Sampling and Analysis of Catecholamines and Metabolites

J. Richard Crout, M.D.*

CATECHOLAMINE BIOCHEMISTRY is discussed with increasing frequency in the clinical literature of almost every specialty of medicine. In view of this, it is pertinent to ask: What basic principles of this field are relevant to human biology and disease? What factors influence the release and metabolism of catecholamines in the body? What can one learn by measuring the catecholamines and their metabolites in urine or blood? Finally, assuming one wants to learn these things, which of the several methods and their modifications are reliable and accurate from the technical point of view? These are the topics explored in this review.

General Principles

COMPARTMENTALIZATION

Three catecholamines are synthesized in the human body: dopamine, norepinephrine (NE), and epinephrine (E).† These compounds have in common a catechol (dihydroxybenzene) nucleus and an amine-containing side chain and are the end products of a synthetic pathway leading from tyrosine to dopa to dopamine to NE to E (fig. 1).† It is important to recognize that figure 1 and others like it in the literature show the overall synthetic and metabolic capabilities of the body as a whole, but they fail to emphasize that no single cell or tissue normally carries out all of the reactions shown. Certain pathways, or even portions of pathways, are localized to some tissues, while other pathways are localized to other tissues—and a firm grasp of the way in which these reactions are compartmentalized in the body is essential to an understanding of current concepts in the field.

For example, the only mammalian cells which carry the synthetic pathway fully to E are, for practical purposes, chromaffin cells, the largest clusters of which are the adrenal medullae. The blood level of E and/or the urinary excretion rate of E therefore may be taken to reflect the release of E from (and therefore the biological activity of) the adrenal medullae. On the other hand, postganglionic adrenergic neurons carry the synthetic pathway only to the level of NE and secrete NE alone as the adrenergic transmitter. Thus, the level of NE in the blood or urine may be taken as a relatively specific index of adrenergic nerve activity.

The enzymes which degrade catecholamines are compartmentalized also; the liver and kidneys are actually the only organs which carry out the full array of metabolic reactions shown in figure 1. Adrenergic neurons contain monoamine oxidase (MAO) in their mitochondria, but they lack catechol-O-methyltransferase (COMT). Adrenergic receptors apparently contain neither enzyme, although COMT may metabolize some NE molecules locally in the tissue at a site close to receptors.† The significance of these different localizations for MAO and COMT will be considered in the following sections.

THE ADRENERGIC NEUROON

NE is stored within the adrenergic neuron not in free solution but rather in a bound form within small membrane-limited intraneuronal

* Associate Professor of Pharmacology and Internal Medicine; Burroughs Wellcome Scholar in Clinical Pharmacology, The University of Texas Southwestern Medical School at Dallas, Dallas, Texas.
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† Abbreviations used in this paper are: NE = norepinephrine, E = epinephrine; NMN = normetanephrine, MN = metanephrine, VMA = vanillylmandelic acid; DOMA = 3,4-dihydroxyphenylactic acid; DOPAC = 3,4-dihydroxyphenylacetic acid; MAO = monoamine oxidase; COMT = catechol-O-methyltransferase.

661
granules (fig. 2). These granules contain a small amount of lipoprotein, a high concentration of NE, and approximately one mol of ATP for every four mols of catecholamine. NE is continuously lost from these granules via two processes: utilization as the neurohumoral transmitter and leakage of NE molecules into the surrounding cytoplasm. The granular NE store is in turn replenished by de novo synthesis and by re-uptake of NE molecules from the surrounding cytoplasm. The adrenergic neuron thus can be visualized as having at least two anatomical pools of NE which are in a steady-state equilibrium—a large intragranular pool of bound NE and a smaller pool of unbound NE which is free in the cytoplasm of the axon (fig. 2). 5 (There is no common agreement on whether NE molecules released as transmitter come from the intragranular or the cytoplasmic store.) An important consequence of this arrangement is that molecules of cytoplasmic NE may encounter mitochondria and there be metabolized by MAO. The MAO metabolites thus formed then diffuse from the neuron, enter the general circulation, and eventually appear in the urine (after metabolism by COMT in the liver and kidneys) as VMA, as shown by the dotted line in figure 2. The intraneuronal metabolism of NE thus provides a pathway whereby NE can be synthesized and appear ultimately in the urine as VMA without having been released as "free" NE, i.e., without ever having produced any physiologic effect as a transmitter. 1, 5

The biological function of this curious and apparently wasteful pathway is not clear. It is possible that more molecules of NE are lost via this mechanism than are released as free transmitter. 1, 5 This pathway is clearly the major route of NE metabolism after the administration of reserpine, a drug which impairs the ability of the granules to hold NE. The entire NE store then leaks into the cytoplasm and subsequently is metabolized by mitochondrial MAO; this accounts for the fact that large amounts of NE can disappear from neurons after the administration of reserpine without producing any sympathomimetic effect. 5

NE released by nerve impulses enters the extracellular space as "free" NE, where it can stimulate adrenergic receptors on the effector cells. As noted in figure 2, some of these transmitter molecules spill over into the venous effluent and eventually reach the systemic circulation, where they are metabolized via the pathways for circulating NE. However, an
important discovery of the last few years is that most of the NE molecules released from nerve endings probably do not reach the circulation; instead, they are taken back into adrenergic neurons by an uptake process for NE located in the axonal membrane. This uptake process is believed to be the major mechanism for removing NE from the region of receptors, i.e., for terminating the action of NE, at least in the heart (although this is not yet established in the case of resistance vessels). Because of this uptake mechanism, the number of NE molecules actually appearing in the venous effluent is substantially smaller than the number of molecules originally released by nerve impulses. At low frequencies of nerve stimulation very few transmitter molecules escape the re-uptake process, while at higher frequencies proportionately more NE may appear in the venous effluent. Also, a low rate of blood flow through the organ favors re-uptake, while a high rate of flow favors washout of NE into the effluent. For these reasons, the concentration of NE in the venous effluent (and consequently its concentration in the systemic circulation and ultimately in the urine) must be viewed as only a semi-quantitative index of the amount of transmitter released by adrenergic neural activity. In spite of this limitation, the measurement of NE concentration in plasma or urine has proven itself to be the most accurate, and most useful, method of appraising the activity of the adrenergic nervous system under a wide variety of experimental conditions.

CIRCULATING CATECHOLAMINES

Although only a portion of the NE molecules released by neural activity find their way to the general circulation, these are the molecules which are degraded and ultimately appear in the urine as metabolites. While some of these molecules may be metabolized by COMT locally within the tissue, this is probably not a major site of inactivation. Most metabolism takes place instead in the liver and kidneys, via the pathways shown in figure 1. The available data indicate that approximately 65 per cent of the E (and presumably also NE) which reaches the circulation is metabolized first by COMT and then by MAO. An important consequence of this fact, as shown in figure 2, is that NE and NMN are generated only from molecules of NE which originally were released as physiologically active "free" NE. In like fashion, though not shown in figure 2, urinary E and MN arise only from physiologically active E.
released into the circulation from the adrenal medulla. However, urinary VMA arises from several sources: circulating NE, circulating E, NE metabolized intraneuronally by MAO, and E metabolized similarly within adrenal medullary cells (fig. 2). For this reason, subtle changes in the release of NE or E are detected most easily and specifically by measuring urinary NE and E (or perhaps NMN and MN), not VMA. Total catecholamine production over a period of time, on the other hand, probably is assessed best by measuring urinary VMA.

Only a tiny fraction (1-4 per cent) of the NE and E which enter the circulation actually appears in the urine as free NE and E. The remainder is metabolized via the general pathways shown in figure 1. The relative proportions of the major urinary metabolites have been measured in several different experimental settings—with somewhat conflicting results (table 1). If one focuses on the urinary excretion values for normal persons, it is clear that endogenously-formed catecholamines appear to be metabolized "more completely" than radioactive catecholamines infused into the circulation; i.e., the endogenous excretion values for normal subjects yield higher ratios of VMA/NE+E and of VMA/NMN+MN than those reported for radioactive NE or E. Perhaps this means that much of the urinary VMA normally is derived from the intraneuronal metabolism of NE rather than from "free" NE (figure 2). This explanation is consistent with the finding that upright posture and the stresses of heart failure and surgery all tend to increase urinary NE relatively more than they increase urinary VMA.

Table 1 also shows that the urinary excretion pattern found in patients with pheochromocytoma differs somewhat from that observed in patients infused with radioactive NE or E, even though both experimental settings supposedly involve the study of circulating catecholamines. While a full explanation for this discrepancy is not available, several factors contribute to it. The first is that the d,l-isomeric mixture of radioactive NE or E may not be metabolized exactly like the l-isomer, in spite of claims to the contrary. (Compare the figures for d,l-E-2-C<sup>14</sup> and d,l-E-7-H<sup>3</sup> with those for l-E-7-H<sup>3</sup> in table 1.) Second, in many pheochromocytomas, particularly those with high total contents of NE+E (table 1), large amounts of NE and E are metabolized within the tumor before they reach the circulation, a phenomenon analogous to the intraneuronal metabolism of NE by MAO. This causes a proportionately greater excretion of VMA and MN+MN in these patients than one would expect from a pure infusion of NE or E. Finally, the pattern of urinary metabolites may be different after a brief infusion of catecholamines (radioactivity studies).

**Table 1. Relative Excretion of Urinary Catecholamines and Metabolites under Different Experimental Conditions**

<table>
<thead>
<tr>
<th>Situation</th>
<th>Reference</th>
<th>Per Cent of Total Excreted as&lt;sup&gt;*&lt;/sup&gt;</th>
<th>VMA</th>
<th>VMA</th>
<th>VMA</th>
<th>MN(M+MN)</th>
<th>MN(M+MN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NE(ED)</td>
<td>NMN(MN)</td>
<td>VMA</td>
<td>Other</td>
<td>NE(ED)</td>
<td>NMN(MN)</td>
</tr>
<tr>
<td>Normal subjects</td>
<td>37</td>
<td>1.1</td>
<td>7.6</td>
<td>91</td>
<td>—</td>
<td>87</td>
<td>12</td>
</tr>
<tr>
<td>Endogenous excretion</td>
<td>38</td>
<td>4</td>
<td>22</td>
<td>32</td>
<td>40</td>
<td>8</td>
<td>1.5</td>
</tr>
<tr>
<td>d,l-NE-2-C&lt;sup&gt;14&lt;/sup&gt;</td>
<td>39</td>
<td>4</td>
<td>47</td>
<td>32</td>
<td>16</td>
<td>7</td>
<td>0.6</td>
</tr>
<tr>
<td>d,l-NE-7-H&lt;sup&gt;3&lt;/sup&gt;</td>
<td>37</td>
<td>6.8</td>
<td>41</td>
<td>41</td>
<td>7</td>
<td>14</td>
<td>1.6</td>
</tr>
<tr>
<td>l-E-7-H&lt;sup&gt;3&lt;/sup&gt;</td>
<td>40</td>
<td>4</td>
<td>34</td>
<td>55</td>
<td>7</td>
<td>14</td>
<td>1.6</td>
</tr>
<tr>
<td>Pheochromocytoma patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;Low&quot; group&lt;sup&gt;**&lt;/sup&gt;</td>
<td>12</td>
<td>5</td>
<td>35</td>
<td>70</td>
<td>—</td>
<td>14</td>
<td>2.8</td>
</tr>
<tr>
<td>&quot;High&quot; group&lt;sup&gt;**&lt;/sup&gt;</td>
<td>12</td>
<td>1.7</td>
<td>27</td>
<td>71</td>
<td>—</td>
<td>42</td>
<td>2.6</td>
</tr>
</tbody>
</table>

<sup>*</sup>In studies of radioactive NE and E, total excretion represents total radioactivity in the urine. In all other studies, total excretion was taken to be the sum of NE+E+NMN+MN+VMA.

<sup>**</sup>"Low" group consists of patients with tumors containing a low total content of NE+E (<100 mg).

<sup>**</sup>"High" group patients had a high tumor content of NE+E (>100 mg).
than during a chronic infusion (pheochromocytoma); insufficient data are available on this point.

For these various reasons, it must be concluded that current understanding of the quantitative aspects of catecholamine metabolism is far from perfect in spite of much careful work. More studies in which urinary NNM, MN, and VMA are measured in addition to NE and E are needed.

**Timing**

The sympathetic nervous system is prepared to function immediately when called upon; i.e., it responds promptly to certain stresses, and returns to baseline soon after the stress is removed. Investigators often are confronted with the problem of documenting these transient changes in sympathoadrenal function. One approach is to determine the blood levels of NE and E repeatedly; except for the sample size (25 ml.) and technical difficulties of the assay, this offers an excellent solution to the problem.

An alternate approach is to measure NE and E in a carefully-timed urine specimen which brackets the period of stress. Because urine is usually easier to collect and assay than blood, this method has become the standard way of appraising sympathoadrenal activity objectively. Urinary NE is derived only from circulating NE, not from adrenergic neurons in the kidney. Also, free NE appears in the urine only during the period of its infusion plus an additional 30-45 minutes (Fig. 3). On the other hand, the metabolites of NE, particularly conjugated NMN and VMA, accumulate slowly in the urine for many hours after the end of an infusion (Fig. 3). This delay in appearance of metabolites means that the assay of these compounds is less likely to be useful than is the assay of urinary NE and E if one wants to measure the sympathoadrenal response to short-term stresses such as anesthesia, anxiety, exercise, etc.

If the period of stress is associated with oliguria, as during shock or very severe exercise, this method of appraising sympathoadrenal function obviously cannot be applied. Under these conditions determination of the blood level of NE and E is the only accurate method of measuring the release of catecholamines into the circulation.

**Techniques and Uses**

**Norepinephrine and Epinephrine**

As noted previously, the measurement of urinary NE and E represents the classical, and still the most widely applied, approach to assessing the activity of the sympathoadrenal system. Urinary NE is a useful index of adrenergic nerve activity, while urinary E reflects the release of E from the adrenal medulla. It should be noted that these comments apply only to free (unconjugated) urinary NE and E. Conjugated NE and E in the urine are derived in part from dietary sources (e.g., norepinephrine in bananas), and the determination of these conjugates introduces a number of artifacts and technical difficulties without yielding additional information of known biological importance. For this reason only free urinary NE and E are assayed in most laboratories.

Catecholamines are extracted and purified from urine by adsorption, onto alumina at pH 8.4 or adsorption onto cationic exchange resins at pH 6.5. Catecholamines are then eluted with acid, and the resulting eluate is assayed fluorometrically. Figure 4 shows the essential
chemistry behind the fluorometric trihydroxyindole method for catecholamines. NE and E are oxidized by potassium ferricyanide to the well-known pink derivatives noradrenochrome and adrenochrome. In alkaline solution these undergo spontaneous rearrangement to 3,5,6-trihydroxyindole and its N-methyl derivative, respectively. Ascorbic acid stabilizes these compounds. Their concentration is then determined with a fluorometer.

All of the modifications used for the determination of urinary catecholamines are variations on this basic theme. In the method used in this laboratory for many years, iodine instead of ferricyanide is used as the oxidizing agent. If the oxidation step is carried out at pH 3.5, only E is oxidized to its corresponding chromo; however, at pH 6.5, both NE and E are oxidized. This method has the advantage of reliability, particularly in the hands of beginners; in addition, the separation of NE from E is quite accurate. Methods employing ferricyanide as the oxidizing agent require more attention to detail, but have the advantage of being faster and more sensitive.

In the method of Anton and Sayre NE and E are separated by oxidation at different pH values. In most of the other ferricyanide methods NE and E are separated by reading the final solution at two different wave lengths, a procedure which is more rapid but less accurate than the oxidation at two different pH values. The fluorometric assay has been automated, although the preparation of eluates still requires the handwork of a technician. Certain fluorescent drugs (particularly tetracycline, quinine, and methyldopa) must be discontinued during collection of the urine, as they produce falsely highly readings in the fluorometer.

The determination or urinary NE and E is of primary value to the clinician in establishing the diagnosis of pheochromocytoma. Urinary NE and/or its metabolites are increased (usually severalfold) in every patient with pheochromocytoma, and the definitive diagnosis of this disease rests on the demonstration of increased values in a 24-hour urine sample. Typical excretion values for normal adults are 20–70 μg. of NE per day and 0–15 μg. of E per day, and patients with pheochromocytoma may have excretion values from 100 to 5,000 μg. of NE + E per day. Both NE and E are increased in perhaps one-half of adults with pheochromocytoma, while NE alone is increased in the remainder.

Urinary excretion rates for NE and E are low in normal adults during sleep (on the order of 10 and 2 mg/min., respectively), and they increase as the sympathoadrenal system is called upon to respond to various stresses. Ambulation ordinarily increases the
excretion rate of NE two- to three-fold, and heavy exercise may increase it further, to the range of 200–300 mg/min. A variety of stresses, including anxiety and fear, increase urinary NE and/or E to some degree; life-threatening events such as burns, shock, and acute myocardial infarction may increase these values dramatically. NE and E also may be assayed in plasma by the trihydroxyindole fluorometric technique. The levels encountered in normal plasma (NE = 0.2–0.5 mg/dl, and E = 0.0 mg/dl) are at the limits of sensitivity of the method; therefore, plasma determinations are intrinsically more difficult and less accurate than urinary assays. The ethylenediamine condensation procedure for plasma NE and E gives "normal" values which are too high due to interfering substances in the blood, and its use is not recommended. Plasma NE and E are measured only for research purposes where urinary assays are unsuitable (e.g., oliguria) or where estimation of the blood level is essential for the research project (e.g., analysis of the venous effluent from a particular organ).

**DOPAMINE**

Dopamine is the third catecholamine present in human urine. Like all catecholamines, it is adsorbed by alumina and is therefore present in eluates prepared for the determination of NE and E. These extracts may be assayed by a fluorometric technique which is quite specific for dopamine, although the chemical nature of the fluorophore is not established clearly.

The most important use of urinary dopamine assays in clinical medicine is in the diagnosis of neuroblastoma. The hallmark of this tumor is an increased excretion of dopamine and its metabolites, in addition to an increase in urinary NE, NMN, and VMA. The determination of either dopamine, homovanillic acid, or VMA will yield a positive diagnosis in 70–80 per cent of cases, and the combination of dopamine and VMA taken together will give a positive diagnosis in nearly every patient. The normal adult excretes 100–350 mg of dopamine per day, and the excretion rate does not seem to correlate well with sympathoadrenal activity. The increases which occur in patients with neuroblastoma are usually easy to detect and sometimes are remarkably high; values 50–100 times the upper limit of normal have been recorded.

**NORMETANEPHINE AND METANEPHINE**

NMN and MN are excreted largely in the form of acid-labile conjugates. These metabolites may be taken as indices of the amount of NE and E which entered the circulation as free vasoactive amine. The most widely used method for the determination of NMN+MN is that developed by Pisano for the diagnosis of pheochromocytoma. In this method the urine is subjected to acid hydrolysis, and the free NMN and MN so formed are then adsorbed onto a cationic exchange resin; these amines are eluted with ammonium and converted to vanillin by periodate ion. The vanillin concentration is then measured spectrophotometrically at 360 mp. This method is particularly useful in screening hypertensive patients for pheochromocytoma because it is technically easier to perform than either a free catecholamine determination or an accurate VMA determination and is, if anything, more accurate in establishing the diagnosis. For these reasons its use in clinical laboratories is increasing. It should be emphasized that the method measures NMN+MN and that the "normal range" is higher than the true normal range for these metabolites. This procedure should not be used for research studies in which the investigator wants an accurate analysis of the normal excretion of NMN and MN.

NMN and MN may be measured individually by fluorometric methods which are modifications of the trihydroxyindole procedure. These methods are relatively tedious, and their use to date has been limited to research studies. The normal adult excretes 100–500 mg of NMN per day and 50–200 mg of MN.

**VANILLYLAMINDELIC ACID**

Urinary VMA is perhaps the best available index of total catecholamine production. A number of accurate methods for its determination are available. In most, the phenolic acids are extracted from acidified urine with ethyl acetate, and this extract is then processed further. In the method of Pisano et al., which is representative of those based on vanillic acid, VMA is converted to vanillin by...
peroxidate ions; the vanillin is then extracted and determined spectrophotometrically. VMA also may be determined by column chromatography, two-dimensional paper chromatography, high-voltage electrophoresis, or gas chromatography; an excellent review of these various techniques has appeared recently. These methods are of comparable accuracy and are all relatively time-consuming to perform; they are suitable for the diagnosis of pheochromocytoma and for research purposes.

The major use of the VMA determination in clinical medicine is in the diagnosis of pheochromocytoma and neuroblastoma. It is interesting that the urinary VMA assay is not necessarily the most practical method of screening hypertensive patients for pheochromocytoma, in spite of the fact that VMA is the major metabolite of NE and E. The normal range for VMA excretion in adults is 2 to 6 mg. per day. While only rare patients with pheochromocytoma have VMA values in this range, perhaps 20 per cent of patients with pheochromocytoma have only a modest increase in urinary VMA (10 mg. per day or less). The sharp separation of normal from abnormal thus requires a high-quality VMA method which does not give normal values higher than 6 mg. per day; this in turn requires as much laboratory effort as the fluorometric analysis of NE. The commercial "Pheset" method in wide use in clinical laboratories meets the requirement of simplicity, but unfortunately it lacks the diagnostic accuracy of other methods, particularly in equivocal cases, because of its high "normal" range (up to 10 mg. per day).

Summary

Noradrenaline (NE) is the peripheral transmitter of the adrenergic nervous system, and epinephrine (E) is the major hormone released from the adrenal medulla in man. The plasma levels of NE and E, or their rates of excretion into the urine, may be taken as relatively good indices of the degree of activity of the adrenergic nervous system and the adrenal medulla respectively. Circulating NE and E are metabolized largely by the liver and kidneys. The 3-O-methylated derivatives normetanephrine (NMN) and metanephrine (MN) are believed, like NE and E, to reflect the amount of "free" NE and E which enter the circulation in a physiologically active form. Urinary vanillylmandelic acid (VMA) is the major end-metabolite of NE and E and is a useful index of total catecholamine synthesis. Urinary VMA is less useful than urinary NE and E as an index of sympathoadrenal function. Accurate methods are available for the determination of all of these compounds in urine and for the determination of NE and E in plasma.

The most important use of these assays in modern clinical medicine is in the diagnosis of pheochromocytoma and neuroblastoma, where their proper application can establish the diagnosis preoperatively in essentially every patient. These methods are also used widely and effectively in research to study sympathoadrenal function in man.

References

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