepinephrine at each time...
NOREPINEPHRINE UPTAKE AND METABOLISM

PERCENT DL NOREPINEPHRINE-7-H² AND T-1824 DYE
LEAVING THE HEART

FIGURE 3
The per cent of DL Norepinephrine-7-H² and T-1824 dye leaving the isolated heart in the first two minutes after their injection into the coronary arterial bed.

interval are expressed as per cent of the amount injected. The bulk of the injected dye appeared in the coronary venous blood during this sampling period in a distribution pattern typical of a non-diffusible indicator. The norepinephrine in the coronary venous blood showed a similar distribution, but the per cent determined at each time interval was much less than that of the dye. The total amount appearing in the venous blood equaled 23% while 87% of the dye was recovered. This corresponded to a norepinephrine extraction by the myocardium of 74%.

RELEASE OF NOREPINEPHRINE AND ITS METABOLITES INTO THE CORONARY CIRCULATION

Coronary venous blood samples were taken periodically over one minute intervals beginning at three minutes and ending at 60 minutes after the injection of the tritiated norepinephrine in five experiments. Since following the injection no further radioactivity was added to the coronary perfusion, the activity observed in these samples represented that which was released spontaneously from the myocardial norepinephrine pool. The results of a typical experiment are shown in figure 4 which represents the activity of norepinephrine and normetanephrine, two fractions which account for the major portion of the total activity in plasma. The activity of the norepinephrine fraction declined over a 30-minute period of time to an essentially constant value, 0.20 to 0.25 mµc/ml. The normetanephrine fraction also showed an absolute decline to a plateau together with the norepinephrine, but the activity relative to norepinephrine increased during this period of time, reaching a value of four to five times that of the catecholamine. In the other four experiments the results were similar with norepinephrine activity falling to between 0.26 and 0.63 mµc/ml while the normetanephrine activity was two to four times greater.

In table 1 a fractionation of the activity in plasma samples in one experiment is given from 20 to 30 minutes, the time at which the activities appeared to level off in these experiments. It may be seen that norepinephrine represented a small fraction of the total activity spontaneously released into the coronary circulation, averaging 11.7%, while normetanephrine activity was two to four times greater.

Circulation Research, Volume XII, February 1968
TABLE 1
Fractionation of Tritium Activity in Plasma

<table>
<thead>
<tr>
<th>Fraction†</th>
<th>20 Minutes</th>
<th>25 Minutes</th>
<th>30 Minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA extract</td>
<td>1.96 mµc/ml</td>
<td>1.74 mµc/ml</td>
<td>1.72 mµc/ml</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>0.23</td>
<td>0.21</td>
<td>0.20</td>
</tr>
<tr>
<td>Dihydroxymandelic acid</td>
<td>0.07</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Normetanephrine</td>
<td>1.18</td>
<td>1.10</td>
<td>1.07</td>
</tr>
<tr>
<td>Residue (Dowex-50 effluent)</td>
<td>0.68</td>
<td>0.65</td>
<td>0.59</td>
</tr>
</tbody>
</table>

*DL Norepinephrine-7-H
†Fractions were obtained as outlined in the text.

TABLE 2
Fractionation of Tritium Activity in Myocardial Tissue

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ventricle*</th>
<th>Atrial appendage*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA extract</td>
<td>47.2 mµc/g</td>
<td>112.2 mµc/g</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>29.0</td>
<td>70.4</td>
</tr>
<tr>
<td>Dihydroxymandelic acid</td>
<td>4.5</td>
<td>5.3</td>
</tr>
<tr>
<td>Normetanephrine</td>
<td>8.9</td>
<td>14.2</td>
</tr>
<tr>
<td>Residue (Dowex-50 effluent)</td>
<td>2.5</td>
<td>15.3</td>
</tr>
</tbody>
</table>

*Samples obtained 60 minutes after administration of tritiated norepinephrine.

Norepinephrine accounted for the largest fraction of the total activity in plasma, averaging 61.9%. Only minimal activity (3.1%) was observed in dihydroxymandelic acid. The residual activity represented some contamination from incomplete recovery of the other fractions plus activity in metabolites which are not catechol and not amine. In the other experiments the results were similar with 13% to 25% of the total activity in the norepinephrine fraction and 39% to 56% in the normetanephrine fraction. In no experiment was the activity present in dihydroxymandelic acid greater than 5% of the total plasma activity.

The effect of the administration of tyramine, an aromatic amine known to release norepinephrine into the coronary venous blood,14 was examined in five experiments. The results of tyramine injection (2 mg free base) in the same experimental preparation depicted in figure 4 are shown in figure 5. The activity of norepinephrine showed a very large increase after tyramine. As a result the amount of activity in norepinephrine was a greater fraction of the total released activity following tyramine (41.0%) than during spontaneous release (11.7%). Normetanephrine activity showed only a small increase after tyramine representing 41% of the total activity. Activity in norepinephrine declined toward the control level over a period of 30 minutes. During this period normetanephrine activity remained relatively constant and only minimally above control. In the other four experiments tyramine produced a 6- to 12-fold increase in norepinephrine activity, so that norepinephrine represented 50% to 80% of the total activity in plasma, whereas the tyramine produced less than 50% increase in normetanephrine activity.

ANALYSIS OF MYOCARDIAL TISSUE

The activity of tritium in tissue obtained from the left atrial appendage and left ventricle 60 minutes after administration of tritiated norepinephrine in one experiment is shown in table 2. Here the total activity of a trichloroacetic acid extract of tissue is shown together with a fractionation of this activity into its components. It may be seen that in distinction to the plasma, activity in the tissue was predominantly in norepinephrine with less than 20% of the total activity in the other metabolites. The atrial appendages...
analyzed before and after the administration of tyramine showed no difference in the distribution of radioactivity in the tissue. The results of this analysis in the tissues taken in the other experiments were similar showing that 60% or more of the total activity in tissues represents unmetabolized norepinephrine.

**Discussion**

Previous studies have demonstrated that isotopically labeled norepinephrine administered to intact animals can be taken up by tissue innervated by sympathetic nerves. In these experiments the manner in which tritiated norepinephrine is extracted in a single circulation through the isolated canine heart has been demonstrated. The norepinephrine concentration relative to the non-diffusible indicator (T-1824 dye) with which it is injected, remains proportionately the same during the passage of 87% of the injected bolus. This is unlike other isotopically labeled diffusible indicators, such as K and Na, which show a back diffusion into the intravascular compartment as the peak concentration of non-diffusible indicator passes by the venous sampling point. This behavior of the norepinephrine indicates that the material which diffuses out of the coronary capillaries fails to return during the time interval required by the non-diffusible indicator to pass through the heart. Such trapping may result from concentration or binding of the material in an extravascular pool so that its diffusion back into the blood is limited. This is the mechanism which is presumed to be responsible for the removal of norepinephrine by the sympathetic nerves in the heart since it is known that norepinephrine stores are maintained at this site against a very large concentration gradient.

The isotopic norepinephrine extracted by the heart is, however, slowly released from storage sites. It appears that the bulk of the norepinephrine so released is metabolized before it reaches the blood and only a small fraction (11.7% to 25%) of the total appears in the coronary venous blood as the catecholamine. The principal metabolite has been demonstrated to be normetanephrine (39% to 61.9% of the total radioactivity released) with smaller amounts of the other metabolites. Thus, it is evident that O-methylation plays a predominant role in the local metabolism in the canine heart of the norepinephrine released spontaneously from sympathetic nerves. Whether this is true in other species is uncertain since it has recently been shown by in vitro assay that the relative activities of catechol-O-methyl transferase and monoamine oxidase in the heart vary in different species.

When the release of norepinephrine is increased by injection of tyramine, the activity of norepinephrine appearing in venous blood is much greater and represents a greater fraction of the total activity in the blood (41%). Although there is also a small increase in the amount of metabolic products appearing at this time in the venous blood, the enzymatic mechanisms responsible for the inactivation of almost 90% of spontaneously released norepinephrine are not capable of handling the increased substrate resulting from tyramine administration. Recently, ex-
Experiments have been carried out in intact dogs to determine the ratio of activity in norepinephrine to total activity in coronary venous blood 60 minutes following the injection of tritiated norepinephrine. In these experiments 9-22% of the total activity in the plasma sample appeared in norepinephrine during the control state and this fraction showed a three-fold increase after cardioaccelerator nerve stimulation, suggesting that the fate of the norepinephrine released by nervous impulses is similar to that released by tyramine. Thus the role of enzymatic activity at the nerve ending in the termination of the action of norepinephrine may be less important than other mechanisms. One such mechanism which has been suggested is return of norepinephrine into the nerve stores. Another possible mechanism for removal of the norepinephrine from the effector area which would be suggested by these studies may be overflow into the blood and metabolism by the liver. This mechanism, however, can be presumed to be of greatest importance with intense adrenergic stimulation when large quantities of norepinephrine are released.

In an attempt to determine if deaminated metabolites were formed, but not released into the blood, an analysis of the tritium activity in the tissues was performed. These studies revealed that the major portion of the activity appeared in norepinephrine, with less than 13% in each of the other fractions. Thus, there is no evidence that there is retention of an important amount of any metabolic product of norepinephrine in the myocardium in these experiments.

Summary

Using an isolated canine heart preparation, the myocardial norepinephrine pool was labeled by injecting tritiated norepinephrine into the blood perfusing the heart. The extraction of the norepinephrine during a single circulation through the coronary bed was shown to be large (74%). As the isotopic material, which was extracted, was released spontaneously from the norepinephrine pool, 75% to 88% of it was metabolized before appearing in the coronary venous blood. The chief metabolite has been demonstrated to be normetanephrine which accounts for 39.0% to 61.7% of the spontaneously released norepinephrine. Because of this it is concluded that catechol-O-methyl transferase is the enzyme primarily responsible for the metabolic inactivation of norepinephrine in the canine heart. When release was increased by the injection of tyramine, more of the released norepinephrine appeared unmetabolized in coronary venous blood, suggesting that the enzymatic process by which norepinephrine is inactivated may be limited. Therefore, the extent to which enzymatic processes contribute to the termination of augmented adrenergic activity in the heart may be questioned.

References
11. Schanker, L. S., Nappiottis, P. A., and...


Uptake and Metabolism of Tritiated Norepinephrine in the Isolated Canine Heart
CHARLES A. CHIDSEY, RICHARD L. KAHLER, LESLIE L. KELMINSON and EUGENE BRAUNWALD

doi: 10.1161/01.RES.12.2.220

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1963 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/12/2/220

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/