Presence of Triiodothyronine, No Detectable Thyroxine and Reverse Triiodothyronine in Human Milk

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Synopsis

Thyroxine (T4), triiodothyronine (T3) and reverse triiodothyronine (rT3) concentrations in human milk were measured by radioimmunoassay in 114 samples obtained from 1 week to 8 months postpartum. Several assay systems applied for the determination of serum thyroid hormone concentration were proved to be unsuitable for human milk, and the method of separating free and antibody-bound hormone by polyethylene glycol was also inappropriate for milk specimens, which tended to give a falsely high value. The binding affinity of T4 to milk was lower than that to serum protein, on which 8-anilino-1-naphthalene sulfonic acid showed no remarkable effect. In spite of the high sensitivity of 100 pg/tube in T4 assay system, no immunassayable T4 was detected in all samples with or without ethanol extraction and trypsin hydrolysates of milk. In contrast, T3 was present in a measurable amount in most of the samples, the mean ± SD value of which was 10 ± 9 ng/100 ml, and those in colostrum were significantly higher than those in matured milk (P < 0.01), whereas rT3 was not detectable in 76 samples tested.

These results indicate that permeability of thyroid hormones through the mammary gland is different between T4 and T3 as well as in placental transport, and human milk cannot be a source of thyroxine supply for the breast-fed infant.

There has been a considerable discrepancy on thyroid hormone concentration in human milk. Man and Benotti (1965) reported that thyroxine-like iodine compounds of milk were extremely low, and recent observation by Varman et al., (1978) also supported this finding although they found variable amounts of triiodothyronine (T3) and reverse triiodothyronine (rT3) by radioimmunoassay. On the other hand, Sack et al., (1977) reported that human milk contained a significant amount of thyroxine (T4) which rose at a level of 4 µg/100 ml between 8 and 48 days postpartum. According to the result, an infant ingesting 150–200 ml/kg/day of breast milk may receive 6–8 µg/kg/day of T4, which is estimated to be the suboptimal therapeutic dose of T4 for an infant with cretinism (Sato et al., 1977). If this is the case, several practical problems arise. First, the content of T4 and T3 in breast milk may be enough to mask the clinical signs and symptoms of cretinism in infancy, and in interpreting serum T4 and/or T3 value of an infant, source of feeding should always be in consideration. Second, the mass screening for congenital hypothyroidism in the newborn period, which has been prevailing over the world, may be influenced considerably by thyroid hormones in the breast milk, so that it would be necessary to change a part of the screening protocol in this respect (Recommendation of the...
American Thyroid Association, 1977). Third, from the therapeutic point of view, breast feeding is recommended to all infant with hypothyroidism especially during the first trimester of infancy, in which thyroid hormones exert the critical effects on the development of the central nervous system (Klein et al., 1972). These problems prompted us to reevaluate the thyroid hormone concentrations in human milk.

Materials and Methods

Human milk was obtained from 114 healthy euthyroid mothers from 1 week to 8 months after delivery. These were arbitrarily divided in four groups by the term postpartum, 0–1 m (n=43, most of them within 1 w), 1–2 m (n=33), 3–4 m (n=22), and 5–8 m (n=16), because the significant difference in T4 and T3 concentrations was reported previously in the lactation period (Sack et al. 1977, Varma et al., 1978). All samples were stored at −20°C in refrigerator until assay.

T4 determination was made by the three different methods; (a) the column T4 method (Oxford), (b) the competitive protein binding method (Tetralute, Ames) and (c) radioimmunoassay (RIA) (RIA-Mat T4 kit, Daichi RI Lab.) (Chopra, 1972). The sensitivity of assay with these methods was 10 ng, 5 ng and 100 pg/tube of T4 respectively. T3 and rT3 concentrations were measured by RIA (RIA-Mat T3 kit, Daiichi RI Lab., T3RIA kit II and rT3RIA kit, Dainabott Lab.) (Chopra, 1974, Lieblich and Utiger, 1972). The efficacy of separating free (F) and antibody-bound (B) fraction of the hormones was tested with three different methods; (a) 25% polyethylene glycol (PEG) (Desbuquois and Aurbach, 1971), (b) 0.25% dextran-coated charcol (DC) (Herbert et al., 1965), (2.5 g charcol and 0.25 g dextran, MW = 200,000 – 300,000, suspended in 0.2 M borate buffer, pH 8.6), and (c) glass-fiber strip coated with anion-exchange resin, Amberlite IRA-400 (resin strip).

Thyroid hormones in milk were extracted with 2 volume of 95% ethanol. Preliminary experiments showed that extraction efficiency of radioactive T4, T3 and rT3 added to milk into 95% ethanol was 86.5±2.2%, 88.6±2.5% and 87.2±4.3% respectively. Assay was done in both extracted and unextracted samples. In the former, the same volume of 63.3% ethanol as samples was added to the standard curves. A double volume of usual serum assay was used with unextracted specimens (20 µl for T4 and 200 µl for T3 and rT3 assay), and thrice or more with ethanol extracts (60 µl for T4 and 300 µl for T3). The final concentration of ethanol was 3.3% in T4 RIA and 13.6% in T3 RIA respectively. rT3 in ethanol extracts was not measured. In addition, T4 was also determined in trypsin hydrolysate of milk. Milk proteins were digested by trypsin (20 mg/ml) (Difco, type I) at 37°C for 20 hr and the whole hydrolysate was extracted with a double amount of 95% ethanol at neutral pH. 60 µl of extract was used for T4 RIA.

To test the recovery of thyroid hormones from milk specimens, authentic preparation of L-T4 (sodium salt, Wako) or L-T3 (Nakarai) was dissolved in a small amount of 0.1 N NaOH, diluted with 0.2 M borate buffer (pH 8.6), which was added to milk at a final concentration of 7.0 and 14.0 µg/dl of T4, and 160 and 320 ng/dl of T3 respectively. T4 concentration was determined with or without ethanol extraction, and T3 concentration was measured with unextracted samples by DC method. As for the comparison of T4 binding affinity to milk proteins with that to serum protein, 10 µl or 100 µl of milk or serum, 1.0 ml of 0.2 M of borate buffer (pH 8.6) with or without 1 mg/ml of 8-aminino-1-naphthalene sulfonic acid (ANS) and 10 µl of 125I-T4 (New England Nuclear, 1200 cpm/µl) were incubated at room temperature for 4 hr. F and B fraction were seperated by 2.5% DC and 2.0 ml of 25% PEG. After centrifugation, radioactivity of total (T) and B fraction were counted with Well-type scintillation counter. Statistical analysis was carried out by Student’s t test.

Results

1) T4 concentration: The column T4 method was found to be unsuitable for milk specimens, because adsorbed components of milk to the resin column made the following elution of T4 fraction difficult, and gave turbidity in eluate, which disturbed colorimetric estimation. The sensitivity of the method (10 ng/tube) also appeared to be inaccurate.

Competitive protein binding assay (Tetrallute) was also proved to be unsuccessful. Adsorption of milk to sephadex particles interfered with chromatography and quantitative 125I-T4 binding to T4-binding globulin (TBG). All estimated values by the method exceeded 20 µg/100 ml.

In T4 RIA system, the addition of 60 µl of 63.3% ethanol to the standard curve did not show any significant impairment in
the sensivity of assay. However, no measurable amount of T4 was detected in all ethanol extracts of milk specimens. Using unextracted milk, there was also no measurable amount of T4 in all samples tested. Furthermore, no significant displacement was observed by adding 20 or 100 µl of unextracted milk directly to the assay system of standard T4 curve (Fig. 1). These indicate the absence of a significant amount of T4 in human milk.

If T4 is really present in a measurable content and yet undetectable in the RIA system, a deduced possibility is that T4 in milk might firmly bind to milk protein, so that no quantitative competition between endogenous and labeled T4 occurs. This possibility was examined by (a) the comparison of T4 binding affinity to milk protein with that to serum protein, and (b) the hydrolysis of milk protein by trypsin. The results were actually against the assumption (Table 1). The binding affinity of T4 to milk protein was much lower than that to serum protein and ANS showed no remarkable effect on the binding. PGE was

![Fig. 1. Effects of the addition of ethanol or unextracted milk on standard curve of T4 RIA. 60 µl of 63.3% ethanol, 20 µl or 100 µl of unextracted milk was added to T4 standard sera. No significant displacement was observed.](image)

### Table 1. Comparison of T4 binding affinity between milk and serum protein

<table>
<thead>
<tr>
<th>F/B separation</th>
<th>Sample</th>
<th>Volume (µl)</th>
<th>No.</th>
<th>Protein-bound T4 without ANS (%)</th>
<th>Protein-bound T4 with ANS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5% Charcol</td>
<td>milk</td>
<td>10</td>
<td>5</td>
<td>17.5±1.0***</td>
<td>18.1±2.0</td>
</tr>
<tr>
<td></td>
<td>serum</td>
<td>10</td>
<td>5</td>
<td>46.6±4.1</td>
<td>17.4±3.0</td>
</tr>
<tr>
<td>25% PEG</td>
<td>milk</td>
<td>100</td>
<td>5</td>
<td>5.3±1.4</td>
<td>4.5±1.3</td>
</tr>
<tr>
<td></td>
<td>serum</td>
<td>100</td>
<td>5</td>
<td>5.2±0.6</td>
<td>4.9±0.8</td>
</tr>
</tbody>
</table>

ANS: 8-anilino-1-naphthalene sulfonic acid (1 mg/ml).
*** P<0.001 vs serum value.

### Table 2. Recovery of thyroid hormones added to human milk

<table>
<thead>
<tr>
<th></th>
<th>No. of samples</th>
<th>Hormone added to milk</th>
<th>Ethanol extraction</th>
<th>Estimated value</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 (µg/dl)</td>
<td>5</td>
<td>7.0</td>
<td>(−)</td>
<td>6.3±0.2</td>
<td>90.0±2.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7.0</td>
<td>(+)</td>
<td>6.3±0.4</td>
<td>90.0±5.3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14.0</td>
<td>(−)</td>
<td>13.6±1.2</td>
<td>97.1±8.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14.0</td>
<td>(+)</td>
<td>12.5±0.9</td>
<td>89.4±6.7</td>
</tr>
<tr>
<td>T3 (ng/dl)</td>
<td>5</td>
<td>160</td>
<td>(−)</td>
<td>159±20*</td>
<td>99.4±12.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>320</td>
<td>(−)</td>
<td>292±40*</td>
<td>91.3±12.4</td>
</tr>
</tbody>
</table>

* The values subtracted endogenous T3 in milk from total T3 concentrations.
found to be an inadequate method for separating F and B fraction. Furthermore, ethanol extracts of trypsin hydrolysates obtained from 20 samples did not contain any detectable T4 by RIA. In contrast, approximately 90% of exogenous L-T4 added to milk could be recovered in this assay system irrespective of the extraction procedure (Table 2). Therefore, on the basis of these findings, it was concluded that no radioimmunoassayable T4 was present in human milk.

2) T3 concentration: Table 3 shows the results of T3 measurement in ethanol extracted and unextracted milk samples by different methods of F/B separation. When 300 µl of 63.3% ethanol was added to T3 assay system (RIA-Mat T3), marked blunting in the standard curve was observed. The value of B0/T decreased from 71.7% to 35.8% and accurate quantitation by this standard curve appeared to be difficult. In samples without ethanol extraction, 13 of 20 specimens contained a measurable amount of T3, the mean ± SD of which was 26 ± 18 ng/100 ml. To confirm this result, another commercially available kit (T3 RIA kit II) was used and efficacy of separating F and B fraction was tested with 25% PGE and 2.5% DC (Fig. 2). As previously mentioned, PGE could not precipitate B fraction quantitatively and most of the estimated samples showed values above 800 ng/100 ml. In contrast, satisfactory separation of F and B fraction was obtained by DC method. Quantitative linearity was noted between 0.1–2.0 ng/ml of T3 concentration. With 200 µl of sample, 0.05 ng/ml of T3 was detectable. By the method, T3 was measurable in 17 of 20
samples, the mean ± SD of which was 22 ± 9 ng/100 ml. This is a comparable value to those obtained by RIA-Mat T3 (Table 3). In addition, recovery of exogenous T3 from milk specimens was also considered to be reasonable by this method (Table 2). Therefore, further determination was done by the DC method with unextracted milk. The mean ± SD value in 114 samples was 10 ± 9 ng/100 ml, ranging from 0 to 40 ng/100 ml (Table 4). Intra-assay variance of duplicated samples was 18.6%, which was relatively great because of low levels of T3 in human milk. Colostrum contained a significantly higher concentration of T3 than matured milk (P<0.01). One month postpartum, no remarkable difference in T3 levels in specimens was observed.

3) rT3 concentration: rT3 was measured with unextracted samples by the same method as T3. The sensitivity of the assay was 30 pg/tube, but rT3 was not detectable in 76 of milk specimens tested.

Discussion

On thyroid hormone concentration in human milk, controversial results have been reported by several investigators. An extremely low level of T4 or T4-like compounds was found by Man and Benotti (1965) and Verma et al. (1978), while similar contents of T4 to serum level in human milk was reported by Strbák et al. (1974) and Sack et al. (1977). The present study demonstrates that several methods for estimating serum T4 concentrations are not always directly applicable to the determination of T4 in human milk. However, in spite of the high sensitivity of the assay system (100 pg/tube), no immuno-assayable T4 was detected in human milk with and without ethanol extraction. In the assumption that this result might be due to some technical error(s), possible causes are listed as follows and their real probability is discussed.

a) Natural degradation of T4 during storage; this is unlikely, because in our experiences, serum T4 is not deteriorated during 2-3 months of storage at -20°C.

b) Low sensitivity of the assay system; the RIA used in the present study can detect 100 pg/tube of T4 in serum.

c) Low specificity of anti-T4 antibody; this possibility is also excluded, because of the good dose-response relation of the standard T4 curve (Fig. 1).

d) The presence of inhibitory factor(s) in milk for binding between T4 and antibody; a possible consequence by inhibitory factor(s) is that 125I-T4 in B fraction decreases, so that an apparent high value should be obtained. This is not the case, and the result of recovery test was also against this possibility.

e) Incomplete F/B separation; in the RIA system used, F fraction is removed by resin strip during the rotating incubation. Incomplete F removal, therefore, results in an apparent low T4 value. However, the direct addition of 100 µl of unextracted milk to the T4 standard curve showed no significant displacement, so that separation of F and B is considered to be quantitative and complete.

f) High binding affinity of T4 to milk; this possibility is also excluded by the compared binding study with serum protein.
(Table 1) and by the result of trypsin hydrolysis of milk proteins.

On the basis of these reasons, we conclude that there exists no radioimmunoassayable T4 in human milk. The previous observation by Sack et al. (1977) was based on using 25% PEG in the separation of B fraction, which is not the acceptable method for quantitative precipitation of B fraction in milk specimens and results in falsely high values. Strbák et al. (1974) suggested that the failure of other authors to find T4 in milk was caused by the use of acid extraction, which produced an alcoholic ester of T4 and T3. This explanation may not be applicable to the failure of detecting T4 in the present study, because no acidification procedure was used in ethanol extraction. At present, the cause of discrepancy is not clear, but it is conceivable that it may be in the difference of methods applied. Their method of T4 determination was the modification of Murphy's protein displacement method (Siersbaek-Nielsen, 1967), the sensitivity of which was reported to be 2 μg/100 ml. This is 100 times lower in sensitivity than the present RIA method. Potter et al. (1959) found that radioactive T4 administered intravenously to rats was excreted in milk, but only in very small amount, one tenth of the plasma level. Tsuyusaki et al. (1978) also reported that no appreciable amount of T4 was detected in human milk by biological assay. These findings were in accordance with our results.

In contrast to negligible T4 concentration, T3 was detectable in human milk at a level of one tenth of serum T3 concentration. Since the addition of ethanol to the standard curve impaired considerably the sensitivity of the assay, and the reasonable recovery of exogenous T3 was obtained without ethanol extraction, later determinations were made with unextracted specimens. By the two different F/B separation methods, that is, the resin strip method, which removed F fraction, and the DC method which measured counts of F, similar values of T3 concentration were obtained. This appears to indicate the adequacy of the determination method. Colostrum contained significantly higher T3 amounts than matured milk, while no appreciable content of rT3 was detected in both. This is contrary to the results described by Varma et al. (1978). They showed 10–500 ng/100 ml of T3 and 1–36.8 ng/100 ml of rT3 concentration in human milk. Their findings suggest that there exists an active secretion process of T3 in the mammary gland. However, this seems rather unlikely because a previous report of Potter et al. (1958) was against this possibility.

In conclusion, we could not find any appreciable amount of T4 and rT3 in human milk, whereas T3 was detectable at a level of one tenth of serum concentrations. This suggests the different permeability between T4 and T3 through the mammary gland as described in placental transport (Fisher et al. 1972). On the basis of the present results, human milk can not be a source of T4 supply for breast-fed infants.

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References