# Free Amino Acids of Different Kinds of Milk

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OVER TWO hundred compounds in milk, including the amino acid composition of the protein hydrolysate, have been extensively studied. There is, however, a paucity of information with respect to the free amino acids of native and heat-treated milk. From studies on other biological fluids, such as plasma, urine and sweat, one might expect a comparatively larger concentration of free amino acids in human milk. It is of interest to note that the concentration of protein in this *nutrient* is about a fifth of that in plasma, and the concentration of free amino acids in milk is less than that in any of the aforementioned biological fluids. However, the importance of free amino acids of milk should not be considered from a quantitative view alone, since the pattern and composition may differ greatly from that of the protein hydrolysate of milk.

This study is concerned with the qualitative and quantitative analysis of different brands of milk and milk substitutes for their content of free amino acids.

## MATERIALS AND METHODS

The materials used in this study consisted of native milk, heat-treated milk and plasma. Five samples of human milk, two samples of cow's milk and one of goat's milk were analyzed by paper and column chromatography. Specimens were obtained on different days of lactation as shown in Table I.

A sample of each of the following products was examined by two-dimensional paper chromatography: (1) pasteurized, homogenized milk; (2) Alacta® (powder); (3) Pet® evaporated (liquid); (4) Enfamil® evaporated (liquid); (5) Modilac® evaporate (liquid); (6) S-M-A New Formula S-26-evaporated (liquid); (7) Carnation® evaporated (liquid); (8) Similac® evaporated (liquid and powder); (9) Bremil evaporated (liquid and powder); (10) Sobee® evaporated (liquid and powder); and (11) Mullsoy® evaporated (liquid and powder) Based on the information obtained from paper chromatography, samples of the following products were further analyzed by ion-exchange column chromatography: (1) Carnation; (2) Pet; (3) Similac; (4) Mullsoy; and (5) Sobee. All samples of heat-treated milk were diluted and prepared according to the prescribed "infant formula" supplying 20 cal. per ounce, except in the case of Alacta which was prepared to supply 18 cal. per ounce. The results are expressed as milligrams per cent of "formula."

Venous blood was obtained in a heparinized tube from a nursing mother whose milk was tested from three different periods of lactation. By adding 4 vol. of 1 per cent picric acid, 20 to 50 ml. of native milk or "formula" were deproteinized. The mixture was centrifuged, and the supernatant was passed through the ion-exchange resin, Dowex 2-X8 (ionic form, $Cl^-$) to remove the picrate. The column was washed with 10 to 30 ml. of 0.01 N HCl, and the combined effluent and wash were prepared for paper and column chromatography.

An aliquot representing 3 ml. of native milk or 5 ml. of "formula" was used for quantitative determination of free amino acids. The samples were evaporated to dryness on a rotary evaporator and applied to the Phoenix Amino Acid Analyzer which is built according to Piez's modification[1] of the technic of Spackman et al.[2] Added routinely to every sample as a standard was 0.5 $\mu$M of $\beta$-alanine.

In order to examine the possible interference of lipid materials with quantitative analysis by column chromatography, the picrate supernatant of two samples was extracted three times with diethyl ether. The extract was analyzed in the same manner as the other samples by column chromatography. The values obtained from the ether-extracted samples were considerably lower. Therefore, the extraction procedure with diethyl ether was omitted.

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**TABLE**
**The Concentrations of Free Amino**

| Data | Human Milk | | | | | Raw Animal | |
|---|---|---|---|---|---|---|---|
| | Colostrum | | Transitional | Mature | | Cow | |
| | 2nd Day | 1st Day* | 8th day | 2 mo.* | 3 wk. | 7 days | 2 mo. |
| Amino acid | | | | | | | |
| Glycine | 2.74 | 0.78 | 0.25 | 0.85 | 0.39 | 0.67 | 1.87 |
| Alanine | 2.65 | 1.04 | 0.18 | 0.80 | 0.88 | 0.06 | 0.18 |
| Valine | 3.25 | 1.22 | 0.18 | 0.65 | 0.30 | 0.07 | 0.19 |
| Leucine | 3.80 | 1.43 | 0.17 | 0.72 | 0.16 | A | 0.10 |
| Isoleucine | 1.99 | 0.70 | 0.10 | 0.39 | 0.06 | A | 0.10 |
| Serine | 2.96 | 1.64 | 0.33 | 0.96 | 0.53 | 0.04 | 0.13 |
| Threonine | 2.82 | 1.52 | 0.28 | 0.64 | 0.76 | Trace | 0.16 |
| Cystine | 0.43 | 0.87 | 0.16 | 0.29 | 0.26 | A | 0.08 |
| Taurine | 0.88 | 6.39 | 1.10 | 3.63 | 6.03 | 2.85 | 0.71 |
| Methionine sulfoxide | 0.21 | A | A | Trace | Trace | A | 1.84 |
| Methionine | 1.08 | 0.41 | Trace | 0.15 | 0.04 | A | Trace |
| Aspartic acid | 2.92 | 1.79 | 0.35 | 0.53 | 1.40 | 0.05 | 0.08 |
| Glutamine | 9.48 | 3.86 | 0.54 | 1.75 | 1.79 | Trace | 0.41 |
| Glutamic acid | 12.88 | 9.58 | 0.62 | 4.20 | 7.66 | 0.82 | 0.64 |
| Lysine | 4.90 | 1.89 | 0.43 | 1.00 | 0.30 | 0.09 | 0.44 |
| Histidine | 1.18 | 0.69 | 0.19 | 0.80 | 0.22 | A | 0.08 |
| Arginine | 2.87 | 0.73 | 0.25 | 0.23 | 0.19 | A | A |
| Phenylalanine | 2.19 | 0.72 | 0.06 | 0.33 | 0.10 | A | 0.40 |
| Tyrosine | 2.71 | 0.97 | 0.06 | 0.45 | 0.11 | A | 0.01 |
| Tryptophane | Trace | Trace | A | A | Trace | 0.22 | 0.43 |
| Proline | 5.79 | 3.59 | 0.44 | 1.68 | 0.23 | 0.19 | 0.44 |
| Citrulline† | 0.39 | 0.42 | 0.06 | 0.68 | 0.09 | A | 0.09 |
| Ornithine† | 0.76 | 0.41 | 0.18 | 0.25 | 1.88 | A | 0.15 |
| Phosphoserine | 2.43 | 2.91 | 0.67 | 0.67 | 0.89 | 0.06 | 0.23 |
| Phosphoglyceroethanolamine† | 0.64 | 1.69 | 0.39 | 0.47 | 5.61 | 2.33 | 2.39 |
| Phosphoethanolamine† | 0.75 | 0.87 | 2.05 | 2.83 | 2.11 | 6.32 | 6.20 |
| α-Amino-n-butyric acid† | 0.08 | 0.06 | Trace | A | 0.10 | 0.03 | 0.03 |
| γ-Amino-n-butyric acid† | 0.22 | 0.55 | 0.55 | 0.84 | 1.19 | 0.14 | 0.65 |
| β-Aminoisobutyric acid† | 0.32 | 0.05 | A | A | A | A | A |
| Unknown compounds | | | | | | | |
| $X_1$ | 0.57 | 1.01 | 0.59 | ... | 0.60 | ... | ... |
| $X_2$ | 0.29 | 0.04 | ... | ... | ... | ... | ... |
| $X_3$ | 0.14 | 0.07 | ... | ... | ... | ... | ... |
| $X_4$ | 0.33 | 0.09 | ... | ... | ... | ... | ... |
| $X_5$ | 0.10 | 0.11 | ... | ... | ... | ... | ... |
| $X_6$ | ... | 0.17 | ... | ... | ... | ... | ... |
| $X_7$ | ... | ... | ... | ... | 0.04 | 0.08 | 0.30 |
| $X_8$ | ... | ... | ... | ... | ... | 0.12 | 0.09 |
| $X_9$ | ... | ... | ... | ... | ... | ... | ... |
| $X_{10}$ | ... | ... | ... | ... | ... | ... | ... |
| $Y_1$ | ... | ... | ... | ... | ... | ... | ... |
| $Y_2$ | ... | ... | ... | ... | ... | ... | ... |
| $Y_3$ | ... | ... | ... | ... | ... | ... | ... |
| $Y_4$ | ... | ... | ... | ... | ... | ... | ... |
| $Y_5$ | ... | ... | ... | ... | ... | ... | ... |
| $Y_6$ | ... | ... | ... | ... | ... | ... | ... |
| $Y_7$ | ... | ... | ... | ... | ... | ... | ... |
| Total | 74.7 | 48.3 | 10.2 | 25.8 | 33.5 | 14.1 | 18.4 |

NOTE: P = present but incalculable; S = presence suspected but value not attainable; A = not detected with the application of the volumes used in this study. All values are expressed in milligrams per cent.
* A specimen of milk of Subject C was tested during the three different periods of lactation.
† Not found in the hydrolysates of different protein fractions of milk.
‡ Calculated as proline; all other unknowns were calculated as leucine.

this study, cysteic acid and phosphoserine completely overlap. The corresponding peak was calculated as phosphoserine, since its presence and predominances were confirmed by paper chromatography and a phosphorous spot test.[3] These additional tests were also applied to confirm the presence of phosphoethanolamine. The peak which appears between phosphoserine and phosphoethanolamine is probably phosphoglyceroethanolamine.[2,4,5]

Appropriate volumes of combined effluent and wash were desalted for paper chromatography by the use of Dowex 50-W-X8 (ionic form, $H^+$). All the amino acids except taurine and some arginine were eluted from the column by passing 40 ml. of 1 N $NH_4OH$ through the column. The eluate was evaporated to dryness on a water bath. The residue was taken up in a suitable volume of water, and its amino acid content was studied by two-dimensional

I
Acids of Different Kinds of Milk

| Milk | Heat-Treated Milk | | | | | Milk Substitutes | | | |
|---|---|---|---|---|---|---|---|---|---|
| Goat | Homogenized Pasteurized Cow's Milk | Carnation | Pet | Similac | | Sobee | | Mullsoy | |
| 1 mo. | | | | Liquid | Powder | Liquid | Powder | Liquid | Powder |
| 0.57 | 0.59 | 0.40 | 0.09 | 0.29 | 0.24 | 0.34 | 0.28 | 0.25 | 0.47 |
| 0.21 | 0.26 | 0.25 | 0.22 | 0.18 | 0.14 | 1.19 | 0.97 | 0.86 | 2.24 |
| 0.11 | 0.21 | 0.15 | 0.14 | 0.10 | 0.08 | 0.48 | 0.55 | 0.53 | 1.16 |
| 0.06 | 0.13 | 0.07 | 0.10 | 0.06 | 0.09 | 0.47 | 0.62 | 0.26 | 0.93 |
| 0.06 | 0.06 | 0.04 | 0.05 | 0.03 | 0.04 | 0.28 | 0.30 | 0.03 | 0.51 |
| 0.19 | 0.08 | 0.05 | 0.08 | 0.09 | 0.05 | 0.42 | 0.39 | 0.74 | 0.42 |
| 0.18 | 0.09 | P | P | 0.05 | 0.07 | 0.52 | 0.72 | 0.56 | 0.21 |
| 0.07 | 0.05 | A | A | 0.07 | 0.05 | A | A | Trace | 1.17 |
| 4.77 | 0.61 | 0.47 | 0.19 | 0.62 | 0.30 | 0.74 | 0.58 | 0.27 | 1.88 |
| A | A | Trace | A | 0.10 | 0.08 | A | A | A | A |
| Trace | Trace | Trace | Trace | 0.03 | 0.04 | 0.22 | 0.19 | 0.05 | 0.28 |
| 0.08 | 0.18 | 0.26 | 0.29 | 0.17 | 0.23 | 2.50 | 2.62 | 2.03 | 4.16 |
| 1.16 | 0.33 | 0.04 | 0.91 | 0.28 | 0.38 | 3.88 | 3.84 | 7.82 | 5.17 |
| 0.56 | 1.77 | 2.47 | 0.65 | 1.48 | 1.13 | 3.14 | 7.32 | 2.49 | 6.75 |
| 0.19 | 0.42 | 0.25 | 0.15 | 0.19 | 0.16 | 0.56 | 1.54 | 0.89 | 0.75 |
| 0.12 | 0.10 | P | 0.08 | 0.05 | 0.08 | 0.61 | 1.98 | 0.96 | 0.98 |
| 0.10 | 0.21 | S | 0.13 | 0.13 | 0.14 | 8.27 | 5.66 | S | 12.11 |
| 0.09 | 0.10 | Trace | 0.04 | 0.05 | Trace | 0.66 | 0.59 | 0.23 | 0.76 |
| 0.06 | 0.08 | Trace | 0.08 | 0.05 | Trace | 0.55 | 0.26 | 0.46 | 0.52 |
| Trace | 0.05 | 1.36 | Trace | 0.06 | 0.09 | A | A | A | Trace |
| 0.28 | 0.17 | 0.10 | 0.36 | 0.14 | 0.17 | 1.24 | 1.06 | 0.29 | 0.78 |
| 0.39 | Trace | Trace | P | 0.03 | Trace | 0.16 | 0.09 | A | 0.14 |
| 0.16 | A | Trace | 0.26 | 0.08 | Trace | 0.46 | 0.15 | 1.09 | 0.22 |
| 0.16 | 0.20 | 0.40 | 0.20 | 0.47 | 0.36 | 0.48 | 0.18 | 0.28 | 0.45 |
| 0.83 | 2.61 | 3.32 | 0.82 | 2.81 | 1.96 | A | A | 1.39 | 1.35 |
| 5.46 | 2.09 | 1.47 | 0.57 | 1.42 | 1.02 | 1.98 | 0.40 | 1.39 | 1.79 |
| 0.03 | 0.03 | 0.02 | A | 0.03 | P | A | A | Trace | P |
| 0.14 | 0.61 | 0.43 | 0.05 | 0.37 | 0.01 | 0.36 | 0.71 | 0.02 | 0.63 |
| A | A | A | A | A | A | A | A | A | A |
| ... | ... | ... | ... | 0.08 | ... | ... | ... | ... | ... |
| ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| ... | ... | ... | ... | ... | ... | ... | ... | ... | ... |
| 0.09 | 0.13 | ... | 0.04 | ... | 0.08 | ... | ... | ... | ... |
| 0.08 | 0.05 | ... | ... | 0.10 | 0.10 | ... | ... | ... | ... |
| ... | ... | 0.14 | ... | 0.09 | 0.03 | ... | ... | ... | ... |
| ... | ... | ... | ... | 0.11 | 0.02 | ... | ... | ... | ... |
| ... | ... | ... | ... | ... | ... | 2.84‡ | 3.48 | 4.38 | 6.60 |
| ... | ... | ... | ... | ... | ... | 0.40‡ | 0.08 | ... | 0.38 |
| ... | ... | ... | ... | ... | ... | ... | ... | 0.14‡ | 1.19 |
| ... | ... | ... | ... | ... | ... | 0.17‡ | 0.13 | 0.24 | 1.42 |
| ... | ... | ... | ... | ... | ... | 0.06‡ | 0.17 | 0.10 | 0.37 |
| ... | ... | ... | ... | ... | ... | Trace | Trace | ... | 1.97‡ |
| ... | ... | ... | ... | ... | ... | ... | Trace | 0.01‡ | 0.25 |
| 16.2 | 11.2 | 11.7 | 5.5 | 9.8 | 7.2 | 33.6 | 34.8 | 27.9 | 58.5 |

paper chromatography using 12 inch by 12 inch Whatman No. 3 MM filter paper with pyridine-acetone-ammonium hydroxide (5:3:2) for the first solvent and isopropyl alcohol-formic acid-water (8:1:1) for the second solvent.

The volume of the eluate applied to the paper represented 500 to 1,000 $\mu$l. of native milk or formula. The chromatograms were stained with ninhydrin (0.25 per cent in acetone).

The blood was centrifuged, and the plasma was treated in the same manner as the milk samples for column chromatography.

## RESULTS

On screening various brands of heat-treated milk by two-dimensional paper chromatography, the differences observed were not striking, except in the case of Bremil which showed a higher concentration of $\alpha$-alanine. However,

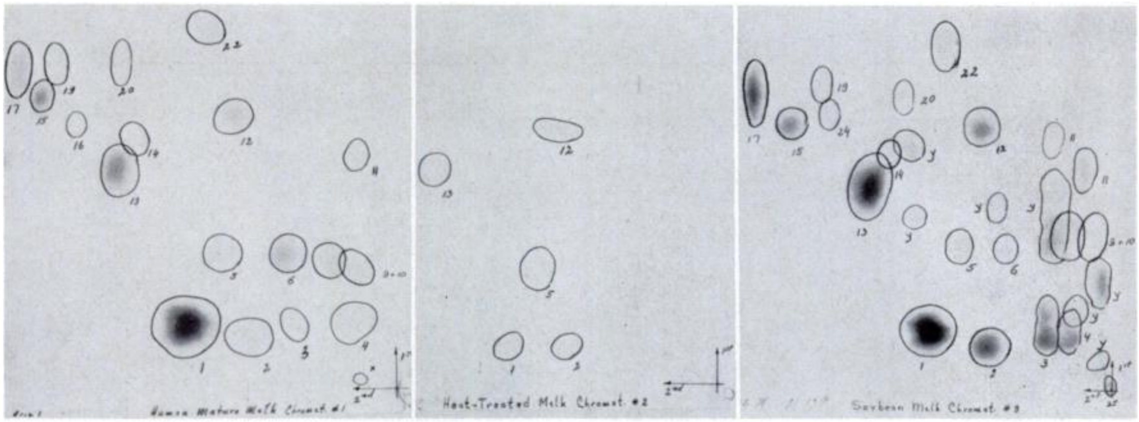

FIG. 1. Two-dimensional chromatograms. *Left,* human milk. *Center,* heat-treated milk. *Right,* soybean milk. The amount applied to each chromatogram represents 500 $\mu$l. of prepared "formula." 1 = glutamic acid; 2 = aspartic acid; 3 = arginine; 4 = cysteine, cystine; 5 = glycine; 6 = glutamine; 9 = lysine; 10 = ornithine; 11 = histidine; 12 = serine; 13 = alpha alanine; 14 = proline; 15 = valine; 16 = beta aminoisobutyric acid; 17 = leucine and isoleucine; 19 = phenylalanine; 20 = tyrosine; 22 = threonine; 25 = phosphoserine; x = unknown compounds in human milk; y = unknown compounds in soybean milk.

quantitative and qualitative differences between native milk, heat-treated milk and soybean milk substitute were obvious by paper chromatography (Fig. 1). The chromatogram of heat-treated milk (Fig. 1, center) shows few amino acids and in low concentrations as compared with the chromatograms of human milk (Fig. 1, left) and milk substitute (Fig. 1, right). The chromatogram of the milk substitute (soybean), on the other hand, reflects an abundance of free amino acids. A number of ninhydrin-positive spots (y) in this chromatogram remain to be identified. These results were further confirmed both qualitatively and quantitatively by column chromatography.

Figure 2 shows the chromatographic record of human mature milk as obtained by ion-exchange column chromatography. Five unknown small peaks are designated as $x_1$ to $x_5$. The unknown peaks observed in the chromatographic record of milk substitute were more distinct and in much higher concentration. They did not correspond to the location of the unknown compounds (x) observed in the chromatographic record of human milk and are reproducible by repeated analysis of the same product. These unknown compounds are, therefore, indicated by the letter "y" in Table I.

The quantitative results of all the samples analyzed by column chromatography are recorded in Table I.

In Figure 3, the concentrations of free amino acids in the colostrum and mature milk of a nursing mother are compared in the bargraph with her plasma free amino acids. The latter prove to be within the expected normal range when compared with the values obtained from eleven women in parturition.

## COMMENTS

Our data (Table I) indicate that the concentration of almost all free amino acids in the colostrum of human milk are higher than the corresponding values in transitional or mature milk. This seems to follow the general pattern of other constituents of the milk. Human transitional milk has the lowest concentration of free amino acids as compared with colostrum and mature milk. We have no data on cow's colostrum. With the exception of taurine, glutamic acid and phosphoethanolamine, the concentration of all amino acids of cow's transitional milk is lower than that of cow's mature milk. The high concentration of free amino acids in colostrum, the sharp decrease in the transitional period and the increase in concentration in mature milk is demonstrated by the study of Subject C (Table I and Fig. 3).

These data also suggest that the concentra-

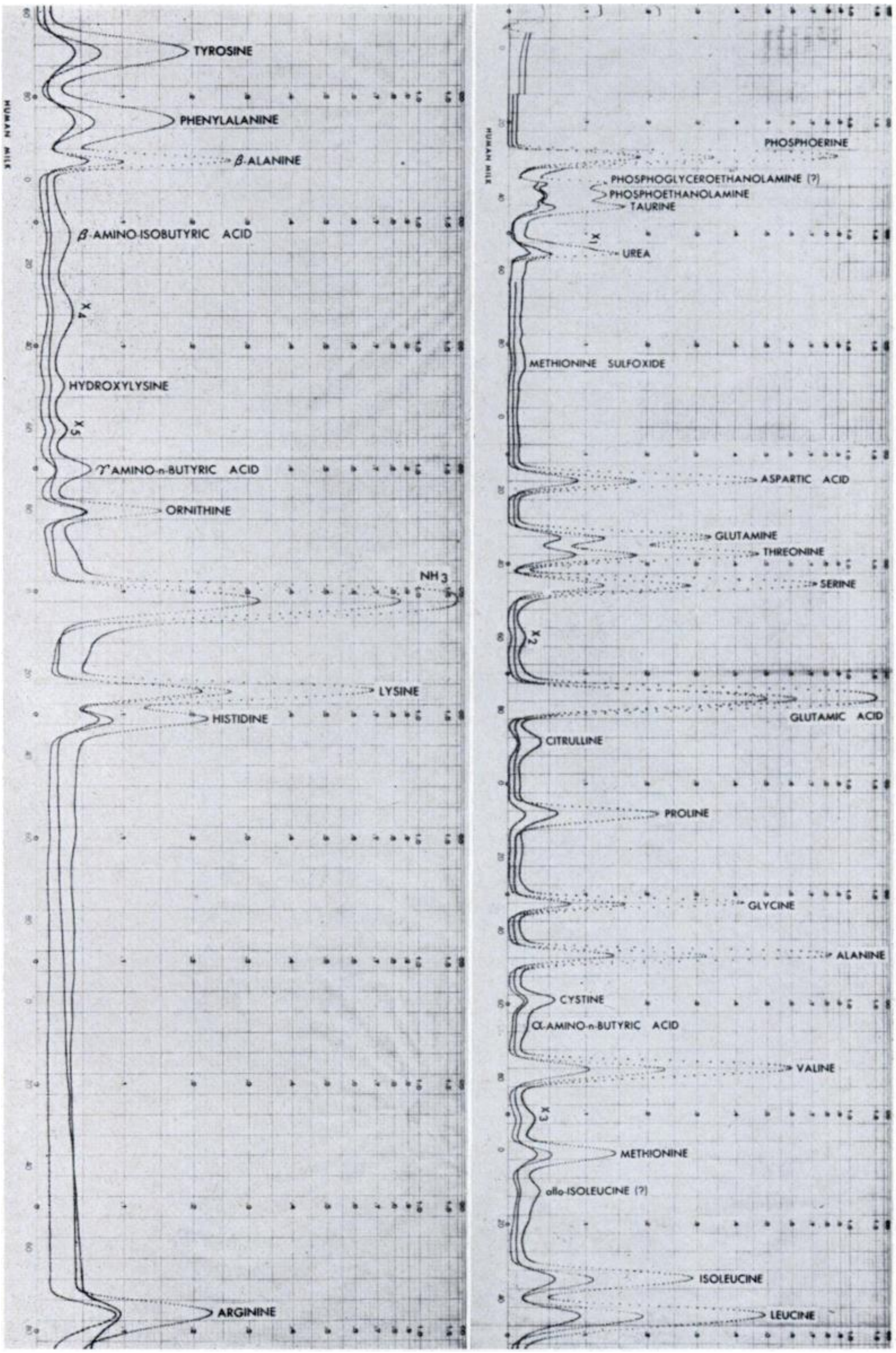


Fig. 2. Chromatographic record of human milk. The β-alanine peak represents the standard which was added to the sample.

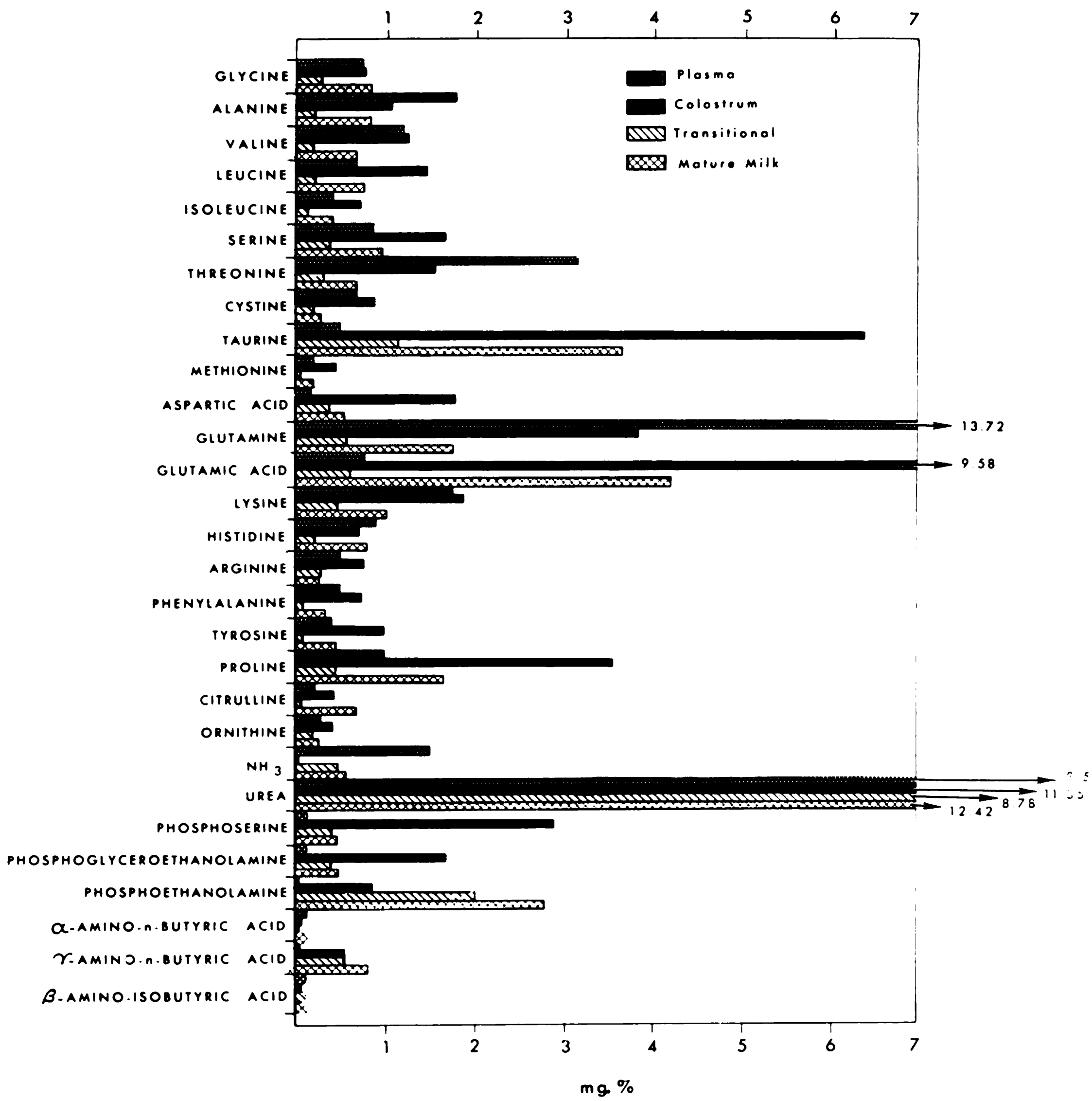


FIG. 3. Free amino acids of human milk from Subject C during the three different periods of lactation compared with the free amino acids of her plasma.

tion of free amino acids of human milk is somewhat higher than that of cow's and goat's milk. In general, the concentration of free amino acids of native milk is much higher than that of heat-treated milk. When the sum of free amino acids of human and cow's mature milk is compared with the corresponding figure for heat-treated milk the ratio ranges from 3:1 to 6:1. The milk substitute prepared from soybeans, commonly used for allergic infants, is exceptionally high in the concentration of free amino acids. This is particularly true in the case of arginine. The free amino acid nitrogen of human milk does not exceed 3 per cent of the total nitrogen of milk. This should not be judged merely from a quantitative point of view, since the free amino acids are readily available for intestinal absorption, and the pattern of the free amino acids of milk differs from that of the protein hydrolysate.

On the other hand, the main source of nitrogen is obtained from protein, and children are being reared on heat-treated milk with no apparent manifestations of deficiency.

Variations in the concentrations of free amino acids during the different periods of lactation as well as the differences in the pattern and concentrations of these free amino acids as compared with the free amino acids in maternal plasma indicate that the excretion of these substances is by an active mechanism rather than by simple diffusion.

## SUMMARY

The free amino acids of native milk from human beings, cows and goats at different periods of lactation were measured by ion-exchange column chromatography.

Heat-treated milk (pasteurized, evaporated and powdered) and a milk substitute made from soybeans were screened by two-dimensional paper chromatography for free amino acids. A representative of each type of heat-treated milk was analyzed by ion-exchange chromatography.

Marked differences were observed between human native milk, heat-treated animal milk and soybean milk substitutes. Soybean milk substitute and colostrum were found to be rich in their free amino acid content.

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