

Analytical Artifacts in Radioimmunoassay of L-Thyroxin in Human Milk

J. Mallol, M. J. Obregón, and G. Morreale de Escobar¹

Previous results are contradictory regarding the concentration of thyroxin in human milk. Using a sensitive radioimmunoassay, we have found a lack of parallelism between the standard curve for thyroxin and the curve for serial dilutions of whole human milk, skimmed milk, or ethanol extracts of milk. Nonspecific binding also indicated the presence of analytical artifacts. Thus we have separated thyroxin from other milk components by means of a strongly basic Bio-Rad anion-exchange resin with quaternary ammonium exchange groups attached to a styrene divinyl benzene copolymer lattice, radioimmunoassaying the fractions eluted with an equivolume mixture of acetic acid and water. Parallelism with the standard curve was good, and results were the same whether or not the resin eluate was further purified by paper chromatography. The range of thyroxin concentration in 21 samples of human milk was 0.29–2.00 $\mu\text{g/L}$ (mean 0.71, SD 0.40, $\mu\text{g/L}$). Such concentrations are unlikely to afford protection to the developing brain of a breast-fed athyreotic baby, as previously claimed.

Additional Keyphrases: *reference interval · disorders of thyroxin production · hypothyroidism · newborns · chromatography, paper · chromatography, anion-exchange*

Montalvo et al. (1) and Štrbák et al. (2) reported, respectively, 7–96 and 15–130 μg of thyroxin (T_4) per liter of human milk, as determined with competitive binding assays of whole milk, skimmed milk, or ethanol extracts of milk. Sack et al. (3) and Tenore et al. (4) reported values of 4–70 and 13–80 μg of T_4 per liter of human milk, respectively, as measured by radioimmunoassay (RIA).² Such concentrations of T_4 might afford a considerable degree of protection to the brain of hypothyroid babies. Indeed, Tenore et al. (4) reported improved mental development in breast-fed, as compared with formula-fed, athyreotic babies and attributed this finding to the higher T_4 content of human milk.

These findings contrast with those of Varma et al. (5), who reported the concentration of T_4 as determined by RIA to be <5 $\mu\text{g/L}$ in 83 out of 88 samples of human milk. Sato and Suzuki (6) also found T_4 concentrations of <5 $\mu\text{g/L}$ in 100 samples of human milk, and in trypsin digests of 20 samples. These results are consistent with the report of Tsuyusaki et al. (7), who found that ethanol extracts of human milk had no biological activity as measured by a goiter prevention bioassay.

The aim of the present work was to ascertain which of these contradictory reports is correct. We used a highly sensitive RIA, developed for serum T_4 by Weeke and Ørskov (8). None of the 21 samples we have studied so far contains >2 μg of T_4

per liter; thus we conclude that the lower values reported are more nearly correct, but still high.

Materials and Methods

T_4 RIA. We used the method of Weeke and Ørskov (8) described for assay of T_4 in human serum, as modified by Obregón et al. (9), in which RIA buffer is used as the solvent for standards used in preparing the T_4 standard curve and polyethylene glycol 6000 (PEG) is used to separate free from antibody-bound labeled T_4 . ¹²⁵I-labeled T_4 (T_4^*) of high specific activity (2000 Ci/g, or higher) was obtained by the method of Weeke and Ørskov (10) as modified by Kjeld et al. (11). The sensitivity is 5 pg of T_4 per tube, 0.5 μL of human serum being sufficient.

In brief, the T_4 standards, prepared in 50 μL of RIA phosphate buffer (40 mmol/L, pH 8.0, and containing 0.6 mmol of thimerosal and 0.5 g of bovine serum albumin per liter), are pipetted into test tubes. Milk samples are pipetted in quadruplicate at each dilution and diluted to 50 μL with the RIA buffer. Three of these quadruplicate tubes receive 100 μL of T_4 antiserum at a 20 000-fold dilution, and the fourth receives 100 μL of buffer. Thus there is an individual "blank" for each triplicate of each dilution of a milk sample. Approximately 7000 cpm of T_4^* is added, in 100 μL of RIA buffer. After 16- to 20-h incubation at room temperature, 1.5 mL of RIA buffer containing 200 g of PEG and 50 mL of normal calf serum per liter is added and the tubes and contents are vigorously vortex-mixed for 20 s. After centrifugation at 2000 rpm for 45 min, the supernatant fluid is aspirated and the radioactivity in the precipitate is counted. RIA buffer "blanks" are used to determine the nonspecifically bound T_4^* for tubes containing T_4 standards, whereas individual "blanks" are used for milk samples.

Sample-preparation procedure A. Whole milk was obtained from volunteers at different times after delivery and stored at -20° until used.

Unless whole milk is used directly for the RIA, add about 5000 cpm of T_4^* to 1.0 mL of whole milk. After 2 h at room temperature, centrifuge the radiolabeled milk for 45 min at 2000 rpm, or extract it with absolute ethanol. In the first case, separate the skimmed milk from the fat layer at 4°C . In the second case, add either the same volume of ethanol (1:2 EtOH extract) or nine volumes of ethanol (1:10 EtOH extracts) to whole or skimmed milk, centrifuge, and use the supernate for analysis. Count the radioactivity in aliquots of whole and skimmed milk and of EtOH extracts to determine the T_4^* partition and extraction coefficients after the usual corrections for counting geometry, efficiency, etc. After centrifugation of whole milk, dissolve the fat recovered in a volume of EtOH corresponding to the initial volume of whole milk.

Assay whole or skimmed milk, or EtOH extracts, at three dilutions. Evaporate EtOH extracts to dryness at room temperature under a stream of air. The radioactivity of these samples was <200 cpm per tube. Follow the above T_4 RIA procedure.

Aliquot volumes indicated in Tables and Figures and in the text are given in "equivalents" of whole or skimmed milk. For example, a "50- μL aliquot" of a 1:2 EtOH extract means that 100 μL of EtOH was actually evaporated for the RIA; in the

Departamento de Endocrinología Experimental, Instituto G. Marañón, C.S.I.C y Facultad Autónoma de Medicina, Arzobispo Morcillo 4, Madrid-34, Spain.

¹ Address correspondence to this author.

² Nonstandard abbreviations: T_4 , L-thyroxin; T_4^* , radiolabeled T_4 ; PEG, polyethylene glycol.

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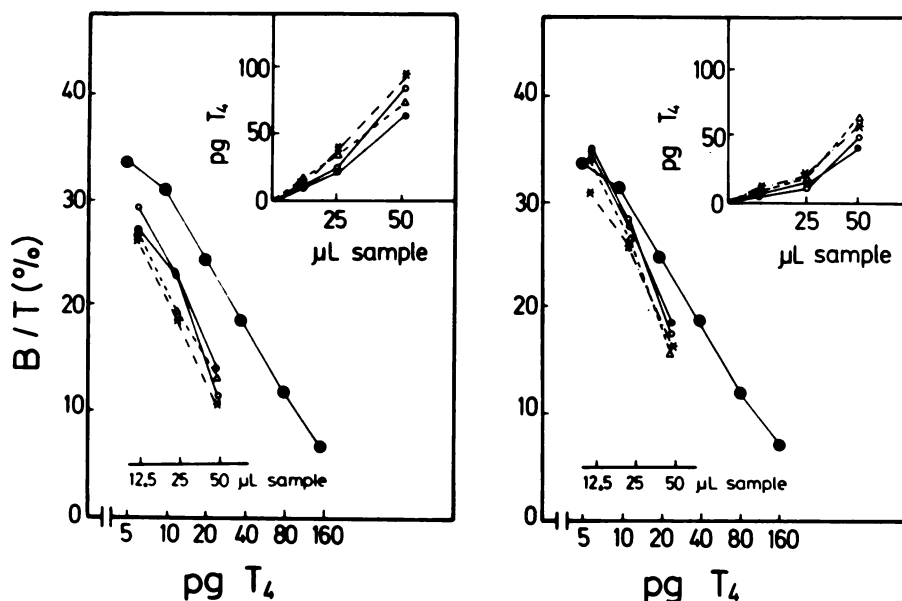


Fig. 1. Serial dilution curves obtained with (left) whole milk and (right) skimmed milk, as compared with the standard curve prepared with use of RIA buffer

Insets: the apparent T_4 contents as a function of the volume of sample used for the RIA. As may be seen, the data usually do not fall on a straight line passing through the origin. Different symbols are used to identify samples from different milk donors. Results are only shown for four donors, but samples for 15 were tested. Most of these 15 samples did not give dilution curves that paralleled the standard curve

case of milk fat, a "50- μ L aliquot" would indicate the fat contained in 50 μ L of the original whole milk.

Sample-preparation procedure B: isolation of T_4 by chromatography on Bio-Rad columns. Add T_4^* (7000 cpm) to 1.5 mL of whole milk (or RIA buffer for the "blank" column). After 1 h at room temperature, pass the mixture through a 1.5-mL column of Bio-Rad 1 \times 2 (200–400 mesh, in chloride form) (12) equilibrated with acetate buffer (0.2 mol/L, pH 7.0). Wash the column with 2 mL of the same buffer and then with 4 mL of EtOH; this removes most of the milk proteins and lipids. Then wash with 2 mL of acetate buffer (0.2 mol/L) at pH 4.0 and 2 mL at pH 3.0, followed by 2 mL of 18 mmol/L (10 mL/L) and 2 mL of 610 mmol/L (350 mL/L) acetic acid. Discard all these eluates. Then pass seven or eight 0.5-mL fractions of 880 mmol/L (500 mL/L) acetic acid through the column, collect the eluates and count the radioactivity of each. Pool the two or three fractions containing the highest radioactivity to obtain "eluate 1." This contains more than half of the T_4^* that was added to the milk sample and is clear and colorless, even when the milk sample has a high lipid content. Count the radioactivity in an aliquot to determine the analytical recovery of T_4^* . Pipet other aliquots (usually 40 and 80 μ L) in quadruplicate into RIA tubes and evaporate them. Add 50 μ L of RIA buffer and follow the T_4 RIA procedure, except add more T_4^* than usual (8000–9000 cpm per tube) to minimize the influence of the 200–300 cpm of T_4^* in the eluate-1 aliquots. Calculate the concentration of T_4 in the initial milk sample from the amount of T_4 found in the aliquot and the recovery of T_4^* in a known volume of eluate 1.

Sample-preparation procedure C: isolation of T_4 by procedure B and paper chromatography. To validate procedure B, evaporate another aliquot of eluate 1, dissolve the residue in small volumes of methanol/ammonia (99/1 by vol), and transfer the solution to Whatman 3MM paper strips, 2.5-cm wide, previously spotted with 50 μ L of EtOH containing propylthiouracil, 10 mmol/L, to protect T_4 from artifactual de-iodination. Submit the strips to descending chromatography in 2-methylbutanol/hexane/ammonia, 2 mol/L (5/1/6 by vol), as described by Bellabarba et al. (13). Dry the chromatograms, cut them into strips 1-cm wide, and count the radioactivity. Pool the two or three strips containing the

highest T_4^* content and elute them with 2.0 mL of methanol/ammonia ("eluate 2"). Count an aliquot for T_4^* recovery. Pipet other aliquots (usually 50, 100, and 200 μ L) in quadruplicate into RIA tubes and evaporate at room temperature under a stream of air. Perform the RIA and calculate the concentration of T_4 in milk, as indicated for procedure B.

Miscellanea: The Bio-Rad resin was washed before the columns were prepared. It was first suspended in 1.2 mol/L acetic acid and then washed several times with distilled water until the pH was 3–4. It then was washed with acetate buffer (0.2 mol/L, pH 7.0). A resin slurry was poured into 2-mL plastic syringes (9 mm i.d.) up to the 1.5-mL mark. The resin was prevented from passing through the syringe by a 6.3-mm dot of Whatman 3 MM paper. The acetate buffer was drained just before the milk sample was added.

In some cases, *Staphylococcus aureus* was used instead of PEG. A 100 g/L suspension of heat- and formaldehyde-killed bacteria, prepared as described by Kessler (14), was kindly obtained for us by Dr. I. Gil from a protein A-rich Cowan strain. We diluted 15 μ L of the suspension to 500 μ L in pH 8

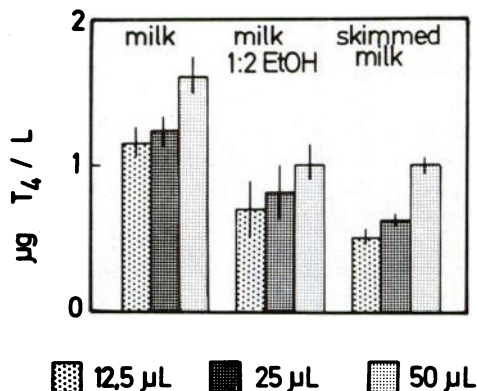


Fig. 2. The apparent T_4 concentrations found on using different aliquots of whole milk, skimmed milk, or 1:2 EtOH extracts of whole milk

The apparent concentration was not independent of the sample volume. The same was true of 1:2 EtOH extracts of skimmed milk or 1:10 EtOH extracts of whole milk. Data are means \pm SEM of the same samples used for Fig. 1

Table 1. Assayed Concentrations of T₄ in Whole Milk, Skimmed Milk, and Milk Fat (col. A), and Calculated T₄ Concentrations in Whole Milk (col. B) ^a

Sample in RIA tube	A: T ₄ , pg/50 μL ^b	B: T ₄ , μg/L ^c
Whole milk	80 (6)	1.60 (0.12)
1:10 EtOH extract	71 (7)	1.57 (0.16)
1:2 EtOH extract	49 (8) ^d	1.07 (0.17) ^d
Skimmed milk	50 (3) ^d	1.42 (0.09)
1:2 EtOH extract	52 (16) ^d	1.48 (0.41)
Fat, EtOH extract	48 (7) ^d	3.22 (0.58) ^d

^a Data are means (and SEM) for samples from the same donors as for Figures 1 and 2. ^b Amount of T₄ found in the RIA tubes, for 50 μL of whole or skimmed milk, the equivalent volumes of EtOH extracts, or the fat corresponding to 50 μL of whole milk. ^c Calculated from the T₄ data in column A and the individual T₄ partition and extraction coefficients. ^d Significantly (*p* < 0.05 or better) different from whole milk, as calculated by the paired *t*-test.

phosphate buffer, added this to the tubes, and centrifuged at 2000 rpm for 45 min. The supernate was aspirated and radioactivity in the pellet was counted.

Statistical analysis. Mean values and SEM were calculated, and Student's *t*-test was performed, as described by Snedecor (15). The paired *t*-test was used to evaluate the significance of differences between results obtained by different procedures, to account for variability in milk from different donors.

Results

If the concentrations of T₄ in human milk were about the same as those in serum (1-4), 0.5 to 1.0 μL of whole milk should suffice for determination of T₄ with the present RIA (8, 9). But we found that a larger sample volume was necessary before we observed displacement of T₄^{*} in the RIA; when we used more sample, however, there was no parallelism between the T₄ standard curve and serial dilutions of most (though not all) of the 15 samples of human milk tested. That is to say, the T₄ concentration was not independent of the volume of sample used in the RIA. Moreover, this problem was not solved by using skimmed milk samples or evaporated EtOH extracts of whole or skimmed milk. This point is illustrated in Figures 1 and 2 for four of the samples tested.

Table 2. Binding of T₄^{*} in the Absence of Anti-T₄ Antiserum in RIA Tubes Containing Buffer or Samples of Human Milk ^a

Sample	% of T ₄ [*] in the precipitate	
	A	B
RIA buffer ^b	5.2 (0.3)	10.1 (0.3)
Whole milk	5.9 (0.1)	19.7 (0.8) ^c
1:10 EtOH extract	7.8 (0.6) ^c	19.9 (1.2) ^c
1:2 EtOH extract	8.5 (0.1) ^c	29.1 (5.3) ^c
Skimmed milk	5.1 (1.3)	13.2 (3.1)
1:2 EtOH extract	7.8 (0.5) ^c	16.2 (3.2) ^c
Milk fat, EtOH extract	7.3 (0.6) ^c	46.3 (2.5) ^c

^a Data are expressed as percentages of the total T₄^{*} added, and are means (and SEM) of four samples (same as used for previous Figures and Table). Data in column A were obtained by using PEG, those in column B by using *S. aureus* to separate free from bound T₄^{*}. ^b The total binding in the presence of anti-T₄ antiserum was 43.4 ± 1.0% on using PEG, and 44.3 ± 1.4% on using *S. aureus*. ^c Difference with respect to the values found for blanks (RIA buffer) are statistically significant (*p* < 0.05 or better) by the paired *t*-test.

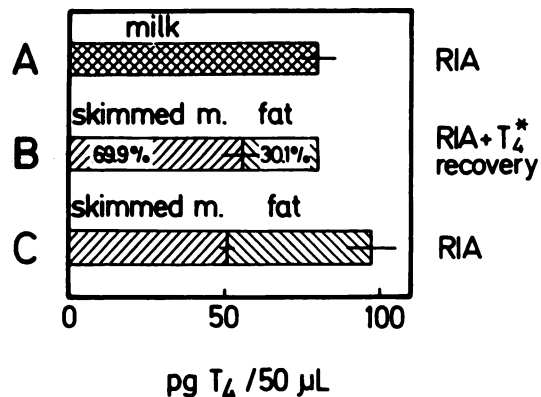


Fig. 3. Comparison of found and calculated T₄

Bar A, the apparent amount of T₄ in 50 μL of whole milk, as determined by RIA. Bar B, amounts of T₄ that should have been found in skimmed milk and in milk fat, as calculated from the data found in A, and the individual partition coefficients of the added T₄ (69.9 ± 4.9% of T₄ in skimmed milk and 30.1 ± 4.9% in fat). Bar C, amounts actually found by RIA on using 50 μL of skimmed milk or the fat in 50 μL of whole milk, corresponding to the four samples used for Fig. 1

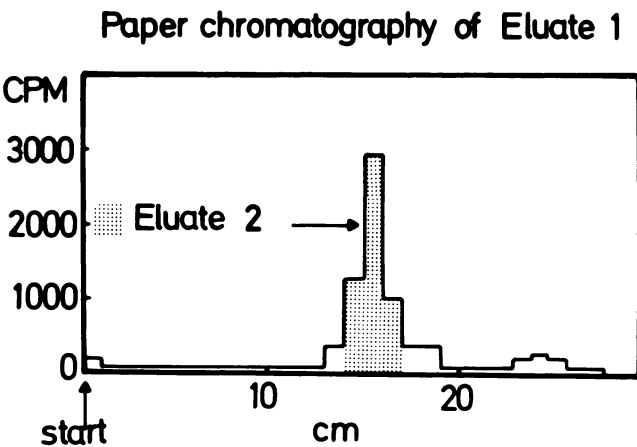
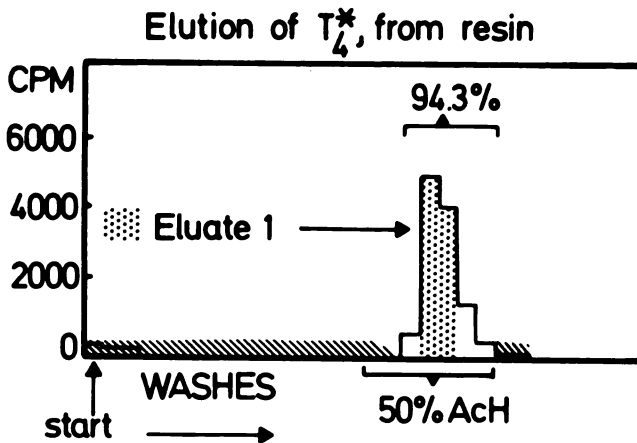


Fig. 4. Upper panel: the pattern of elution of T₄^{*} added to human milk, after passing it through Bio-Rad columns and the successive washes described here

Practically all of the T₄^{*} is eluted with acetic acid (AcH), 500 mL/L. Fractions are of 0.5-mL volume. Eluate 1 is formed by pooling the two fractions with the highest T₄^{*} content so that 40-80 μL of milk equivalents will require minimum volumes of Eluate 1. The shaded area indicates fractions with high turbidity, or opalescence. Most of the "milky" components are eluted in the first washes, but the first fractions eluting with AcH are not completely clear. When the column is further washed with 700 mL/L AcH, the first eluates are again opalescent. Care is taken to choose for eluate 1 those fractions with a high T₄^{*} content that are most transparent. No visible residue remains in the RIA tubes after evaporation of 40-80 μL of eluate 1

Lower panel: the pattern of distribution of T₄^{*} contained in eluate 1 after ascending paper chromatography (13)

The T₄^{*} in the strips indicated by the shaded area is eluted and pooled to obtain eluate 2

We observed that the apparent T_4 concentration also depended on the pretreatment given the sample (Table 1): the lower apparent T_4 values found for 1:2 EtOH as compared with 1:10 EtOH extracts could not be accounted for by the T_4 extraction coefficient, which exceeded 91% in both cases. Thus the difference persists when the T_4 concentration in whole milk is calculated; the fat also appeared to contain significantly more T_4 than expected from the T_4 partition coefficient (column B of Table 1). We noticed that a layer of fat was visible in 1:2 EtOH extracts of whole milk, but not in the 1:10 EtOH extracts; evidently more fat was being dissolved in 1:10 than in 1:2 EtOH extracts. Milk fat separated from whole milk by centrifugation was also completely dissolved in EtOH.

The sum of the apparent amounts of T_4 found in skimmed milk and milk fat exceeds that found in whole milk (Figure 3). When the T_4 partition coefficients were applied to the T_4 data found for whole milk, the calculated amount of T_4 and that actually measured by RIA coincided for the skimmed milk fraction. In contrast, the amount measured by RIA for the milk fat significantly exceeded the calculated one.

We considered the possibility that these problems were caused by the presence of moieties binding the labeled antigen, especially when the lipid content of the sample transferred to the RIA is high, because the use of skimmed milk did not solve all the problems encountered. To assess such possibilities we evaluated the nonspecific binding of T_4 . Table 2 shows results obtained with both PEG and *S. aureus*. Nonspecific binding of T_4 was often greater in samples derived from milk than in RIA buffer alone, and often appeared related to the probable fat content of the sample (column B of Table 2). Nonspecific binding by *S. aureus* could be as great as total binding in samples likely to contain a high proportion of milk fats.

The lipophilic nature of T_4 made it unlikely that solvent-extraction procedures would adequately separate it from lipidic milk components. Paper chromatography was initially attempted with whole milk, skimmed milk, and EtOH extracts, but good separations were not consistently obtained. Bio-Rad resin columns (12) were used and preliminary ex-

periments done to define the appropriate volumes of milk relative to the amount of resin and the procedure that would elute most of the T_4 in a clear, non-opalescent fraction. Procedure B was developed and initially validated with procedure C, which involves paper chromatography of the resin eluate.

Figure 4 shows elution patterns of T_4 after its adsorption on a Bio-Rad resin column and after paper chromatography of the resin eluate. Good parallelism was found between the T_4 standard curves and serial dilutions of both eluates 1 and 2 (Figure 5). The eluates from the "blank" columns did not interfere in the T_4 RIA. Figure 6 confirms, for both eluates 1 and 2, that the T_4 concentration is no longer dependent on the volume of sample in the RIA tube. The nonspecific binding of T_4 as evaluated with eluate 2 was the same as that of the standard curve blanks (Table 3), but specific binding with eluate 1 was somewhat higher. Despite this, when we used the individual blanks to calculate the T_4 concentration, the results obtained with eluates 1 and 2 were the same (Figure 6).

Individual blanks increase with the amount of eluate 1 used in the RIA. If large volumes of eluate 1 are needed because of a low T_4 concentration in the milk sample, procedure C should be followed. The same is advisable if individual blanks for eluate 1 are unusually high. Procedure B should not be altered without previous validation. Thus, an increase in the volume of milk relative to that of the resin bed would decrease the proportion of T_4 retained on the column and later eluted with acetic acid/water (1/1 by vol). A decrease in the amount of milk used relative to that of resin bed would result in the use of larger aliquots of eluate 1, a procedure that should be avoided for the reason indicated above.

Figure 7 shows our preliminary frequency distribution of the T_4 concentrations in human milk. Most of the samples were obtained two or three weeks postpartum. As may be seen, in none of the samples tested so far did the T_4 concentration exceed $2 \mu\text{g/L}$.

Discussion

Our results show that human milk contains substances that interfere with T_4 RIA in proportion to the volume of milk

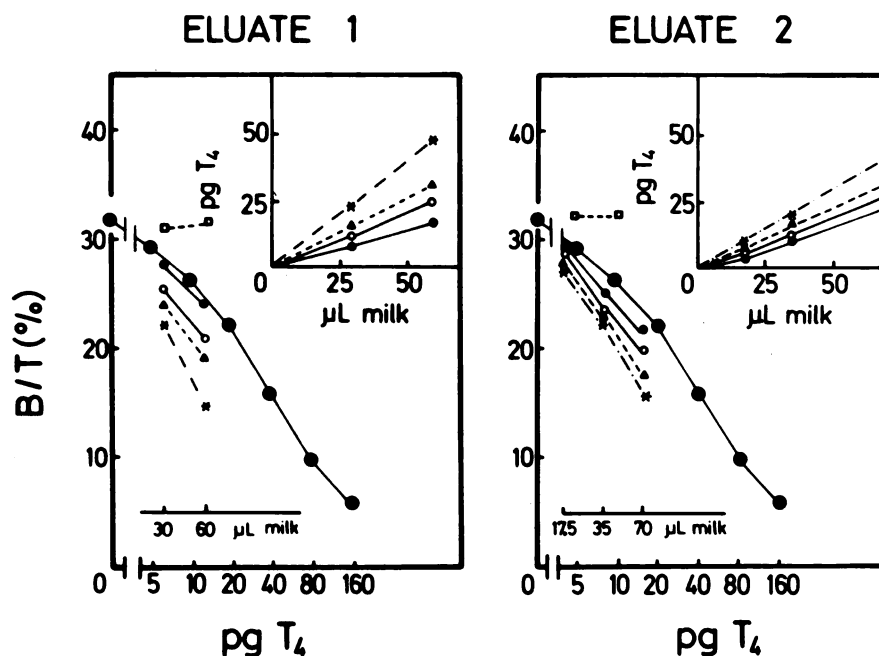


Fig. 5. Serial dilution curves obtained with eluates 1 and 2

For the sake of simplicity, the milk volumes shown are the approximate means corresponding to 40 and 80 μL of eluate 1, or 20, 100, and 200 μL of eluate 2, as assessed from individual T_4 recoveries. The actual mean values are given in Table 3. Different symbols are used to identify milk specimens (different from those used for previous Figures and Tables). (\square - - - \square), results obtained for eluates 1 and 2 from "blank" columns

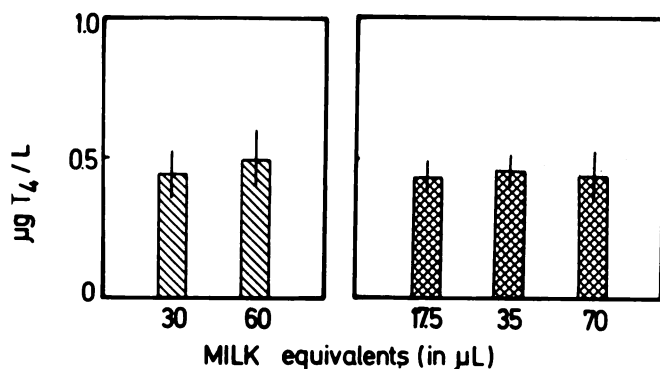


Fig. 6. T_4 concentrations found on different volumes of milk equivalents for the RIA

Results no longer depend on sample volume, and are the same whether the resin eluate is further purified by paper chromatography (right, eluate 2) or not (left, eluate 1)

required for the RIA. Previous studies reporting T_4 concentrations $>5 \mu\text{g/L}$ (1-4) were done with less-sensitive competitive binding or radioimmunoassays that required larger sample volumes (100 μL or more). Thus it is likely that those results were artificial, a possibility apparently not excluded by the authors, because they report no validation tests, such as serial-dilution curves and the like. The use of skimmed milk or EtOH extracts as samples would not necessarily obviate these artifacts, as shown here.

Tenore et al. (16) reported that there might be analytical problems with the determination of T_4 by RIA: T_4^* -displacement activity in the T_4 RIA did not coincide, after chromatography on Sephadex LH 20, with the fractions eluting the radiolabeled T_4 added to the milk sample as tracer. The RIA activity was eluted in the void volume, where no labeled T_4 was found, whereas no RIA activity was found in the peak containing tracer T_4 . In view of our results perhaps the amounts of T_4 eluted in the fraction containing the tracer T_4 were too small to be detected by RIA, and the "radioimmunoassayable activity" they found in the void volume actually reflected the presence of moieties interfering in the T_4 RIA.

The interpretation suggested by these authors was, however, that endogenous T_4 did not equilibrate with the added labeled T_4 . If this were so, values obtained with our procedures B and C might be underestimated. But we believe that the data in Figure 3 show this to be unlikely, because the amount of T_4 in the skimmed-milk fraction coincided with the one calculated from the whole-milk T_4 content and the T_4^* partition coefficient. In contrast, the amount of T_4 in the fat layer

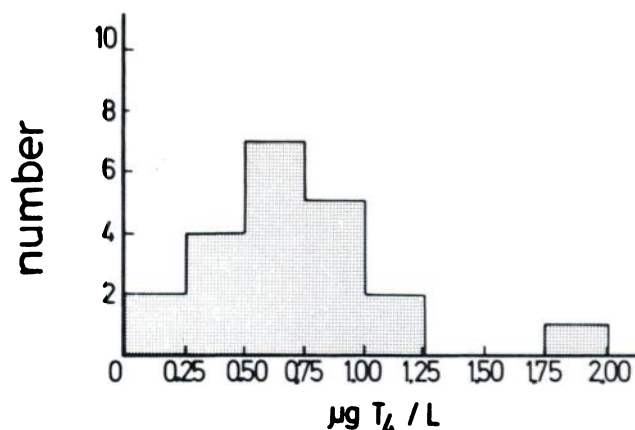


Fig. 7. Distribution curve of T_4 concentration found in 21 samples of human milk, most of which were obtained two to three weeks postpartum

The mean value (\pm SEM) was 0.71 ± 0.09 (range 0.24-2.00) $\mu\text{g/L}$

did not correlate, probably because of the presence of interfering moieties. The sum of the T_4 found in the skimmed milk plus that in the fat layer should have been the same as the T_4 found in whole milk if the only problem were a lack of equilibration of added T_4^* with the endogenous T_4 .

Apart from interferences apparently related to its high lipid content, human milk might contain thyroxin-binding proteins; corticosteroid-binding globulins have been identified in human and rat milk, and are apparently identical to the binding proteins in serum (17, 18). This might also affect the RIA results, because the amounts of "blockers" added for the determination of T_4 in serum might not be adequate.

Procedures B and C are currently being validated for the determination of T_4 in sera and tissues with a high lipid content.

Our results are in essential agreement with the reports by Varma et al. (5), Sato and Suzuki (6), and Tsuyusaki et al. (7). In view of the results by Sato and Suzuki (6) for trypsin digests of human milk, it is unlikely that the total T_4 content exceeds 5 μg of T_4 per liter. Thus, unless human milk contains high concentrations of T_3 , which is unlikely (6), the thyroid hormone content of human milk would not afford adequate protection to the developing brain of athyreotic babies.

The reported improved mental development of breast-fed as compared with formula-fed hypothyroid babies (4) might be related to some other nutritional difference, such as the iodine content (19). The human baby depends totally on the iodine in milk for thyroid function during the suckling period; ectopic thyroid tissue might function longer if the iodine supply is adequate.

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References

1. Montalvo, J. M., Wahner, H. W., and Christie, R. J., Triiodothyronine and thyroxine levels in breast milk of humans and primates. *56th Meeting of the Am. Endocrine Soc.*, Atlanta, GA, 1976, p A-91. Abstract.
2. Štrbák, V., Macho, L., Kováč, R., et al., Thyroxine by competitive protein binding analysis in human and cow milk and in infant formulas. *Endocrinol. Exp.* 10, 167-174 (1976).

Table 3. Binding of T_4^* in the Absence of Anti- T_4 Antiserum in RIA Tubes Containing Different Volumes of Eluates 1 and 2 from Seven Different Milk Specimens^a

Sample	Eluate evaporated, μL	μL of milk equivalent ^b	% of T_4^* (nonspecific binding)
RIA buffer ^c	—	—	7.0 (0.2)
Eluate 1	40	28.4 (4.2)	8.5 (0.5) ^d
Eluate 1	80	56.8 (4.2)	10.0 (0.4) ^d
Eluate 2	50	17.8 (5.9)	7.1 (0.1)
Eluate 2	100	35.7 (5.9)	7.4 (0.5)
Eluate 2	200	71.4 (5.9)	7.8 (1.3)

^a Data are means (and SEM), with separation by PEG. ^b The mean (and SEM) of the equivalent amounts of milk, based on the individual T_4^* recovery data. ^c The total binding in the presence of anti- T_4 antibody was $39.1 \pm 0.4\%$. ^d Difference with respect to the RIA buffer (standard curve blanks) is statistically significant.

3. Sack, J., Amado, O., and Lunenfeld, B., Thyroxine concentration in human milk. *J. Clin. Endocrinol. Metab.* **45**, 171-173 (1977).
4. Tenore, A., Parks, J. S., and Bongiovanni, A. M., Relationship of breast feeding to congenital hypothyroidism. In *Recent Progress in Pediatric Endocrinology (Serono Sympos., Vol. 12)*, 1977, pp 213-218.
5. Varma, S. K., Collins, M., Row, A., et al., Thyroxine, triiodothyronine and reverse-triiodothyronine concentrations in human milk. *J. Pediatr.* **93**, 803-806 (1978).
6. Sato, T., and Suzuki, Y., Presence of triiodothyronine, no detectable thyroxine and reverse triiodothyronine in human milk. *Endocrinol. Jpn.* **26**, 507-513 (1979).
7. Tsuyusaki, T., Sasaki, N., Miyamoto, S., et al., *12th Annu. Meeting Jpn. Pediatr. Endocrinol. Soc.*, 1978.
8. Weeke, J., and Ørskov, K., Evaluation of thyroid function. *Rec. Adv. Clin. Biochem.* **1**, 111-128 (1978).
9. Obregón, M. J., Pascual, A., Mallol, J., et al., Evidence against a major role of L-thyroxine at the pituitary level: Studies in rats treated with iopanoic acid. *Endocrinology* **106**, 1827-1836 (1980).
10. Weeke, J., and Ørskov, K., Synthesis of ¹²⁵I monolabeled 3:5:3'-triiodothyronine and thyroxine of maximum specific activity for radioimmunoassay. *Scand. J. Clin. Lab. Invest.* **32**, 357-360 (1973).
11. Kjeld, J. M., Kuku, S. K., Diamant, L., et al., Production and storage of ¹²⁵I-thyroxine and ¹²⁵I-triiodothyronine of high specific activity. *Clin. Chim. Acta* **61**, 381-389 (1975).
12. Galton, V. A., and Pitt-Rivers, R., A quantitative method for the separation of thyroid hormones and related compounds from serum and tissues with an anion-exchange resin. *Biochem. J.* **72**, 310-318 (1959).
13. Bellabarba, D., Peterson, R., and Sterling, K., An improved method for the chromatography of iodothyronines. *J. Clin. Endocrinol. Metab.* **28**, 305-307 (1968).
14. Kessler, S. W., Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: Parameters of the interaction of antigen-antibody complexes with protein A. *J. Immunol.* **115**, 1617-1624 (1975).
15. Snedecor, G. W., *Statistical Methods*, 5th ed., Iowa State University Press, Ames, IA, 1956.
16. Tenore, A., Koldovsky, O., Parks, J. S., et al., Problems in the measurement of thyroid hormones in milk. 60th meeting Am. Endocrine Soc., 1978, Miami, FL. Abstract 91.
17. Rosner, W., Beers, P. C., Awan, T., and Khan, M.S., Identification of corticosteroid-binding globulin in human milk: Measurement with a filter disk assay. *J. Clin. Endocrinol. Metab.* **42**, 1064-1073 (1976).
18. Pearlman, W. H., Skrzynia, C., Hampel, M. R., et al., The level of corticosterone-binding proteins in rat milk and coincidental serum, and the dissociation rates of the corticosterone-protein complexes. *Endocrinology* **108**, 741-746 (1981).
19. Mallol, J., Obregón, M. J., and Morreale de Escobar, G., Determinación de yodo y tiroxina en leche humana y maternizada. XX Reunión de la Sociedad Española de Endocrinología, Palma de Mallorca, 1981.