Thomas, Springeld, Ill., 2nd ed., 1951.

3. Harris, J. O., *J. Bact.*, 1948, v56, 271.

4. Follis, R. H., Burnett, J. M., Lasheuer, Z. E., *Bull. Johns Hopkins Hosp.*, 1952, v91, 463.

5. Sevag, M. G., Miller, Ruth E., *J. Bact.*, 1948, v55, 381.

6. Vargues, R., Grenier, B., Lortholary, J., *C. R. Soc. Biol.*, 1961, v155, 1379.

7. Vargues, R., Grenier, B., Moraud, B., *Ann. Inst. Pasteur*, 1961, v101, 56.

8. Husain, S. S., Burrows, W., *J. Infect. Dis.*, 1956, v99, 90.

9. Treffers, H. P., Yaw, K. E., *Fed. Proc.*, 1948, v7, 311.

10. Muschel, L. H., Treffers, H. P., *J. Immunol.*, 1956, v76, 1.

11. Muschel, L. H., Carey, W. E., Baron, L. S., *ibid.*, 1959, v82, 38.

12. Michael, J. G., Braun, W., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 422.

13. Wardlaw, A. C., *J. Exp. Med.*, 1962, v115, 1231.

14. Muschel, L. H., *Fed. Proc.*, 1963, v22, 673.

15. Brenner, S., *et al.*, *Nature*, 1958, v181, 1713.

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Free Amino Acids in Milk.* (28460)

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Although a large body of data is available on the total amino acid content of milk, there is little information about the free amino acids. Investigations on amino acid excretion of infants showed that at least one, later shown to be homocitrulline(1,2), must originate in milk, and it seemed possible that other unusual amino acids might be present in small amounts, either free or in proteins. It was, therefore, of interest to examine the free amino acids in milk for 2 reasons: first, to see if there are any new or unique components and, second, because some information might be gained about the intracellular environment of milk-secreting cells. This note reports values for the concentration of free amino acids in milk from 3 healthy, nursing mothers under fasting conditions, with concomitant measurements of the amino acids in serum and urine collected at the same time. Values obtained with cow's milk are also included.

Experimental procedures. Three mothers were used as subjects; samples were collected from subject A 4 weeks, and from K and S 3 months after delivery. They had not eaten for 14 hours and their infants had not nursed for 12 hours before the samples were taken. Milk was expressed manually and approximately 30 ml was obtained from each breast. The milk was centrifuged immediately, the lipid layer was removed and discarded, and a protein-free filtrate was prepared with an ARAFLO (Applied Research Corporation, N. Y.) cell using nitrogen pressure. One drop of 3 *N* HCl was added to a 10 ml sample of the filtrate which was then concentrated *in vacuo* at room temperature to a volume of about 1 ml. This was made up to a volume of exactly 5.0 ml by addition of pH 2.2 citrate buffer, 0.2 *N* in Na^+ and stored at −15° until the analyses were made. Blood was drawn from the antecubital vein, allowed to clot at room temperature for 7 hours, and a serum filtrate was prepared for analysis in the manner described for milk. The subjects voided when they arrived at the laboratory, and a urine sample was collected after the blood was taken. The creatinine content was determined and the urine was stored at −15° until the analyses were made.

Milk was collected directly from Jersey cows, with no attempt to regulate time of collection, centrifuged just after collection to separate lipids, and filtrates were prepared immediately. In addition to pressure filtration, sulfosalicylic acid filtrates were prepared by a modification of the method of Hamilton (3).

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Amino acid analyses were made with a Beckman/Spinco model 120 amino acid analyzer using the conditions described by Spackman *et al.*(4). For analysis, 4 ml loads of milk and serum filtrates from the women, and amounts of urine which contained 2 mg of creatinine were used. A 10 ml load of filtrate from cow's milk was used. The results of the analyses are presented in Table I.

Discussion. Because of the wide variation of the amounts of individual amino acids present in the different samples of milk and the small number of subjects, the values for each subject are listed, rather than an average. Factors which influence the variations in the free amino acids in milk from the same or different individuals provide an obvious subject for research on milk formation. Human milk contains a higher level of most of the common free amino acids than does cow's milk. An exception is that cow's milk has much more glycerophosphoethanolamine, phosphoethanolamine, and phosphoserine. Values are not listed in Table I for glycerophosphoethanolamine and phosphoserine because they were not well separated with the conditions used, and a standard sample of glycerophosphoethanolamine was not available. It could be judged from the analyzer record, however, that cow's milk contained 2 to 4 times more of these components than did human milk, perhaps because the dairy cows have been bred selectively to produce milk with increased amounts of lipids and casein. The phospho compounds are of importance for the synthesis of both substances.

A distinctive feature in the occurrence of the common amino acids is the high level of glutamic acid. It is improbable that a significant proportion of this glutamic acid was formed by hydrolysis of glutamine after the milk was collected, because of the rapidity with which samples were prepared for analysis. Also, as shown by the values for the ratio of concentration of amino acids in milk to those in serum, glutamic acid is markedly concentrated in milk. The presence of a high level of free glutamic acid in milk might well be beneficial, since glutamic acid occupies a key position in the metabolism of amino acids and provides a source of α-ketoglutaric acid for the tricarboxylic acid cycle. Free glutamic acid would be especially useful in milk, since digestive enzymes are not fully developed during early life, and an infant might not have a ready supply of this important metabolite.

Since the mammary gland is a modified sweat gland and citrulline has been reported to occur in large amounts in sweat(5), the possibility that citrulline and also homocitrulline might occur in milk seemed likely. Citrulline has been reported also to be a component of hair follicle protein(6). Deutsch and Samuelsson(7) reported 1.3 mg per 100 ml of free citrulline in cow's milk, but in the present work citrulline could not be detected in human milk, and only a trace, less than .015 mg per 100 ml, was present in cow's milk. Deutsch and Samuelsson used dialysis to prepare their milk for analysis, and it is possible that some bacterial contamination might have been responsible for the presence of citrulline in their preparation. Alternatively, a bacterial infection in the udders of the cows could have led to its presence, or a species difference could be responsible. In any event, the virtual absence of free citrulline in the samples of human and cow's milk reported on here is certain. This result suggests that the origin of the citrulline in sweat might be examined under conditions whereby the action of microorganisms actually living in skin could be eliminated as a possible source for it. Free homocitrulline could not be detected in either human or cow's milk, and all the observations made in this laboratory are in agreement with the finding of Gerritson, *et al.*(2) that it is an artifact formed during the processing of milk.

Because sweat contains a very high concentration of arginine and a high concentration of histidine(8), some concern was felt because the basic amino acids were not present in high concentration in the ultrafiltrates used here, and the possibility was considered that the basic amino acids might have been bound by the phosphoproteins in milk so that they did not appear in the ultrafiltrate. To test this possibility, a sulfosalicylic acid filtrate of milk was prepared(3). The presence of sulfosalicylic acid should free any

TABLE I. Free Amino Acids in Milk, Serum, and Urine.

Amino acid	Milk (mg/100 ml)			Serum (mg/100 ml)			Urine (mg/g creatinine)			Conc in milk / Conc in serum			Cow's milk (mg/100 ml)			
	K	A	S	K	A	S	K	A	S	K	A	S	Lit.(7)	1	2	3
Phosphoethanolamine	.95	.53	.14	.07	.02	.07	7.98	32.4	—	13	27	2.0	8.3	.72	3.3	—
Taurine	4.10	6.10	2.44	2.66	2.25	2.65	83.8	3.75	100	1.55	2.70	.92		.33	3.5	.69
Urea	36.5	26.7	52.2	28.7	29.7	56.5	12,080	9,200	10,700	1.27	.92	.92	31.0	35.0	52.0	22.5
Hydroxyproline	.03	.13	.01	.06	.20	.16	0	0	0	.50	.65	.06		0	.01	0
Aspartic acid	.43	.73	.30	.53	.36	.53	—	—	—	.81	2.03	.57	.29	—	.39	.07
Threonine	.77	1.07	.36	1.45	2.05	1.73	11.9	14.3	11.9	.53	.52	.21	.10	.08	.09	.08
Serine	1.21	1.00	.37	2.29	2.13	1.79	29.4	29.4	15.8	.53	.47	.21	.22	.08	.16	.07
Glutamine + asparagine	6.65	4.97	.69	6.27	7.43	7.74	45.3	43.8	42.4	1.06	.67	.09	.13*	.32	.53	.24
Proline	.29	.23	.18	1.78	1.64	2.59	0	0	0	.16	.14	.07	.54	.25	.28	.26
Glutamic acid	16.9	18.4	13.8	1.65	1.80	2.13	2.94	2.94	5.88	10.2	10.2	6.50	3.2	.42	2.8	1.0
Citrulline	0	0	0	.57	.56	.31	—	—	—	—	—	—	1.3	0	tr	tr
Glycine	.86	.66	.24	2.61	3.27	2.70	71.3	37.6	49.6	.33	.20	.09		.21	1.53	.45
Alanine	1.09	1.85	.62	2.94	3.65	3.43	6.24	13.4	14.2	.37	.50	.18	.29	.17	.23	.14
α-Aminobutyric acid	.23	.18	.05	.33	.26	.26	1.03	1.03	2.06	.89	.69	.19		.02	.04	.01
Valine	.53	.53	.20	2.43	2.46	3.20	2.34	2.34	4.68	.22	.22	.06	.15	.06	.06	.07
Cystine	.57	.15	.33	.30	.45	.39	2.40	4.81	—	1.90	.33	.85		0	.03	0
Methionine	.07	.04	.04	.37	.37	.37	—	—	—	.19	.11	.11		.03	.05	.04
Isoleucine	.13	.10	.06	.69	.69	.72	—	—	—	.19	.15	.08	.09	.02	.04	.04
Leucine	.36	.30	.10	1.55	1.44	1.55	2.62	—	—	.24	.21	.06	.09	.03	.03	.06
Tyrosine	.23	.23	.14	.77	1.31	1.04	7.25	10.9	3.62	.30	.18	.13		.03	.01	.05
Phenylalanine	.16	.12	.08	.83	.87	.91	4.96	3.30	3.30	.19	.14	.09		.01	.02	.03
β-Aminoisobutyric acid	0	0	0	.05	.05	.02	144	52.6	3.09	—	—	—		0	0	0
Ornithine	.03	.03	.03	1.32	1.85	1.32	1.32	1.32	1.32	.02	.02	.02	.31	.04	.03	.06
Ethanolamine	.46	.52	.53	.08	.08	.06	28.1	22.6	17.7	5.7	6.5	8.9		—	.05	.39
Lysine	.33	.26	.18	2.95	2.74	2.89	11.7	11.7	16.1	.11	.10	.06	.81	.55	.30 (.18)†	.50 (.36)†
1-Methylhistidine	0	0	0	0	.08	0	8.46	49.1	15.2	—	—	—		0	0	0
Histidine	.35	.37	.08	1.32	1.24	1.36	82.2	41.9	18.5	.26	.30	.06	.55	—	tr (.07)†	tr (.08)†
3-Methylhistidine	0	0	0	.04	.04	.08	32.1	23.7	23.7	—	—	—		0	0	0
Arginine	.17	.13	.17	1.09	1.22	1.13	1.74	5.23	1.74	.16	.11	.15	.45	.20	.14	.19

* Asparagine only.

† Amount present in sulfosalicylic acid filtrate.

amino acids which might be bound in this fashion. A filtrate prepared in this manner did not contain an increased amount of any of the amino acids in human milk or of arginine in cow's milk. Histidine, however, must be present in a bound form in cow's milk, since only a trace could be detected in the ultrafiltrate, but an amount comparable to that of many of the other amino acids was present in the sulfosalicylic acid filtrate.

No unexpected components could be detected among the ninhydrin-positive substances of human milk. The samples of cow's milk, however, contained 3 unidentified materials: with the system of Spackman, Moore and Stein(4) all were detected in the eluate from the 50 cm column. One is a minor component which emerges 7 ml before phenylalanine and tyrosine, the major substance emerges 18 ml before ornithine, and the remaining one is eluted with lysine. This last material is detectable because it alters the usual ratio of 570 mμ to 440 mμ absorption given by lysine, and is not present in the sulfosalicylic acid filtrate.

Summary. The concentrations of the free amino acids in human and cow's milk are reported.

1. Gerritsen, T., Waisman, H. A., Lipton, S. H., Strong, F. M., *Arch. Biochem. Biophys.*, 1962, v97, 34.

2. Gerritsen, T., Vaughn, J. G., Waisman, H. A., *ibid.*, 1963, v100, 298.

3. Hamilton, P. B., *Ann. N. Y. Acad. Sci.*, 1962, v102, 55.

4. Spackman, D. H., Stein, W. H., Moore, S., *Anal. Chem.*, 1958, v30, 1190.

5. Ghadimi, H., Stern, M., Shwachman, H., *A.M.A. J. Dis. Child.*, 1960, v99, 333.

6. Rogers, G. E., *Biochim. Biophys. Acta*, 1958, v29, 33.

7. Deutsch, A., Samuelsson, E.-G., *Intern. Dairy Congr., Proc., 15th Congr., London*, 1959, v3, 1650.

8. Hier, S. W., Cornbleet, T., Bergeim, O., *J. Biol. Chem.*, 1946, v166, 327.

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Hypoglycemic Activity of 3,5 Dimethylisoxazole. (28461)

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Although numerous chemicals and extracts of biological materials have been reported to possess oral hypoglycemic activity(1,2,3), the discovery of an orally effective agent with an insulin-like mechanism has remained an elusive goal. Sulfonylureas and biguanides have offered partial success but are not effective in all diabetics. Therefore, the need still remains for agents with oral activity *via* different mechanisms with the possibility of a broader spectrum of action than the presently available oral agents.

This report describes the hypoglycemic action of 3,5 dimethylisoxazole* (U-21221, Fig. 1) which is a highly potent orally active compound with a mechanism of action different from that of available antidiabetic drugs.

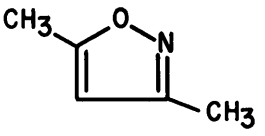

FIG. 1. Structure of 3,5 Dimethylisoxazole (U-21221).

Methods. Blood sugar of intact rats. This method has been described(5) and consists of measuring ability of compounds to depress blood sugar of glucose-primed fasted intact rats 2 hours after treatment. Potency ratio was calculated by standard USP method(4).

Oxidation of glucose-U-C^{14} by intact rats. This method(6) consists of intraperitoneal injection of glucose U-C^{14} (1.4 μc) one-half hour after oral administration of compound. Animals were placed in glass metabolic units and expired CO_2 trapped and sampled periodically, precipitated as $BaCO_3$ and counted

* Compound synthesized by Dr. J. B. Wright, Chemistry Dept., The Upjohn Co.