AS RECENTLY AS 1929 the first correct preoperative diagnosis of a pheochromocytoma was made and followed by resection. Between 1945 and 1954, the pharmacological tests and the determination of urine catecholamines were described and found to improve the accuracy of preoperative diagnosis of chromaffin tumors significantly. Despite these advances, the majority of such tumors continued to be discovered at autopsy. Even the best of the pharmacological tests, those using histamine and phentolamine, were subject to occasional misleading results and caused discomfort to the patient. Catecholamine determinations offered significantly greater reliability but they required complex equipment and highly trained laboratory personnel. Until recently, none of these tests fulfilled the requirements of general availability, absence of untoward side effects, accuracy, and the economy necessary for a screening procedure.

While systematically studying the excretion of phenolic acids in the urine of human subjects, Armstrong and his co-workers were impressed by the occurrence of O-methylated derivatives of these acids. Amine oxidation of the side chain of epinephrine and norepinephrine, with the formation of a mandelic acid derivative, had been suspected previously (fig. 1). Further modification of this structure by O-methylation of the hydroxyl group in the meta position to the side chain would have been expected to yield 3-methoxy-4-hydroxymandelic acid (VMA, for vanillylmandelic acid). In 1957, Armstrong and his co-workers succeeded in demonstrating that normal human subjects excreted this substance and that patients with pheochromocytomas excreted abnormally large quantities of VMA in their urines. Shortly thereafter Axelrod described the transferase responsible for catechol-O-methylation and suggested that O-methylation preceded amine oxidation of epinephrine and norepinephrine, with the consequent formation of their respective 3-O-methyl congeners, metanephrine and normetanephrine (fig. 1). Methods for the determination of metanephrine, normetanephrine, and their respective conjugates were too unwieldy to be clinically useful for the detection of pheochromocytomas. Moreover, recent studies demonstrated that tritium-labeled VMA (H\textsuperscript{3}-VMA) far exceeded tritium-labeled normetanephrine (H\textsuperscript{3}-NM) excretion after intravenous administration of tritium-labeled norepinephrine (dl-beta-H\textsuperscript{3}-NE).

In 1958, a comparative study of epinephrine, norepinephrine, and VMA excretion in normal human subjects and in those with essential hypertension and pheochromocytomas was initiated. Attempts were made to (a) determine the value of the urine VMA assay for the diagnosis of pheochromocytoma and (b) develop a new and simple technique suitable for the detection of these tumors by routine screening of large numbers of hypertensive subjects. This report is an evaluation of the degree of success attained, as well as a review of the results of such studies performed upon almost 300 hypertensive subjects.

Methods

Semi-quantitative determinations of urinary VMA can be accomplished most simply by bidirectional paper chromatography of ethyl acetate extracts of acidified urine aliquots. It yields a reproducible accuracy of ± 10 per cent and has
DIAGNOSIS OF PHEOCHROMOCYTOMA

GLUCURONIDE AND/OR SULFATE CONJUGATES

NOREPINEPHRINE (NE)

CATECHOL O-METHYL TRANSFERASE

MONAMINE OXIDATION + ALDEHYDE OXIDATION

CH_3 O-< -C-C-N

NORMETANEPHRINE (NM)

MONAMINE OXIDATION + ALDEHYDE OXIDATION

3.4 DIHYDROXYMANDELIC ACID

CATECHOL O-METHYL TRANSFERASE

METANEPHRINE (M)

MONAMINE OXIDATION + ALDEHYDE OXIDATION

GLUCURONIDE AND/OR SULFATE CONJUGATES

3-METHOXY,4-HYDROXY-MANDELIC ACID (VMA)

Figure 1

Metabolic pathways of epinephrine and norepinephrine.

The ratio (R) of D^{430}/D^{350} reflects the VMA concentration in a semiquantitative manner when the patient follows a diet free of coffee, fruit, and vanilla for 48 hours prior to the collection of the urine. Ingestion of coffee gives rise to a urinary product tentatively identified as 3-methoxy, 4-hydroxyphenylhydracrylic acid, which yields a violet color with diazotized p-nitroaniline. This substance, as well as those urinary phenolic acids derived from ingestion of fruit, cake, candy, or ice cream, can give rise to an R value (< 1.30) suggesting the presence of an abnormally large quantity of VMA (> 4 to 5 µg./mg. creatinine). Such false positive results can be accurately evaluated by paper chromatography, but this entails a time-consuming procedure for every patient who fails to follow the prescribed diet.

A recent modification of this colorimetric test discards or destroys enough of the exogenously derived phenolic acids to permit semiquantitative assay of VMA in the urine of a patient who has not observed any dietary restrictions. A volume of urine equivalent to 1.0 mg. creatinine is acidified with hydrochloric acid to a pH of 2 and placed in boiling water for 10 minutes. The pH is then adjusted to exactly 4.0 by means of a 0.5 N acetate buffer, and the sample is extracted three times with ethyl acetate. The latter is discarded, the sample is acidified with hydrochloric

a sensitivity of 0.2 to 0.3 µg. The recovery of VMA added to urine is 90 per cent. Routine aliquots are urine volumes equivalent to 0.5 mg. creatinine. For greater accuracy, samples with high VMA content are re-assayed using urine volumes equivalent to 0.25 mg. creatinine or less. Under ordinary circumstances, any random urine specimen will suffice for a VMA determination by this technique. No immediate acidification or re-frigeration of the urine specimen is necessary, and urine VMA is stable at 10 C. for months or years.

Since the chromatographic technique is too cumbersome for routine screening of hypertensive patients for the detection of chromaffin tumors, it is preferable to use a simple colorimetric procedure, which was developed for this purpose in 1959.

A volume of urine equivalent to 0.5 mg. creatinine is acidified and extracted with ethyl acetate. The extract containing the phenolic acids is reduced to dryness, taken up in dilute K_2CO_3, and coupled with diazotized p-nitroaniline. The violet AZO-VMA* thus formed is extracted into n-amyl alcohol and the densities at 450 and 550 mÅ are determined with a Beckman DU spectrophotometer.

At the suggestion of Dr. A. Smith, the formula previously depicted for AZO-VMA was found to be in error. Studies with beta-H^-VMA reveal that the side chain of VMA is lost and that the coupling likely takes place at this point.

Circulation Research, Volume IX, May 1961
Table 1
Preoperative Studies on 41 Patients with Pheochromocytomas

<table>
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<tr>
<th>No.</th>
<th>µg VMA/mg. creatinine*</th>
<th>R value with single extraction</th>
<th>R value with double extraction</th>
<th>µg. VMA/24 hrs.</th>
<th>µg. NE/24 hrs.</th>
<th>µg. E/24 hrs.</th>
<th>µg. NE/mg. creatinine</th>
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* Determined by paper chromatography
Table 2

VMA Excretion of Normal Subjects and Patients with Pheochromocytomas Determined by Bidirectional Paper Chromatography

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<tr>
<th>Investigators</th>
<th>Normal</th>
<th>Urinary VMA excretion</th>
<th>Phaeochromocytoma</th>
<th>No. subjects</th>
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<td>1. Armstrong, M.D., McMillan, A., and Shaw, Kin.F.</td>
<td>0.5 ± 0.16 mg./24 hours; 1.5—3.0 µg./mg. of creatinine</td>
<td>9.5—17.0 µg./24 hours</td>
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<td>2. Kraupp, O., Stannam, H., Bernheimer, H., and Obermaus, H.</td>
<td>0.5—3.5 µg./mg. of creatinine</td>
<td>9—90 µg./mg. of creatinine; mean = 28 µg./mg. of creatinine</td>
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<tr>
<td>3. Armstrong, M.D., and McMillan, A.</td>
<td>1—3 µg./mg. of creatinine</td>
<td>7.5—40 µg./mg. of creatinine; 4.4—185 µg./24 hours</td>
<td>24</td>
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<td>4. Gitlow, S.E., Khassis, S., Cohen, G., and Mendelowitz, M.</td>
<td>1.0—2.5 µg./minute; 1.4—2.6 mg./24 hours</td>
<td>7.5—11.0 µg./minute</td>
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<td>5. Robinson, R., Ratcliffe, J., and Smith, P.</td>
<td>0.5—3.5 µg./mg. of creatinine</td>
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<td>6. Kiernar, A.A.</td>
<td>&lt;4.0 µg./mg. of creatinine</td>
<td>5.0—62.0 µg./mg. of creatinine; mean = 23.3 µg./mg. of creatinine; 3.1—80.7 mg./24 hours</td>
<td>6</td>
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</table>

Results

The paper chromatographic technique reveals that normal subjects as well as those with primary hypertension excrete between 0.5 and 4.0 µg VMA/mg. of creatinine (about 2 to 3 mg VMA/day). Epinephrine and norepinephrine excretion average 0.002 and 0.013 µg./mg. creatinine respectively in these groups. Their mean VMA excretion exceeds the mean catecholamine excretion by 100-fold. Those subjects who observe the dietary restrictions are found by the simplified screening test to have R values in the range of 1.42 to 2.25. Practical experience with this test, however, demonstrated that approximately 10 per cent of patients failed to observe the diet. Their R values were therefore less than 1.30, necessitating paper chromatography in order to demonstrate that their VMA excretion was indeed normal. Of 14 such patients, only one had an R value less than 1.30 when tested by the modification of the screening test in which the additional extraction at pH
was employed (hereafter referred to as the double extraction technique). Among approximately 300 urine samples, the screening test never failed to demonstrate an R value less than 1.30 when more than 4 to 5 µg. VMA/mg. creatinine was present. In this same group, the double extraction method revealed only a single instance of an R value less than 1.30 associated with a normal VMA excretion. The range of R values by either of the screening methods was found to be the same for normal subjects as for those with primary hypertension.

Table 3 presents the paper chromatographic VMA, catecholamine, and VMA screening-test results of 41 patients prior to the removal of the pheochromocytoma. The mean VMA excretion for these patients is 16.5 µg./mg. creatinine (range, 5 to 40 µg. VMA/mg. creatinine). The R values for either the single or double extraction screening test are less than 1.16 in all but one case. Because of the relatively low VMA excretion of subject 41, five preoperative urine samples were studied. One of these revealed an R value greater than 1.30, the only known instance in this series of a normal VMA excretion in the presence of a pheochromocytoma. On the other hand, six subjects (nos. 14, 17, 18, 25, 27, and 42) had normal or near-normal catecholamine excretion in the face of elevated VMA excretion. Although the double extraction test is more sensitive and the double extraction technique more specific than the single extraction test, the double extraction technique is not necessary when the single extraction test is negative. The VMA excretion of patients prior to the removal of the pheochromocytoma was 16.5 µg./mg. creatinine (range, 5 to 40 µg. VMA/mg. creatinine). The R values for either the single or double extraction screening test were less than 1.16 in all but one case. Because of the relatively low VMA excretion of subject 41, five preoperative urine samples were studied. One of these revealed an R value greater than 1.30, the only known instance in this series of a normal VMA excretion in the presence of a pheochromocytoma. On the other hand, six subjects (nos. 14, 17, 18, 25, 27, and 42) had normal or near-normal catecholamine excretion in the face of elevated VMA excretion. Although the double extraction test is more sensitive and the double extraction technique more specific than the single extraction test, the double extraction technique is not necessary when the single extraction test is negative. The VMA excretion of patients prior to the removal of the pheochromocytoma was 16.5 µg./mg. creatinine (range, 5 to 40 µg. VMA/mg. creatinine). The R values for either the single or double extraction screening test were less than 1.16 in all but one case. Because of the relatively low VMA excretion of subject 41, five preoperative urine samples were studied. One of these revealed an R value greater than 1.30, the only known instance in this series of a normal VMA excretion in the presence of a pheochromocytoma. On the other hand, six subjects (nos. 14, 17, 18, 25, 27, and 42) had normal or near-normal catecholamine excretion in the face of elevated VMA excretion. Although the double extraction test is more sensitive and the double extraction technique more specific than the single extraction test, the double extraction technique is not necessary when the single extraction test is negative. The VMA excretion of patients prior to the removal of the pheochromocytoma was 16.5 µg./mg. creatinine (range, 5 to 40 µg. VMA/mg. creatinine). The R values for either the single or double extraction screening test were less than 1.16 in all but one case. Because of the relatively low VMA excretion of subject 41, five preoperative urine samples were studied. One of these revealed an R value greater than 1.30, the only known instance in this series of a normal VMA excretion in the presence of a pheochromocytoma. On the other hand, six subjects (nos. 14, 17, 18, 25, 27, and 42) had normal or near-normal catecholamine excretion in the face of elevated VMA excretion. Although the double extraction test is more sensitive and the double extraction technique more specific than the single extraction test, the double extraction technique is not necessary when the single extraction test is negative.
atropine, or by the ingestion of coffee, tea, fruit, or vanilla-containing substances. In fact, ingestion of as much as 8 mg. epinephrine fails to raise the urine VMA from the normal to the pheochromocytoma range.

Seriously ill patients with severe pulmonary insufficiency, metastatic tumors, or shock may have an abnormally elevated VMA excretion which overlaps with that quartile of patients with pheochromocytomas who excrete the least VMA. Patients with metastatic carcinoid also demonstrate minimally elevated VMA excretion (about 5 μg. VMA/mg. creatinine). Their high 5-hydroxyindoleacetic acid (5-HIAA) excretion is readily apparent on the paper chromatograms, making this test diagnostically useful for detecting the carcinoid syndrome. The high 5-HIAA excretion may produce a false positive result with the single extraction VMA screening test, but it fails to affect the double extraction test.

None of these patients offered serious diagnostic difficulties insofar as chromaffin tumors were concerned. On the other hand, one young subject suffering from accelerated hypertension with severe hypertensive encephalopathy had elevated VMA excretion after the intravenous administration of phentolamine. Abdominal exploration failed to reveal a pheochromocytoma, and permission for a post-mortem examination was not obtained when he died a few months later. This represents the only subject who was suspected of having a pheochromocytoma, whose VMA excretion was elevated by the chromatographic technique, but who failed to reveal that tumor upon exploration.

Drugs that interfere with the formation or metabolism of the catecholamines, such as monamine oxidase inhibitors, antihypertensive agents, dehydrogenase inhibitors, and insulin, may be expected to modify VMA excretion, but only iproniazid has thus far been reported to diminish VMA excretion to a significant degree.

Discussion

Tables 2 and 3 review the findings of other investigators who have assayed VMA excretion. Those using similar methods are in good agreement, whereas the electrophoretic and column chromatographic procedures yield higher values. None of the tests offers greater reliability or equals the simplicity of the colorimetric techniques used in this study.

Although all of the methods appear to separate the VMA excretion of normal subjects from that of patients with pheochromocytomas, Bollman et al. studied two patients with elevated VMA and normal catecholamine excretion in whom chromaffin tumors were apparently not found. Kraupp et al. described a patient with a pheochromocytoma who excreted abnormally large quantities of VMA and catecholamines in the presence of paroxysmal hypertension but only large amounts of VMA between paroxysms. The intravenous administration of labeled epinephrine or norepinephrine is followed by brief excretion of the unmodified catechol but prolonged excretion of labeled VMA. The normal catecholamine excretion and elevated VMA excretion in a few of our patients with pheochromocytomas may be explained on this basis.

As in the present study, Robinson, Ratcliffe, and Smith found no significant difference in VMA excretion between normal subjects and those with primary hypertension. von Studnitz demonstrated slightly increased VMA excretion associated with the carcinoid syndrome. The same investigator also found that the monoamine oxidase inhibitor, iproniazid, interfered with the formation of VMA. Increased VMA excretion has been described in children with ganglioneuromas or neuroblastomas.

Despite the apparent reliability of the VMA determination for the detection of pheochromocytomas, alternative methods for the diagnosis of this tumor are both desirable and, at times, essential. Nevertheless, neither pharmacological testing nor the catecholamine determination fulfills all of the requirements for a practical screening procedure. Even Hingerty's recent catecholamine screening test, while meeting the need for simplic-
ity, would have missed about 10 per cent of the patients in the series presented here (those excreting < 180 µg catecholamine/24 hours). On the other hand, the simple colorimetric urine test for VMA should facilitate routine screening of hypertensive patients for the detection of pheochromocytomas.

Summary

Methods for the determination of urinary vanillylmandelic acid (VMA) have been reviewed and variations in VMA excretion in different disease states summarized. The determination of VMA excretion is believed to represent a reliable method for the detection of a pheochromocytoma. Colorimetric screening tests for the detection of elevated VMA excretion have been reviewed and evaluated as laboratory aids in the diagnosis of pheochromocytoma.

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**Discussion**

*Dr. Sjoerdsmma:* Examination of the normal excretion pattern of catecholamine metabolites shows that VMA [MOMA] is much the larger in terms of absolute quantities. Since it is the per cent change that is the most useful index in diagnosis, I wonder if you have had more trouble with VMA because of the rather high normal values than you would have had with catecholamines in which a small absolute increase may represent a hundred or more per cent elevation? What is the upper limit of normal values as determined by your assay?

*Dr. Gitlow:* Our normal values all have been between 1 and 4 µg./mg. of creatinine. True, seriously ill patients, such as those with metastatic neoplasms or in shock, can excrete more at times. Occasionally these patients may be the very ones you wish to test. That was the case with the boy who had hypertensive encephalopathy and was in coma at the time the urine specimen was obtained. Whether his increased VMA excretion was due to a functioning tumor or to his moribund condition, I don’t know.

The VMA analysis has accurately detected 41 tumors. Thus far no one has found a chromaffin tumor in any of our patients who had a VMA excretion in the normal range. I imagine it is only a matter of time until that happens. I am sure there are occasions when analyzing for metanephrine or normetanephrine might be of definite assistance. However, the technique of Pisano, which is the only one I am aware of, requires the use of the IRC 50 column, and clinical laboratories are loath to use such techniques. Moreover, this method requires oxidation to vanillin, and I suspect that it measures other substances as well. I wonder whether the vanillin technique for VMA doesn’t measure more than the single phenolic acid also?

*Dr. Helmer:* In the past few years, we have tested about 7,000 hypertensive patients by the aortic strip technique. We have found only 47 cases of pheochromocytoma, so you are catching up with us quickly. With this screening procedure, a determination can be made every five minutes.

I was much interested in your comments about paroxysmal hypertension. Determination of VMA between paroxysms would have a real advantage because the detection of catecholamines generally is limited to the height of the response. Patients with essential hypertension have a lower than normal excretion of catecholamines because these are excreted mainly by the renal tubules and damage to the kidney impairs this excretory mechanism.

*Dr. Gitlow:* We have expressed urinary VMA per milligram of creatinine for just that reason. In the hypertensive group, variation in VMA excretion appears to be slightly greater than normal, but the mean VMA excretion is the same as that of the normal group. In regard to catecholamine determinations as a screening test for pheochromocytoma, I applied Hingerty’s technique to our data and I would say that four or five patients in this group might have been missed because their excretion of catecholamines was almost normal.
Diagnosis of Pheochromocytoma by Assay of Catecholamine Metabolites
STANLEY E. GITLOW, MILTON MENDLOWITZ, ELIZABETH KRUK and SARAH KHASSIS

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