STUDIES ON THE FORMATION OF 3-METHOXY-4-HYDROXY-D-MANDELIC ACID, A URINARY METABOLITE OF NOREPINEPHRINE AND EPINEPHRINE[1]

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Until quite recently little was known concerning the nature of the end-products of the metabolism of norepinephrine and epinephrine. Reviews by Blaschko (9) and von Euler (12) discussed the various chemical and enzymic transformations of the amines which had been studied, but both reviewers made clear that the physiological importance of these reactions was not established. The most definite information available was the work by Schayer *et al.* (18–20), which demonstrated that after the administration of norepinephrine-α-C^{14} or epinephrine-α-C^{14} to rats essentially all the administered radioactivity could be found in the urine in the form of three radioactive metabolites. The same three metabolites were observed after the administration of either of the amines.

The work to be discussed in the present paper had its origin in research on the metabolism of aromatic compounds in patients with phenylketonuria. In order to understand the abnormalities in the pathological condition, it was necessary to examine the phenolic acids in the urine of normal humans. Many unknown acids were characterized and several of them were identified as the work progressed (4). In the early attempts to gain information on the structure of the unknown phenolic acids many approaches were used. One was to feed to humans phenolic substances which were suspected to occur in the course of normal endogenous metabolism. Because hydroxytyramine had been reported to occur in urine (13) and is known to be a substrate for amine oxidase (9), homoprotocatechuic acid, the end-product of the oxidative deamination of hydroxytyramine, was prepared, ingested and the urine was examined to see if any of the unknown phenolic acids appeared in an increased amount. A large amount of one of these acids, which is always present in urine in small amounts, was found to be excreted after the ingestion of homoprotocatechuic acid. The same material appeared after the ingestion of L-dihydroxyphenylalanine (21). This acid, which had chromatographic properties corresponding to those of authentic homovanillic acid, was isolated and proved to be homovanillic acid. Work on the preliminary identification of the other phenolic acids in urine showed that there were several which had the 3-methoxy-4-hydroxyphenyl nucleus, so it seemed possible that the methylation of the 3-hydroxyl of homoprotocatechuic acid might be an example of a more general type of reaction. The possible gen-

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erality of the methylation of the 3-hydroxyl group of 3,4-dihydroxy compounds was indicated further by preliminary experiments in which the ingestion of protocatechuic and caffeic acids was found to give rise to the excretion of several substances containing the 3-methoxy-4-hydroxyphenyl nucleus. At about this time, De Eds, Booth and Jones (10, 11), who were working on the metabolism of flavonoid substances, reported the biological methylation of these same compounds.

It was a natural extension of these findings to suspect that a similar reaction might occur with norepinephrine and epinephrine as well as with 3,4-dihydroxymandelic acid, which would be formed by oxidative deamination of the amines, and that the methylated acid, 3-methoxy-4-hydroxymandelic acid, might be found in urine. Actually, it seemed quite likely in advance that methylation of the amines themselves probably occurred, on the basis of observations now over 50 years old. These were by Professor Abel (1), who proposed and defended an incorrect structure for epinephrine on the basis of degradation results. He found that after oxidative breakdown of his isolated epinephrine he was able to detect vanillin by its characteristic odor and with specific qualitative tests, although he could never isolate it. He proposed for epinephrine a structure which contained the 3-methoxy-4-hydroxyphenyl nucleus. He commented that either his epinephrine contained that grouping or that some rearrangement, unknown at that time to organic chemistry, resulted in the transfer of a methyl group to a phenolic hydroxyl group. In the light of the biological methylation of the 3-hydroxyl of catechols, it now seems likely that his isolated epinephrine was contaminated with a small amount of the 3-O-methyl ethers of epinephrine and norepinephrine, which have now been shown to occur in adrenal glands (7, 23), and that this led him to his erroneous deduction.

The phenolic acids in the urine of many people were examined, and, in addition, more detailed studies were carried out with a few individuals. A substance which has the same chromatographic properties as authentic 3-methoxy-4-hydroxymandelic acid (VMA)[3] was found to occur in all samples of urine in amounts that do not vary greatly between individuals or in the same individual under widely varying dietary regimens. Small doses of epinephrine and norepinephrine were then ingested and the amount of VMA excreted was determined. There was no significant increase. This was not unexpected, however, since the pharmacologic ineffectiveness of the amines when administered orally is well known, and it seemed reasonable to suppose that a different metabolic pathway or route of excretion would occur with endogenously produced as contrasted with orally administered amines.

Through the collaboration of Dr. Harold Brown of the Veterans' Administra-

[3] Because of the cumbersome nature of the proper name for this compound, which will probably be referred to frequently in the future, the trivial name "vanilmandelic acid" is proposed, and the abbreviation "VMA." This name makes clear the relation of the aromatic nucleus to that in vanillin and of the molecule to mandelic acid. The other abbreviations used in this paper are DMA for 3,4-dihydroxymandelic acid, and PA for protocatechuic acid.

tion Hospital, Salt Lake City, we were able to obtain urine from patients in shock whose blood pressure was being maintained by the parenteral administration of norepinephrine. Here, a substantial portion of the norepinephrine administered, about 30%, could be accounted for as extra VMA in their urine. This finding added support to the idea that the VMA in urine actually originates from norepinephrine.

The preparation of 3,4-dihydroxymandelic acid was then undertaken, in order to make certain that it does undergo methylation *in vivo*. Rather strangely, this acid, of obvious interest as the end-product of the action of amine oxidase upon epinephrine, had not been synthesized previously, though Barger and Ewins (8) in 1909 reported an attempted synthesis and described a grossly impure product. Dihydroxymandelic acid was prepared (22), ingested and, as expected, led to an increase in the VMA in urine.

For a final demonstration that VMA in the urine represents an end-product of norepinephrine metabolism, an attempt was made to locate patients with pheochromocytomas, since these tumors produce considerable amounts of norepinephrine and epinephrine. If it could be established that a patient with a pheochromocytoma excreted a large amount of VMA before surgery, and a normal amount after surgical removal of the tumor, the combination of the different types of evidence that VMA is an end-product of norepinephrine metabolism would be convincing. Urine from many patients with hypertension was examined until finally a patient with an adrenal pheochromocytoma was located. This patient excreted VMA at the level of 90 μg/mg creatinine before surgery, and, after a large tumor was removed, produced only 2.7 μg/mg creatinine. Urine samples from a total of 8 patients with surgically confirmed pheochromocytomas have been examined up to the present time. The results of the VMA estimations are listed in Table 1. It was possible through the collaboration of Dr. Brown and his staff to obtain urine from one of these patients (DS) for a period of several days. This urine served as starting material for the isolation and conclusive identification of the urinary material which has the chromatographic properties of VMA. The isolated substance proved to have the same physical properties as synthetic 3-methoxy-4-hydroxy-D-mandelic acid.

It is apparent from the data listed in Table 1 that examination of urine for the presence of excess VMA will provide a convenient means for the diagnosis of pheochromocytomas as well as information on the success of surgical removal of the tumors. Previously, the estimation of urinary catecholamines has proved to be a reliable method for detecting the tumors (14). However, far smaller amounts of the amines than of VMA are excreted, the catechol compounds are inherently unstable, and technical difficulties in their determination make their detection unsuitable for routine use in laboratories where a considerable background of experience is not available. VMA, however, is chemically quite stable under most conditions, and the chromatographic estimation may be carried out readily without the use of specialized and expensive equipment by people without previous experience with paper chromatography. Urine from many healthy individuals and from patients suffering from hypertension, and from a variety of

TABLE 1

VMA excretion by patients with surgically confirmed pheochromocytomas

	VMA Excretion (μg/mg creatinine)	
	Before surgery	After surgery
HN	90	2.7
CD	31	4.4
DC	23	4.4
JT	19	—
DS	12	1.5
DG*	11	13 (2 weeks) 6 (2 months)
MR†	9	2.2
MN	9	—
Normal range 1.5–3.5		

* Pheochromocarcinoma of peri-renal area.

† Paraganglioma of celiac plexus.

other diseases has been examined for VMA content in our laboratory. The creatinine content of the urine has been used as the basis for determining the amount of VMA present. As may be noted in Table 1, 8 patients with surgically confirmed tumors excreted from 9 to 90 μg of VMA per mg creatinine, whereas the amount normally found in urine is 1.5 to 3.5 μg/mg creatinine. Several hypertensive patients who had clinical symptoms which warranted surgical exploration, despite the finding of normal amounts of VMA in their urine, were found not to have pheochromocytomas. Some difficulty does remain in the interpretation of results when patients excrete VMA at the level of 4 to 7 μg/mg creatinine. Many acutely ill persons, including some with terminal illness, have been found to excrete this amount, and subsequent autopsy has failed to reveal any signs of a pheochromocytoma. It seems likely that severe physiological stress may result in an increased production of norepinephrine and epinephrine, and a resulting increased excretion of VMA in the amounts which might be expected if a small tumor is present.

In Table 1 it should be noted that patients DG and MR had tumors which were not of the adrenal medulla. Further, DG had a diffuse tumor which had possibly metastasized to other areas. Surgical removal of the obviously pathological tissue not only did not result in marked clinical improvement, but her excretion of VMA actually increased in the postsurgery period, and remained much higher than normal two months later.

After it had been definitely established that VMA occurs in human urine and that it probably arises from norepinephrine and epinephrine it was of interest to continue with some simple metabolic experiments in order to gain more information on the manner in which VMA and one of its precursors, 3,4-dihydroxymandelic acid (DMA) are handled in the body. For these experiments, epinephrine, norepinephrine, DL-DMA, and D- and L-VMA were ingested, and the

phenolic acid metabolites which then could be detected in urine were examined. These metabolites were VMA, DMA and protocatechuic acid (PA). The results of these experiments are presented in abbreviated form in Table 2. In addition to these compounds, a small amount of vanillic acid and vanilloylglycine appeared whenever protocatechuic acid was present. The rigorous dietary control necessary to eliminate completely small amounts of vanillic acid precursors was not undertaken for these experiments, however, and since the percentage of the ingested compounds excreted in this form was small, no attempt was made to quantitate vanillic acid production.

Attempts to detect glucuronide or sulfate derivatives of VMA or DMA in human urine were fruitless. Since both of these acids are unstable to acid hydrolysis, urine collected after the ingestion of either acid was treated with glucuronidase and sulfatase preparations. No extra VMA or DMA was released by such treatment. The white rat, on the other hand, normally excretes no free VMA, but treatment of rat urine with β-glucuronidase results in the release of an amount of VMA comparable, on a weight basis, to that produced by humans. Even after the ingestion of large amounts of VMA, the rat excretes practically no free VMA, but mainly the glucuronide.

It should be noted that a low recovery of urinary metabolites was obtained after the ingestion of either D-VMA (natural form), L-VMA, or DL-VMA. There was no indication of a marked change in the recovery of metabolites of DMA with variation in the amount ingested. This behavior of VMA and DMA is in marked contrast to that of the corresponding derivatives without an α-hydroxyl group, since high recoveries of homovanillic acid and homoprotocatechuic acid were made after the ingestion of either acid or of L-dopa (21). Only about 10% of ingested DMA could be accounted for, mostly in the form of its methylated derivative, VMA. A smaller amount of unchanged DMA was excreted along

TABLE 2

Metabolism of vanilmandelic and dihydroxymandelic acids in man

Compound ingested	Amount ingested (mg)	Urinary Metabolites (% of compound ingested)			
		VMA	DMA	PA	Total recovery
D-VMA	10	23	0	0	23
	100	18	0	0	18
L-VMA	100	29	0	0	29
DL-DMA	10	8	0	0	8
	20	6	0	1	7
	50	9	3	1	13
	100	8	2	2	12
	200	5	4	2	11
	500	4	3	2	9
(−)-Epinephrine	10	4	0	0	4
	50	1	0	0	1
(−)-Norepinephrine	10	3	0	0	3
	50	2	0	0	2

with, surprisingly, about an equal amount of protocatechuic acid. This formation of protocatechuic acid from DMA appears to be unique, since no evidence exists for this type of conversion of other mandelic acids. This is of some importance since, if mandelic acids could undergo degradation to benzoic acids, vanillic acid might occur as a metabolic product of VMA. Carefully controlled experiments with both isomers of VMA itself, however, failed to give evidence for even a small conversion of VMA to vanillic acid.

Two points remain to be considered in connection with the data presented in Table 1. First, since the recovery of VMA itself is so low in these experiments, only about 20%, it seems probable that a considerably larger proportion of DMA is metabolized by methylation than would appear from the actual amount of VMA excreted after ingestion of DMA. As much as 40 to 50% might undergo methylation.

The second point is whether any information bearing on the quantitative importance of the methylation versus the amine oxidase pathway for the metabolism of norepinephrine can be gained from these observations. Axelrod (5–7) has demonstrated that brain and liver tissue contain enzymes which are very active in methylating the 3-hydroxyl of epinephrine and norepinephrine to form

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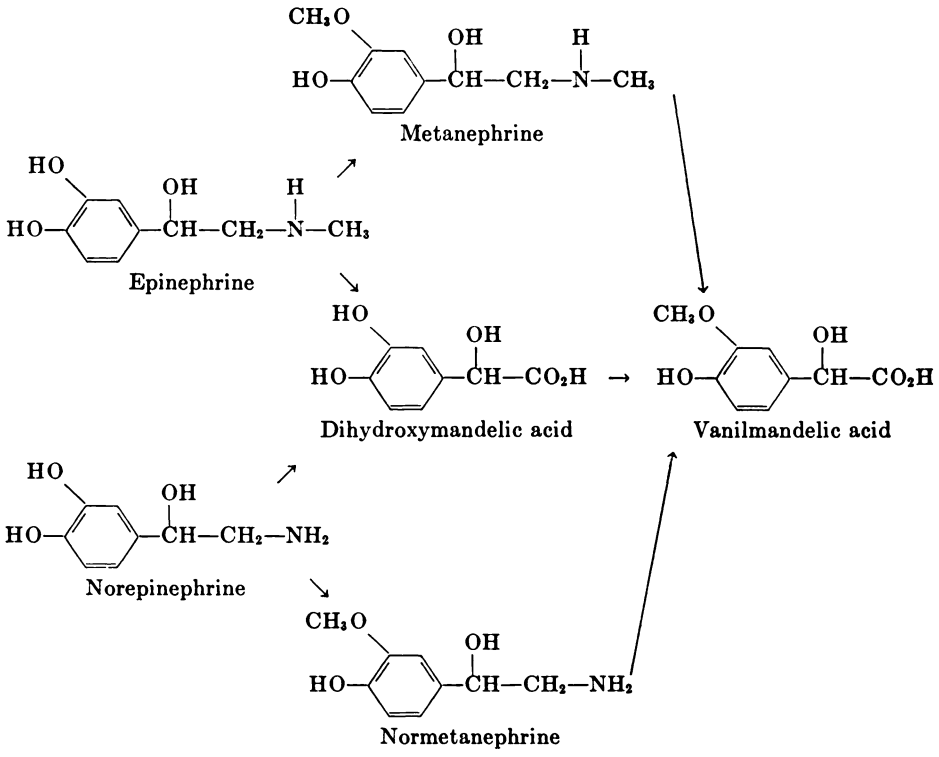

FIG. 1.

new amines, which he has designated metanephrine and normetanephrine, respectively. In addition, these new amines have been found to occur naturally in spleen and adrenal glands of rats (7), and it has been suggested that methylation of epinephrine and norepinephrine prior to oxidative deamination is a principal route for their metabolism. Oxidative deamination must still be considered as a potentially important reaction by which the amines may be converted to physiologically inactive substances, however. After the infusion of carbon-14-labeled epinephrine into humans, Resnick *et al.* (17) and Goodall, Kirshner *et al.* (15, 16) observed in urine a radioactive metabolite which has the properties of DMA. In addition, one of the pheochromocytoma patients reported in Table 1, DG, excreted DMA in addition to the large amount of VMA. Since the proportion of DMA excreted unchanged after ingestion of it is so much smaller than that recovered as VMA, a considerably greater amount of DMA must have been produced in the above cases than was detected. These observations indicate that at least some endogenous norepinephrine and epinephrine undergoes destruction by amine oxidase before methylation occurs. It seems likely that the proportion of the amines degraded by either pathway will probably vary in different tissues, and may even vary from one individual to another in the same site.

In closing, the reactions and compounds shown in Figure 1 outline reactions which now appear to be involved to a major extent in the metabolism of epinephrine and norepinephrine. Further work will be required to delineate more clearly the relative importance of these pathways.

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