

- Evans, R. J., Davidson, J. A., Bauer, D. H., Bandemer, S. L., *Poultry Sci.* 37(1), 81 (1958).
- Feeney, R. E., Abplanalp, H. A., Clary, J. J., Edwards, D. L., Clark, J. R., *J. Biol. Chem.* 238, 1732 (1963).
- Feeney, R. E., Clary, J. J., Clark, J. R., *Nature (London)* 201, 192 (1964).
- Gandhi, S. K., Schultz, J. R., Boughey, R. W., Forsythe, R. H., *J. Food Sci.* 33, 163 (1968).
- Kloos, G., Schmidt, I., *Arch. Lebensmittelhyg.* 18(8), 180 (1967).
- Koehler, H. H., Jacobson, M., Agricultural Experiment Station Bulletin 760, Washington State University, Pullman, Wash., 1972.
- Lawrence, J. M., Herrick, H. E., Grant, D. R., *Cereal Chem.* 47, 98 (1970).
- Osuga, D. T., Feeney, R. E., *Arch. Biochem. Biophys.* 124, 560 (1968).
- Radhakrishnamurthy, B., Chapman, K., Berenson, G. S., *Biochim. Biophys. Acta* 75, 276 (1963).
- Weber, L., Osborn, M., *J. Biol. Chem.* 244(16), 4406 (1969).

Received for review July 5, 1972. Resubmitted September 24, 1973. Accepted November 12, 1973. Scientific Paper No. 4000, Washington Agriculture Research Center, College of Agriculture, Pullman, Wash. 99163, project 1807.

## Conjugated Compounds in Cow's Milk. II

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An additional 28 compounds existing in cow's milk as conjugates, presumably detoxification conjugates of glucuronic and sulfuric acid, were identified. The compounds included 13 saturated and unsaturated *n*-aliphatic acids, three aromatic acids, four guaiacols, three catechols, three phe-

nols, and aceto- and propiovanillone. The relative amounts of the compounds are given. A comparison of the amount and nature of compounds found in milk and compounds isolated from urine by other workers is discussed.

In a previous investigation (Brewington *et al.*, 1973) the presence and identification in cow's milk of numerous compounds existing as detoxification products of glucuronic and sulfuric acid were demonstrated. This report is a continuation of that work.

### EXPERIMENTAL SECTION

**Isolation and Enzymatic Hydrolysis of Conjugates.** The procedures used were the same as previously described (Brewington *et al.*, 1973), with the exception that double volumes of redistilled methylene chloride were used for extractions.

**Fractionation of Free Compounds.** The methylene chloride solution of free compounds, obtained after enzymatic hydrolysis, was evaporated on a steam bath under nitrogen to approximately 1 ml and treated at room temperature overnight with 5 ml of a 1.5% solution of sulfuric acid in methanol. Five milliliters of H<sub>2</sub>O then were added, and the phenolic compounds and methyl esters formed were extracted with 2 × 20 ml of methylene chloride. The phenolic compounds were separated from the methyl esters by extraction in the cold with 2 × 30 ml of a 5% NaOH solution, which was acidified to pH 1 with concentrated HCl and extracted with 3 × 120 ml of methylene chloride. Both methylene chloride solutions then were dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to a small volume suitable for analysis.

**Gas Chromatographic and Mass Spectrometric Methods.** The five columns employed for the gas chromatographic analysis of the two methylene chloride fractions obtained are listed in Table I. Column E was programmed from 80 to 190° at 2.5°/min; column C from 50 to 220° at 4°/min; column S from 75 to 220° at 4°/min; column X from 70 to 150° at 4°/min; and column O from 70 to 200° at 5°/min. The instrument used was the Perkin-Elmer 900, equipped with a flame ionization detector and having an injection port and detector temperature of 230°. Certain areas were trapped, as before, and reinjected into a LKB 9000 combination gas-liquid chromatograph-mass

spectrometer using the same columns. The operating conditions of the mass spectrometer were the same as previously described (Brewington *et al.*, 1973).

**Reference Compounds.** The compounds were identified by comparison with the mass spectra and retention times of authentic compounds. 4-Methylguaiacol, 4-ethylguaiacol, and 4-vinylguaiacol were kindly supplied by Dr. Aaron Wasserman, Eastern Regional Research Center, Wyndmoor, Pa. 3-*n*-Propylphenol was prepared by reduction of isosafrole with sodium in ethanol (Cousin and Lions, 1937). Propiovanillone was synthesized by oxidation of 4-hydroxy-3-methoxyphenyl-1-propanol (Pearl, 1956). 4-Allylphenol was made by alkylation of phenol with allyl chloride in the presence of ZnCl<sub>2</sub> (Buu-Hoi *et al.*, 1954). Fries rearrangement of catechol diacetate and subsequent reduction with zinc amalgam of the 4-acetylcatechol formed gave 4-ethylcatechol (Miller *et al.*, 1938). All other compounds were purchased commercially.

### RESULTS AND DISCUSSION

Table II lists all compounds found to date, the relative amount of each found in the methylene chloride extract and the column(s) used in their isolation and identification. The Carbowax column was the most versatile for the array of phenols present and was particularly useful for the catechol compounds. The EGSS-X and EGA columns were satisfactory for methyl esters. The EGA gave better resolution for the short-chain methyl esters, whereas the EGSS-X column was more suitable for the long-chain methyl esters.

Table III gives mass spectral peaks most useful in diagnosis of those compounds whose spectra are not published or easily located.

A comparison of the amounts of conjugated compounds found in milk by us and in urine (Suemitsu *et al.*, 1970) leads to the conclusion that urine is the preferred medium for elimination of such compounds. However, a comparison of the nature of the compounds present and their relative amounts reveals many differences. One important difference is the absence in urine of aliphatic acids, which occur in appreciable quantities in cow's milk. Milk, on the other hand, contained only the aromatic acids benzoic, phenylacetic, and hippuric, whereas Suemitsu *et al.* (1970) found a host of methoxy and hydroxy derivatives of

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**Table I. Glc Columns<sup>a</sup> Employed**

E.	5 ft × 1/8 in. (o.d.) 7.5% ethylene glycol adipate (EGA) and 2% H <sub>3</sub> PO <sub>4</sub> on 90–100 mesh Anakrom ABS
C.	5 ft × 1/8 in. (o.d.) 1% Carbowax 20M on 60–80 mesh Gas Chrom Q
S.	4 ft × 1/8 in. (o.d.) 20% SE-30 on 60–80 mesh Chromosorb W (AW-DMCS)
X.	5 ft × 1/8 in. (o.d.) 15% EGSS-X on 100–120 mesh Gas Chrom P
O.	8 ft × 1/8 in. (o.d.) 3% OV-1 on 100–120 mesh Chromosorb Z

<sup>a</sup> All columns were stainless steel and silanized.

**Table II. Compounds Identified as Conjugates in Cow's Milk**

Compound	Relative amount <sup>a</sup>	Glc column(s) used in isolation
Phenol <sup>b</sup>	m	E, C, S, O
<i>p</i> -Cresol	vl	E, C, S, O
4-Ethylphenol	vl	E, C, S, O
3- <i>n</i> -Propylphenol <sup>b</sup>	l	E, C, S, O
4-Allylphenol <sup>b</sup>	vs	E, C
Catechol <sup>b</sup>	vs	C
4-Methylcatechol <sup>b</sup>	s	C
4-Ethylcatechol <sup>b</sup>	m	C
Guaiacol <sup>b</sup>	vs	C
4-Methylguaiacol <sup>b</sup>	vs	C, O
4-Ethylguaiacol <sup>b</sup>	s	C, O
4-Vinylguaiacol <sup>b</sup>	vs	C, O
Vanillin	vs	E
Acetovanillone <sup>b</sup>	m	E, C, S, O
Propiovanillone <sup>b</sup>	s	E, C, S, O
Methyl vanillate	vs	E, S
Ethyl vanillate	vs	E, S
<i>p</i> -Hydroxyacetophenone	m	E, O
δ-Decalactone	vs	E
γ-Dodecalactone	vs	E
δ-Dodecalactone	vs	E
Indole	vs	C, E, O
Benzoic acid <sup>b</sup>	s	E, X
Phenylacetic acid <sup>b</sup>	l	E, X
Hippuric acid <sup>b</sup>	vl	E
Butyric acid <sup>b</sup>	s	E
Caproic acid <sup>b</sup>	s	E
Caprylic acid	l	E, X
Nonanoic acid <sup>b</sup>	vs	E, X
Capric acid	m	E, X
Undecanoic acid <sup>b</sup>	vs	E, X
Lauric acid	s	E, X
Tridecanoic acid <sup>b</sup>	vs	E, X
Myristic acid <sup>b</sup>	m	E, X
Pentadecanoic acid <sup>b</sup>	vs	E, X
Palmitic acid	l	E, X
Palmitoleic acid <sup>b</sup>	m	X
Heptadecanoic acid <sup>b</sup>	vs	X
Stearic acid <sup>b</sup>	s	X
Oleic acid <sup>b</sup>	m	X
Linoleic acid <sup>b</sup>	s	X
Linolenic acid <sup>b</sup>	vs	X

<sup>a</sup> vl, very large; l, large; m, medium; s, small; vs, very small. <sup>b</sup> Compounds identified in present work.

benzoic, phenylacetic, and phenylpropionic acids in urine. Hippuric acid, though not found in urine, was present in overwhelming quantities in milk, much more than *p*-cresol. Another important difference was the presence in urine of only a few phenols, namely, phenol, *p*-cresol, guaiacol, *p*-ethylphenol, 3-propylphenol, and catechol. Catechol in particular was present in large quantities in

**Table III. Mass Spectra of Compounds Identified**

Compound	Mass spectrum, <i>m/e</i> (relative intensity)
3- <i>n</i> -Propylphenol	136 (M <sup>+</sup> ) (35), 121 (14), 108 (45), 107 (100), 94 (8), 91 (9), 79(10), 77 (25)
4-Allylphenol	134 (M <sup>+</sup> ) (100), 133 (71), 119 (16), 117 (18), 115 (19), 107 (51), 105 (32), 103 (11), 91 (21), 79 (18), 78 (15), 77 (39), 65 (11)
4-Ethylcatechol	138 (M <sup>+</sup> ) (43), 123 (100), 91 (15), 77 (17), 65 (11), 51 (13), 39 (13)
4-Methylguaiacol	138 (M <sup>+</sup> ) (100), 123 (93), 105 (7), 95 (34), 77 (20), 67 (19), 55 (17)
4-Vinylguaiacol	150 (M <sup>+</sup> ) (100), 135 (86), 107 (38), 79 (12), 78 (10), 77 (31), 53 (11), 51 (13), 43 (28)
Acetovanillone	166 (M <sup>+</sup> ) (46), 152 (11), 151 (100), 123 (26), 108 (11), 77 (9), 65 (10)
Propiovanillone	180 (M <sup>+</sup> ) (23), 152 (10), 151 (100), 123 (19), 108 (9), 77 (6), 65 (7)

urine, but only in trace amounts in milk. The reasons for these differences are beyond speculation except that different mechanisms are involved in the elimination of compounds into urine and milk.

With the exception of phenol, *p*-cresol, vanillin, and guaiacol, none of the phenolic compounds identified have been found in milk systems before. Most of the phenolic compounds found are degradation products of plant lignin (Pearl, 1967) and this is their most probable source. Cobb *et al.* (1963) and Scanlan *et al.* (1968) have suggested that vanillin, which is an important flavor compound in heated milks, is formed by the action of heat on precursors arising from the degradation of lignin. The fact that vanillin was found as a conjugate indicates that vanillin is formed before any heat treatment. However, it may be that more than one mechanism is involved in the formation of vanillin as well as in the formation of other compounds. If the possible precursors of vanillin could be found free in milk, additional merit could be given their suggestion. Likewise, if many of the phenols identified as conjugates could be found free, more plausibility could be given to our speculation that these compounds may also originate from the action of enzymes. It is doubtful, though, that enzymatic hydrolysis of the vanillin conjugate, which apparently occurs in a very small amount, would release enough vanillin to cause any significant flavor change.

## LITERATURE CITED

- Brewington, C. R., Parks, O. W., Schwartz, D. P., *J. Agr. Food Chem.* **21**(1), 38 (1973).
- Buu-Hoi, N., Eckert, B., Demerseman, P., *J. Org. Chem.* **19**, 726 (1954).
- Cobb, W. Y., Patton, S., Grill, H., *J. Dairy Sci.* **46**(6), 566 (1963).
- Cousin, S. G., Lions, F. J., *Proc. Roy. Soc. N. S. Wales* **70**, 413 (1937).
- Miller, E., Hartung, W. H., Rock, H. J., Crossley, F. S., *J. Amer. Chem. Soc.* **60**, 7 (1938).
- Pearl, I. A., *J. Amer. Chem. Soc.* **78**, 4433 (1956).
- Pearl, I. A., "Chemistry of Lignin," Marcel Dekker, New York, N. Y., 1967.
- Scanlan, R. A., Lindsay, R. C., Libbey, L. M., Day, E. A., *J. Dairy Sci.* **51**(17), 1001 (1968).
- Suemitsu, R., Fujita, S., Yoshimura, M., Gen, H., Yuasa, A., Ushijima, J., *Agr. Biol. Chem.* **34**(6), 957 (1970).

Received for review July 17, 1973. Accepted October 15, 1973. Mention of brand or firm names does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.