Identification of Vanillic Acid as a Catabolite of Noradrenaline Metabolism in the Human.* (27646)

LAWRENCE ROSEN AND MCC. GOODALL (Introduced by S. Rogers)
Memorial Research Center and Hospital, University of Tennessee, Knoxville

On the basis of elevated urinary levels of vanillic acid (VA) obtained under stress conditions in man, Smith suggested that VA might be an endogenous metabolite of adrenaline and/or noradrenaline(1,2); however, the evidence presented by Smith is not entirely convincing since the source of VA could also be exogenous, *i.e.*, coffee, chocolate, vanilla, etc.(3,4).

The purpose of this paper is to report the isolation of radioactive urinary VA following intravenous infusion of radioactive noradrenaline in man.

Materials. dl-Noradrenaline-2-C^{14} was obtained from Volk Radiochemicals, Skokie, Ill. (specific activity 20 mc/mM); Dowex-1-2X resin (200-400 mesh) from Calbiochem, Los Angeles, (Bio-Rad, AG-1-2X grade). Amberlite IRC-150, XE-64 resin (200-400 mesh) from Rohm and Haas, Philadelphia; vanillic acid from Light and Co., London, England.

Methods. Infusion and collection of urine. Four normal subjects were infused over a one-hour period with 9.5 μc dl-noradrenaline-2-C^{14}; urine was collected for 24 hours post-infusion.

Separation of catabolites and isolation of vanillic acid. The VA present in the urine was separated in 2 ways:

Procedure I. The basic catabolites were removed by passing an aliquot of the 24-hour urine through an Amberlite IRC-50 column. A portion of the combined effluent and water washings (containing approximately 10,000 cpm) was then fractionated on a 1 X 10 cm Dowex-1 acetate column using ammonium acetate buffer, pH 4.8 of varying molarity (0.05, 0.3, 1.0, and 3.0 *M*) as described previously(5,6). Five ml fractions were collected; the radioactivity of each fraction was followed by using an end-flow Geiger tube counter. The radioactive peak which was eluted from the Dowex column at the beginning of the 3 *M* buffer contained VA. The fractions containing VA obtained from each of the 4 subjects were then combined. The combined peaks were acidified (pH 1-2; 6 *N* HCl) and the phenolic acids extracted with ethylacetate (0.5 volume, 5 times). The ethylacetate extract was evaporated under nitrogen to a small volume and then chromatographed on Whatman 3mm paper using *iso*propanol:ammonia:water(8:1:1) as the developing solvent. The radioactivity chromatographed as 2 distinct peaks, the faster of which was VA and the slower was 3,4-dihydroxymandelic acid (DOMA). The radioactive peak containing VA was eluted from the paper with water and concentrated to a small volume and acidified to pH 2. This aqueous solution was then added dropwise to an ethanolic solution of carrier VA. The precipitate was filtered, washed with cold water, and dried to constant weight. The precipitate was recrystallized 2 more times from ethanol-water. The melting points of the recrystallized precipitates and of the starting material were identical, 211-212° (uncorr. MEL-TEMP hot block). Two aliquots from each recrystallization were assayed for radioactivity. These aliquots were assayed in the scintillation spectrometer using a solvent composed of 10 parts toluene (PPO, 4 g/l and POPOP, 0.100 g/l) : 2 parts ethanol.

Procedure II. As for the second method of separating VA, the Amberlite IRC-50 effluent and washings from selected urine samples were acidified (pH 1-2, *N* HCl) and extracted with ethylacetate (0.5 volume, 5 times). The extracts containing VA and other free phenolic acids were evaporated under nitrogen and taken up in water. Carrier VA, DOMA, and 3-methoxy-4-hydroxymandelic acid (MOMA) (0.5 mg each) were added to the aqueous solution. After adjustment of the pH to 6.1 with 0.2 *N* NaOH, the solution was passed through a 1 X 10 cm

* Supported by U.S.P.H.S. grant and U. S. Air Force contract.

Dowex-1 acetate column. The column was washed with 150 ml of water and then attached to a gradient elution system. The elution system was composed of 4 reservoirs connected in series, *i.e.*, 175 ml of water, 175 ml of water, 175 ml of 2 *M* ammonium acetate (pH 4.8), and 175 ml of 6 *M* ammonium acetate (pH 4.8). The order of elution from the Dowex-1 acetate column by this technic is MOMA, DOMA, and VA. Five ml fractions were collected and measured for optical density (279 mμ, Zeiss Spectrophotometer PMQ II), and assayed for radioactivity. The radioactive fractions which were eluted from the Dowex column and contained Carrier VA, were concentrated, acidified, and extracted with ethylacetate. The concentrated extracts were chromatographed (ascending) on Whatman No. 4 paper in either chloroform:glacial acetic acid:water (2:2:1) (Solvent System A) or in benzene:propionic acid:water (10:9:1) (Solvent System B); or on Whatman 3mm paper (descending) in *n*-butanol: glacial acetic acid : water (4:1:1) (Solvent System C). After development, the chromatograms were cut into 1 cm strips and the radioactivity assayed. Chromatograms were assayed by either measuring the activity of an aliquot in the end-flow counter or by immersing the strip directly into a counting vial for use with a scintillation spectrometer (Packard Tri-Carb).

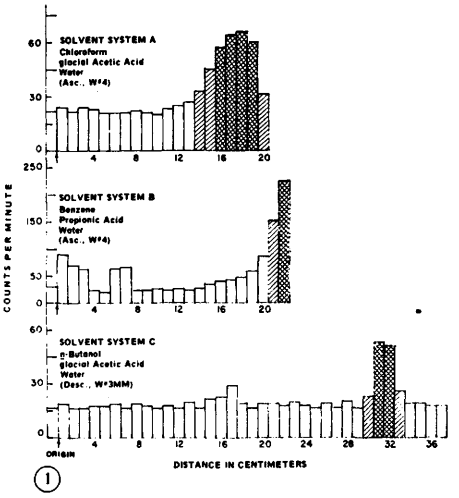
Results. Urine containing 42,000 cpm was placed on 4 Dowex columns, *i.e.*, one for each of the 4 subjects. Results indicate that 1,200 cpm may be accounted for as radioactive VA;

TABLE I. Recrystallization of Carrier VA with the Ethylacetate Soluble Radioactive Fraction Containing VA-C^{14} (Procedure I).

Recryst'n No.	Net c/100 min., mg	S.D.,* %	mg/vial
1	325 ; 315	(2)	22.3, 20.5
2	318 ; 287	(11)	2.9, 3.4
3	292 ; 297	(10)	4.1, 3.2

Total radioactivity used was 42,000 cpm. 400 mg VA used. See text for details.

* Derivation of the standard deviation (S.D.) is given by a sample calculation: Recryst'n 1, 22.3 mg/vial, gross C = 8878 ± 94 c/100 min., background = 1860 ± 43 c/100 min. Net c/100 min./22.3 mg = 7018 ± 137 (2%) or 315 c/100 min./mg.



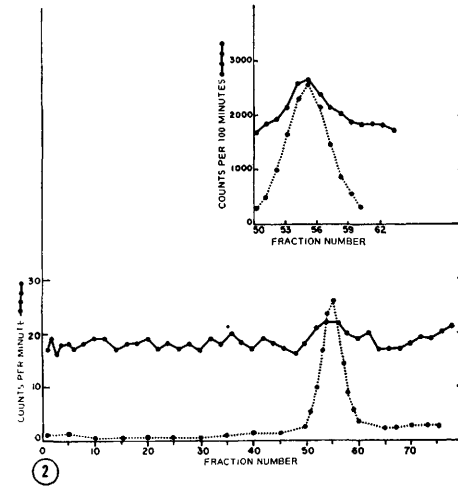


FIG. 1. Paper chromatographic movement of radioactive material eluted from the Dowex column with Carrier VA. The presence of Carrier VA is indicated by hatching (cross-hatching indicates that more Carrier VA is present in that area).

FIG. 2. Elution from a Dowex-1-2X acetate column of the chromatographic peak from Solvent System C (see Fig. 1). A total volume of 400 ml of the gradient system was used and 5 ml fractions were collected. Inset shows results when radioassay of fractions 50 to 63 were made in a Packard Tri-Carb Liquid Scintillation Spectrometer, bg = 1860 ± 43 counts per 100 min.

this was calculated from the data presented in Table I (400 mg × 3 cpm/mg). Thus, VA represents 2.9% of the non-basic catabolites. Since 10% of total radioactivity recovered in the 24-hour period following infusion is composed of basic catabolites(5,6), the final value for vanillic acid accounts for 2.6% of

the radioactivity recovered in the 24-hour period following infusion of the 4 subjects. The amount of VA was not determined for each individual.

After acidification and extraction with ethylacetate of the radioactive material eluted with Carrier VA using Procedure II, it was found that the major portion of this radioactivity co-chromatographed with Carrier VA in the 3 different solvent systems used (Fig. 1). Solvent Systems A and B are extremely useful in separating free phenolic acids such as MOMA, DOMA, and VA since additional hydroxy groups, whether aromatic or aliphatic, markedly slow down the rate of movement(7). The chromatographic peak from System C (Fig. 1), containing Carrier VA, as indicated by optical density, and radioactivity, was collected, adjusted to pH 6.1, placed on a Dowex column and subjected to gradient elution (400 ml total volume; 100 ml per reservoir). The presence of VA-C^{14} is indicated by the elution pattern of the radioactivity and Carrier VA from this column (Figure 2).

Discussion. The ultimate formation of a small amount of VA from noradrenaline involves oxidative degradation of the side chain; therefore, the most likely immediate precursor is MOMA. The recent findings(8) that the perfusion of rat liver with MOMA led to formation of VA supports this concept; however, Pellerin and D'Iorio(7) found that incubation of DOMA with rat liver or kidney slices led to formation of both MOMA and VA; thus, it is possible that DOMA may undergo degradation to 3,4-dihydroxybenzoic acid which could then be methylated to yield VA. Two other compounds formed from noradrenaline(9), 3-methoxy-4-hydroxy-mandelaldehyde, and 3-methoxy-4-hydroxyphenylethylglycol, could also be degraded to VA. The contribution of the various possible pathways leading to VA requires further study.

Summary. VA has been identified as a urinary catabolite of noradrenaline in man. In the 24-hour period following infusion of dl-noradrenaline-2-C^{14} into 4 subjects, the urinary VA accounted for 2.6% of the recovered C^{14}.

1. Smith, P., Bennett, A. M. H., *Nature,* 1958, v181, 709.

2. Hill, G. A., Ratcliffe, J., Smith, P., *ibid.,* 1958, v182, 1160.

3. Booth, H. N., Emerson, O. H., Jones, F. T., DeEds, F., *J. Biol. Chem.,* 1957, v229, 51.

4. Shaw, K. N. F., Trevarthen, J., *Nature,* 1958, v182, 797.

5. Goodall, McC., Kirshner, N., Rosen, L., *J. Clin. Invest.,* 1959, v38, 707.

6. Rosen, L., Goodall, McC., *Am. J. Physiol.,* 1962, v202, 883.

7. Pellerin, J., D'Iorio, A., *Can. J. Biochem. Physiol.,* 1958, v36, 491.

8. Discherl, W., Thomas, H., Schriefers, H., *Acta Endocrin.,* 1962, v39, 385.

9. Axelrod, *J., Physiol. Rev.,* 1959, v39, 751.

Received June 11, 1962. P.S.E.B.M., 1962, v110.

The Price Virus. An Unclassified Enterovirus Isolated from Patients with Central Nervous System Disease.* (27647)

EDWIN H. LENNETTE, NATHALIE J. SCHMIDT, ROBERT L. MAGOFFIN, JUANITA DENNIS AND ANNA WIENER

Viral and Rickettsial Disease Laboratory, California State Department of Public Health, Berkeley

Over the past 5 years viral isolation attempts on stool specimens from patients with symptoms of CNS disease have led to the recovery of 6 immunologically related strains of virus which appear to be distinct from any of the currently recognized enterovirus types. This report describes certain properties of these viruses and presents evidence confirming their human origin.

* This work was supported by a grant from Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S., Dept. of H.E.W.

Materials and methods. Viral isolation attempts. All of the isolates were recovered in