Occurrence of Ethylvanillic Acid in Human Urine and its Metabolism.* (25646)

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Many laboratories are applying technics of paper chromatography to study of compounds in body fluids of patients with various diseases in the hope that new abnormalities of metabolism may be found. A pointed example of the caution which must be exercised in interpreting results of an exploratory chromatographic screening procedure is provided here by the finding that a phenolic acid which had been observed only in urine of severely mentally retarded institutionalized children is not only not of endogenous origin but is not even a naturally occurring substance. The phenolic acid, compound 28(1), was observed in urine of a few severely mentally retarded patients. It was excreted consistently by these, but not by other patients nor by normal persons. Much later, in connection with another problem, the metabolites excreted after ingestion of vanilla flavoring included a material which had the same chromatographic properties as compound 28. These vanilla preparations contained ethylvanillin (3-ethoxy-4-hydroxybenzaldehyde) in addition to vanillin, so ethylvanillic acid (3-ethoxy-4-hydroxybenzoic acid) was prepared; it has the same chromatographic properties as compound 28. The vanilla flavoring used in the kitchen of the institution was a solution of pure ethylvanillin in propylene glycol. The very severely retarded patients, among whom were those who excreted ethylvanillic acid, were maintained on semi-solid diets, which included considerable amounts of milk preparations and puddings, all highly flavored with vanilla. Thus, only a group of severely retarded patients were given enough synthetic flavoring agent to make it a prominent urinary metabolite. After ingestion of artificial vanilla flavoring or ethylvanillic acid another new phenolic acid, in addition to ethylvanillic acid, was found in urine. This material had properties identical with those of synthetic ethylvanilloylglycine. In addition to these 2 substances, a glucuronide of ethylvanillic acid was present.

Methods. Preparation of compounds. 3-Ethoxy-4-acetoxybenzoic acid (m.p. 154-155‡, Lit. 152-153°(2)) and 3-ethoxy-4-hydroxybenzoic acid (ethylvanillic acid) (m.p. 165°, Lit. 164-165°(2)) were prepared as described by King(2). 4.5 g of 3-ethoxy-4-acetoxybenzoic acid and 2.8 g of glycine ethyl ester hydrochloride were coupled by the method of Vaughn and Eichler(3). The resulting crude 3-ethoxy-4-acetoxybenzoylglycine ester was not purified but was saponified without further treatment. The 1.9 g (40% theor.) of crude 3 - ethoxy - 4 - hydroxybenzoylglycine (ethylvanilloylglycine) was recrystallized from 30 ml of hot water to yield 1.7 g of product; white needles, m.p. 161-162°. For analysis the product was again recrystallized from water: m.p. 162-163°. Analysis§ Calculated: for $C_{11}H_{13}O_5N$: C, 55.23%; H, 5.48%; N, 5.85%. Found: C, 54.81%; H, 5.64%; N, 5.71%. For routine screening, urine samples were extracted and the extracts subjected to 2-dimensional paper chromatography as described previously(1). Chromatographic properties of ethylvanillic acid and ethylvanilloylglycine are presented in Table I; properties of vanillic acid are included for comparison. For chromatographic identification of ethylvanillic acid and ethylvanilloylglycine, aliquots of solutions of the authentic compounds were added to extracts of control urine which contained neither, and chromatography was carried out in usual 2-dimension solvent pairs. Location of com-

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‡ Melting points were taken in open capillary tubes and are uncorrected.

§ Analyses made by Weiler and Strauss Microanalytical Lab, Oxford, England.

TABLE I. Chromatographic Properties of Ethylvanillic Acid and Ethylvanilloylglycine.

Compound	R_F Ipr-NH_3	Bz-Prop. acid	aqu. KCl	Bu-Ac. acid	Color reactions Dz SA	Dz PNA	PDAB/Ac_2O
Vanillic acid	.20	.70	.64	.89	Orange	Purple	
Ethylvanillic acid	.32	.84	.64	.93	"	"	
Ethylvanilloylglycine	.23	.28	.71	.83	Pink	"	Greenish-yellow

Solvent systems: Ipr-NH_3, isopropyl alcohol - aqu. ammonia - H_2O, 8:1:1; Bz-Prop. acid, benzene - propionic acid - H_2O, 100:70:5; aqu. KCl, 20% aqu. KCl; Bu-Ac. acid, *n*-butanol - acetic acid - H_2O, 8:2:2.

Reagents: Dz SA, diazotized sulfanilic acid(7); Dz PNA, diazotized *p*-nitroaniline(8); PDAB/Ac_2O, *p*-dimethylaminobenzaldehyde in acetic anhydride(9).

pounds as well as color reactions described in Table I were compared with those on chromatograms prepared from extracts of urine obtained from patients and after ingestion of ethylvanillic acid and artificial vanilla flavoring, respectively. For quantitative estimation of free and conjugated ethylvanillic acid and ethylvanilloylglycine, urine samples were processed as described for determination of dopa metabolites(4). Suitable aliquots of ethyl acetate extracts of urine were applied to paper along with appropriately located graded amounts of authentic compounds. The chromatograms, containing both extract and standard compounds, were developed with usual solvent pairs and sprayed with diazotized *p*-nitroaniline; amount of compound in the extract was estimated by visual comparison of the spot produced by the unknown with the standards. The accuracy of this procedure was at least ± 15%. Completeness of recovery with the extraction procedure was tested by adding known amounts of authentic compounds to urine and determining recovery, which was quantitative for ethylvanillic acid and 85% for ethylvanilloylglycine. The values listed in Table II are corrected for incomplete extraction of ethylvanilloylglycine.

Results. Metabolic exp. Intake of tea, coffee, fruits, vegetables, and flavored beverages was restricted to minimize interference by phenolic acids of dietary origin. 100 mg and 500 mg portions of ethylvanillic acid dissolved in bicarbonate solution were taken by an adult male. Urine was collected for first 8 hours after compound was ingested, then for a second 8 hour period. Examination of second 8 hour collection showed that it contained less than 2% of ethylvanillic acid taken; values reported are only for the first 8 hour period. Amount of free ethylvanillic acid, ethylvanillic acid released by acid hydrolysis, and ethylvanilloylglycine excreted are listed in Table II. The acid labile conjugate was a glucuronide, since amount of ethylvanillic acid released by hydrolysis with β-glucuronidase (Ketodase, Warner-Chilcott) corresponded closely with that released by acid hydrolysis.

Recently, Hill, Ratcliffe, and Smith(5) commented upon the occurrence of ethylvanillic acid along with vanillic acid in urine of persons under stress. Although they acknowledged the fact that ethylvanillin is used as a flavoring agent and gives rise to ethylvanillic acid, they held open the possibility of an endogenous origin of the ethylvanillic acid they detected, based upon the fact that subjects undergoing driving-stress experiments and eating "virtually identical" meals excreted different total amounts and proportions of vanillic and ethylvanillic acids. Our experience on the origin of the 3-hydroxyphenyl acids in urine and a possibility of a relation between

TABLE II. Metabolism of Ethylvanillic Acid.

Ethylvanillic acid ingested (mg)	Ethylvanillic acid excreted: Free (mg)	As glucuronide (mg)	As glycine conjugate (mg)	Total (mg)	Total (% of ingested)
100	13.4	21.	1.5	35.9	35.9
500	129.2	61.	23.8	214.0	42.8

their excretion and mental diseases(6) offer a plausible explanation for the results of Hill, *et al.* Although it was possible to assure reasonably similar intake of meals with patients and control subjects, it was difficult to control intake of beverages and refreshments such as coffee, tea, cola beverages, milk shakes, ice cream, sweets, etc., between meals. These foods, unfortunately, represent a major source of chromogenic phenolic substances in urine. Thus, in the driving-stress experiments, even though consumption of meals was controlled, a variable intake between meals could account for appearance of variable total amounts of vanillic and ethylvanillic acids. The varying proportions might be due to the fact that commercial vanilla preparations contain varying mixtures of vanillin and ethylvanillin, and different materials eaten could have been prepared with different flavoring preparations. In the absence of rigorous experimental proof of a new type of metabolic reaction, biological ethylation, it would seem safer to ascribe the appearance of ethylvanillic acid in urine to intake of ethylvanillin.

Summary. Ethylvanillic acid (3-ethoxy-4-hydroxybenzoic acid) occurs occasionally in human urine. Its presence follows ingestion of ethylvanillin, a component of some artificial vanilla flavorings. After ingestion of ethylvanillin or of ethylvanillic acid, free ethylvanillic acid, a glucuronide conjugate, and a glycine conjugate are excreted.

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Multiplication of Single Mammalian Cells in a Nonbicarbonate Medium. (25647)

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It is standard practice to use a CO_2-bicarbonate system to buffer mammalian cell cultures, even in single cell plating technics where one would expect the other constituents of the medium such as amino acids and serum protein to exert an adequate buffering action. The result of this practice is that cultures must be incubated in a high pCO_2 to control pH. If bicarbonate could be omitted from the medium, incubation could be in air instead of the usual 5% CO_2, and plating out of single cells and manipulation of such cultures would be considerably simplified. This paper describes attempts to attain this objective. It was found that L cells did not form colonies consistently under these conditions unless a Krebs cycle intermediate, oxalacetic acid (OAA), was added to the medium.

Methods. Stock cultures were grown in defined medium CMRL-1066(1) with 20% dialyzed horse serum for the L strain(2) and 20% whole sheep serum for HeLa(3). Both strains were clonal lines. Primary cultures of

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