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Capillary blood
hypothyroidism, congenital
radioimmunoassay
thyroxine (T_4)

Thyroxine (T_4) Immunoassay Using Filter Paper Blood Samples for Screening of Neonates for Hypothyroidism

P. REED LARSEN[18] AND KATHY BROSKIN

Division of Endocrinology and Metabolism, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

Extract

A rapid, sensitive radioimmunoassay for thyroxine (T_4) is described which requires a specimen of dried blood on filter paper. One milliliter of glycine-acetate buffer containing anti-T_4 antibody, tracer T_4, and sodium salicylate is added to a tube containing a 1/8-inch dot of the filter paper specimen. After incubation overnight, bound and free hormone are separated by addition of dextran-coated charcoal. Quantitation is obtained using a standard curve prepared from dots of dried blood samples with known T_4 content. The dot remains in the solution throughout the procedure. Recovery of T_4 is 95% and intra- and interassay coefficients of variation are both less than 10%. The mean T_4 content of 983 samples from 3-day-old infants was 189 ± 48 pg T_4/dot (mean SD). This corresponds to the T_4 in 1.5 μl plasma, and thus the estimated plasma T_4 in these infants is 12.6 ± 3.2 μg T_4/100 ml. Nine neonates had repeated samples in which the T_4 content was lower than 2 SD below the mean. All of these infants had normal cord thyroid-stimulating hormone (TSH) concentrations and thus presumably do not have primary hypothyroidism. The method

should be useful for screening neonates (and older infants), since it can be adapted for use with the punch-index machine for automated processing, no prior extraction of T_4 from the dot is required before quantitation, and the small size of the sample allows repeated tests of suspicious results.

Speculation

A method for measuring T_4 in dried blood collected on filter paper is presented which should be useful in large scale screening programs for the detection of congenital hypothyroidism.

The recent development of immunoassays for measurement of T_4 in small quantites of serum has led to the application of these techniques to neonatal screening for hypothyroidism. A frequency of 1 in 8,500 has been suggested for this condition from the recent experience in Pittsburgh reported by Klein *et al.* (4) and a frequency of approximately 1 in 10,000 from the data of Dussault *et al.* (2) in the Quebec population. This relatively high incidence, similar to that reported for phenylketonuria (PKU), would appear to justify intensive efforts at early recognition of the disease so that treatment may be instituted to prevent or ameliorate the mental retardation which is part of the natural course of the untreated disease. The recent reports of Dussault *et al.* (2, 3) and O'Donnell *et al.* (6) have already demonstrated the feasibility of using a measurement of T_4 in capillary blood samples collected on filter paper to screen for hypothyroidism. The present report deals primarily with the methodology of a T_4 assay for screening purposes which may have certain technical advantages over those reported previously.

METHODS AND MATERIALS

SOURCE AND TYPE OF SPECIMEN ANALYZED

With the cooperation of the Magee-Womens Hospital in Pittsburgh, Pennsylvania and after informed consent from each mother, three additional ½-inch filter paper circles were filled with blood from each infant at the time of heel puncture for PKU testing. The samples were usually collected on the third day of life in full term infants but at various ages in premature infants. The filter papers were received in our laboratory with 1–3 days of obtaining the blood samples and were stored either at room temperature or more recently, at 4°.

PROCEDURE FOR ELUTING AND MEASURING T_4 IN DRIED BLOOD SAMPLES

After considerable experimentation, the following procedure was adopted. Some of the considerations used in arriving at this method are discussed in greater detail under *Results*. The basic principles of the immunoassay are those described previously for assay of T_4 in human serum (5).

SOLUTIONS

Glycine-Acetate Buffer. Glycine-acetate buffer (GAB), pH 8.6, was comprised of 0.2 M glycine, 0.13 M sodium acetate.

Antibody Solution. Antibody solution was comprised of GAB containing anti-T_4 antibody (obtained from rabbits immunized with bovine thyroglobulin as described previously (5) at a dilution of 1:15,000, 5 mg/ml of sodium salicylate (to block T_4 thyroxine-binding globulin (TBG) binding, and 4,000–8,000 cpm/ml (but <75 pg) of [^{125}I]T_4 (7).

Preparation of Standard Dots. In this procedure thyroxine (free acid (8)) was dissolved in 0.04 N NaOH at a concentration of 32 μg/ml. The concentration was verified by spectrophotometry using the molar extinction coefficient as described (5). Heparinized blood from a patient with myxedema was enriched with T_4 to yield the following concentrations of exogenous T_4: 0, 2, 4, 6, 8, and 10 μg/100 ml whole blood. After mixing well by inversion, aliquots of each of these standards and the unenriched blood were spotted on filter paper identical with that used in collecting the neonatal specimens. An aliquot of each sample was then centrifuged and the T_4 concentration was determined in the plasma supernatant by immunoassay as described previously (5). In addition, aliquots of the standards were enriched with [^{125}I]T_4 and placed on filter paper to allow determination of the quantities of T_4 eluted.

Charcoal Solution. The following reagents were added to 1 liter of distilled deionized water; 7.65 g sodium chloride, 12.5 g decolorizing neutral carbon (9) and 1.25 g T80 dextran (10). This solution was mixed well and stored at 4°. Dextran-coated charcoal solution for separation of bound and free T_4 was prepared by appropiate dilution of this solution. The optimal dilution is determined experimentally from time to time since different results are obtained with different lots of charcoal. Currently, 200 ml dextran-charcoal solution described above is mixed with 150 GAB.

PROCEDURE FOR ASSAY

A standard ⅛-inch paper punch was used to punch dots of both standard and unknowns (in duplicate) into clean polystyrene tubes, 16 × 125 mm (11). Two additional spots from one of the standards were also punched for use in tubes to which GAB with only salicylate and tracer T_4 added to serve as a control for nonspecific binding. With a Micro-Medic automatic dispensing pipetter (12), 1 ml diluent containing antibody, tracer, and sodium salicylate was added to each tube. The tubes were then vortexed briefly and it was ascertained that the paper dot remained in the solution. The tubes were stored overnight at 4° and the total number of counts in each tube was determined by counting 10 tubes at random. Separation of bound and free [^{125}I]T_4 was obtained by adding 1 ml diluted, cold dextran-charcoal solution, vortexing briefly and allowing the tubes to remain at 4° in an ice-bath for 45 min after addition of the charcoal. In practice, dextran-coated charcoal was added to tubes in groups of 80 (the limit of the centrifuge) using a Bio-Pet (13). The addition of the charcoal solution should take no longer than 5 min. The tubes were then centrifuged at 4° for 15 min at 2,000 rpm in a Sorvall RC-3 refrigerated centrifuge. The supernatant was decanted and a 1-ml aliquot transferred to a polystyrene tube, 16 × 125 mm and counted in an automatic γ counter.

Quantitation was obtained from the binding-displacement curve constructed using the standard dots.

RESULTS

RATIONALE FOR USE OF ⅛-INCH DOT

In Figure 1 are shown samples of specimens received in our laboratory. It has been our experience that the ½-inch circles preprinted on the filter paper are often incompletely filled with blood. For this reason, we elected to use smaller dots and, at first, adapted the method for the ¼-inch dot shown in the middle of the upper row. It became apparent subsequently that even greater sensitivity was both possible and desirable and a method was developed which required only a ⅛-inch dot. Figure 1 shows that even small quantities of blood such as those seen in the specimens on the bottom row still provide adequate samples for at least two, and sometimes three, specimens. Other advantages were found using the smaller dots. First, whereas recovery from the ½-inch spot containing about 38 μl blood was only 77%, approximately 95% of the T_4 can be eluted from a ⅛-inch dot. It was also found that it is not necessary to remove the ⅛-inch dot from the reaction mixture during the process of the assay and thus a time-consuming extraction step is avoided.

In Table 1 are depicted the quantities of blood contained on the ⅛-inch dots using four samples of normal adult blood and five samples of cord blood, both spotted after heparinization. In the normal adult human samples, 2.83 μl whole blood is contained in the dot. The amount of whole blood was determined by prelabeling the blood with [^{125}I]T_4, counting an appropriate aliquot and comparing these counts with those in a ⅛-inch dot. The results shown are the means of quintuplicate samples. Since the distribu-

tion of T_4 is limited to plasma, the results reflect the quantity of plasma contained in the ⅛-inch dot. We have assumed that there is no segregation of the blood cells from the plasma for the purpose of these calculations. In the third column are presented the quantities of [^{125}I]T^4 in microliter equivalents found in the supernatant after overnight incubation under the conditions described under *Methods*. In normal human serum this amounts to quantities of T_4 equivalent to that found in 1.46 μl plasma and represents extraction of 94.9% of the [^{125}I]T_4 present in the dot. In the lower half of Table 1 are presented data obtained from cord blood. Cord blood has a higher hematocrit than that found in adult blood and there appears to be a rough correlation between the hematocrit and the amount of blood found on the ⅛-inch dot (again determined as the quantity of [^{125}I]T_4 per dot). Thus, while there is 2.83 μl whole blood/dot in the four samples of normal blood with a mean hematocrit of 45%, there are 3.45 μl whole blood/dot in the five samples of cord blood. This difference is significant whereas the quantity of plasma per dot is not different. This suggests that it is the quantity of plasma which determines the area of filter paper occupied by the blood rather than the total volume of blood. In any case, the extraction of [^{125}I]T_4 is approximately 95%, indicating that the higher quantities of TBG present in cord blood do not interfere with elution of T_4 from the paper.

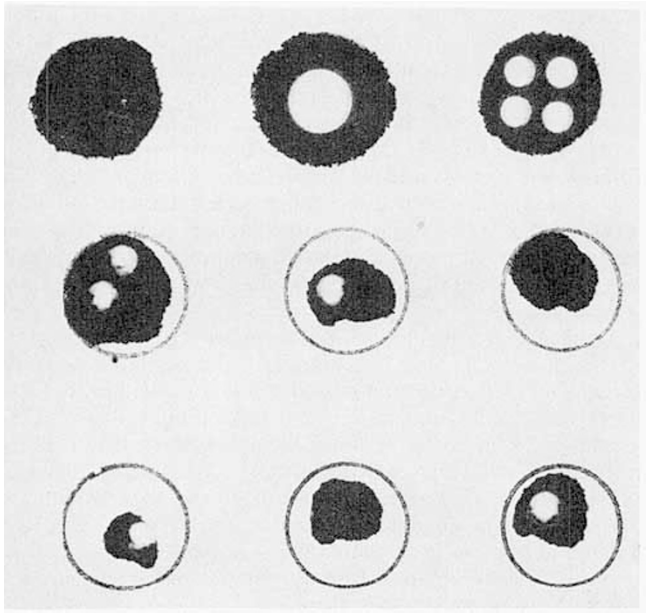

Fig. 1. Representative samples of filter paper containing dried capillary blood specimens received in our laboratory. The printed circle seen in the middle and bottom rows is ½ inch in diameter. The hole in the middle circle of the top row is ¼ inch diameter while the remainder are ⅛-inch dots.

A major factor which interferes with elution of T_4 is preheating of the sample. In samples autoclaved for 5 min, a procedure used locally before PKU testing, only 5–10% of T_4 is eluted from the filter paper. However, at room temperature or at 4°, we have not observed any decrease in the percent of T_4 eluted from a ⅛-inch dot over a period of as long as 3 months and after 5 months at room temperature the recovery was 84.9%.

STANDARD CURVE OBTAINED USING ⅛-INCH DOTS

In Table 2 are presented data obtained from the standard curve prepared as described under *Methods*. The antibody was diluted to allow more precise estimates of T_4 values in the lower portion of the normal range than at higher levels. Thus, the displacement curve slope decreased at higher T_4 levels. The plasma T_4 concentrations of these various standards are shown in the right-hand column. The amount of plasma T_4 eluted from the dot corresponded to that contained in 1.5 μl plasma.

The use of whole blood in the measurement of T_4 results in an increase in the nonspecific binding of T_4 due to some substance, presumably hemoglobin, contained in or on the erythrocytes. This appears as an absolute increase of approximately 10% in the blank tubes (containing no antibody). When 1.5 μl serum (as opposed to whole blood) are added to each tube, the blank is 4—5%. The difference in nonspecific binding is not caused by the presence of

Table 2. *Displacement curve using standards prepared from 1/8-inch dots*

T_4/tube, pg	% [^{125}I]T_4 bound, mean ± SD	Plasma T_4, μg/100 ml
22	68 ± 2	1.5
80	53 ± 1	5.3
132	45 ± 2	8.8
184	40 ± 1	12.3
236	36 ± 1	15.7
283	35 ± 1	18.9
Blank (no antibody)	14 ± 1	

Table 1. *Quantities of blood and T_4 extraction using 1/8-inch dots*

Sample	Hct, %	Whole blood, μl	[^{125}I]T_4 extracted, μl plasma	Recovery [^{125}I]T_4, %
Heparinized normal blood				
1	44	2.68	1.44	95.9
2	46	2.90	1.49	94.5
3	46	2.91	1.47	93.9
4	45	2.83	1.45	95.4
Mean ± SD	45 ± 1	2.83 ± 0.11	1.46 ± 0.02	94.9 ± 0.9
Heparinized cord blood				
1	50	3.25	1.54	95.2
2	51	3.38	1.56	93.4
3	51	3.26	1.54	93.8
4	57	3.54	1.46	95.4
5	62	3.81	1.43	96.4
Mean ± SD	54 ± 5[1]	3.45 ± 0.23[2]	1.51 ± 0.06 (NS)[3]	94.8 ± 1.2 (NS)

[1] $P < 0.025$ for significance of difference between heparinized normal and cord blood by unpaired t test.
[2] $P < 0.005$.
[3] Not significant.

the paper dot *per se* since removing the dot before separation of bound and free T_4 has no effect on the blank and addition of blank paper dots plus serum is also without effect. The percent bound figures presented in Table 2 have not been corrected for nonspecific binding, but this is not different whether the dots are obtained from adults or neonates, nor does it differ from sample to sample.

INTRA- AND INTERASSAY VARIATION

In Table 3 is shown the coefficient of variation within and between assays. The coefficient of variation is 7—10% both within and between assays and is similar to the coefficient of variation of 8% in T_4 immunoassays using serum or plasma (5). Analysis of heparinized cord blood T_4 concentrations using the dots agreed well with the predicted value determined by T_4 immunoassay of an aliquot of plasma. In Table 4 are presented data from a systematic study of the correlation between T_4 concentrations determined directly from plasma with the calculated value based on the dot T_4. Plasma and dried capillary blood (from a fingerstick) were obtained simultaneously from normal adults or from patients with thyroid disease. The plasma T_4 was computed from the dot T_4 content (C) and also measured directly in plasma by radioimmunoassay (M) (5). In 24 samples, from subjects with a broad range of plasma T_4 concentrations, there is an excellent correlation between the results obtained by the two techniques. The mean difference of 0.4 μg T_4/100 ml between the measured and calculated values suggests that there is a tendency to underestimate the T_4 value using fingerstick specimens, but the difference is small. It can be seen that the estimates of T_4 in patients with hypothyroidism are clearly lower than results obtained from euthyroid and hyperthyroid subjects.

RESULTS OBTAINED TO DATE IN SAMPLES FROM NEONATES

The mean T_4 in 983 neonates is 189 ± 48 pg of T_4/dot (mean ± SD). However, 7% of the values are greater than 283 pg although they were entered as such in the calculation. The estimated plasma T_4, assuming 1.5 μl plasma equivalent/dot is 12.6 μg/100 ml. The standard deviation corresponds to 3.2 μg T_4/100 ml and thus 2 SD below the mean would be equivalent to a plasma T_4 concentration of 6.2 μg T_4/100 ml (93 pg/dot). In these first approximately 1,000 samples duplicate analyses have been carried out with excellent agreement. Specimens in which at least one value was less than 93 pg have been repeated and the results of both assays on these specimens are presented in Table 5. The results are grouped based on initial individual and final mean T_4 results which were concordant and both less than 93 pg/dot. Of those, seven had low values on repeat testing, although *subject* A_7 had a single high value in the second assay. It is of interest that *infants* A_3, A_5, and A_7 were all premature. The other four infants on repeated testing proved to have results that were >93 pg. In the case of the 14 infants whose initial results were discrepant, that is, one value above and one value below 93 pg/dot 2 were found to be persistently below 93 pg on repeat testing whereas 11 had mean T_4 values on subsequent testing which placed them within 2 SD of the mean. One subject, E_1, had persistently discrepant results and although the mean T_4 was >93 pg/dot, no reason for the discrepancy was apparent. In the last column of the table are shown cord TSH values in these infants measured in a parallel screening program (4). Despite T_4 values which are less than 93 pg/dot in nine subjects (*groups A* and *C*) no TSH values were elevated. In addition, during the period of time covered by these assays, no infants with suspected congenital primary hypothyroidism have been recognized using the cord serum TSH screening procedure. We have not observed any T_4 values which were lower than 3 SD below the mean (<45 pg/dot).

Table 3. *Intra- and interassay variation*

Blood sample	*n*	pg/dot (mean ± SD)	CV, (%)[1]
Intra-assay			
Cord 1	9	150 ± 11	7.3
Cord 2	9	175 ± 19	9.2
Cord 3	9	186 ± 15	8.0
Interassay,			
Normal	11	175 ± 15	8.6

[1] Coefficient of variation, SD/mean.

Table 4. *Recovery of thyroxine using dried capillary blood obtained by fingerstick from adults*[1]

	Thyroxine			
Sample	pg/dot	Calculated[2] plasma T_4, μg/100 ml (C)	Measured plasma T_4, μg/100 ml (M)	ΔM − C, μg/100 ml
1	<22	<1.5	<1.0	0
2	<22	<1.5	<1.0	0
3	<22	<1.5	<1.0	0
4	<22	<1.5	<1.0	0
5	38	2.5	2.2	−0.3
6	47	3.1	4.8	+1.7
7	60	4.0	4.4	+0.4
8	70	4.7	4.6	−0.1
9	73	4.9	5.8	+0.9
10	78	5.2	4.5	−0.7
11	84	5.0	5.0	+0.9
12	86	5.7	5.8	+0.1
13	120	8.0	8.6	+0.6
14	130	8.6	8.9	+0.3
15	133	8.9	8.1	−0.8
16	134	8.9	7.8	−1.1
17	140	9.3	9.1	−0.2
18	148	9.9	10.1	+0.2
91	162	10.8	11.6	+0.8
20	170	11.3	10.8	−0.5
21	172	11.4	10.2	−1.2
22	200	13.3	15.6	+2.3
23	232	15.5	19.1	+3.6
24	265	17.6	19.1	+1.5

[1] Mean ± SEM of M − C = +0.4 ± 0.2 μg T_4/100 ml.
[2] Assuming T_4 equivalent to 1.5 μl plasma.

DISCUSSION

The T_4 method described above is rapid, sensitive, and reproducible. The features which are of interest with regard to a screening program are first, the use of a ⅛-inch dot which permits synthesis of this test with current automated methods available for processing filter paper specimens. Second, the smaller quantities of blood required allow frequent repetition of suspicious values. In addition, we have experienced a considerable variation in the completeness of filling of the circles. A ⅛-inch dot eliminates the necessity for prior extraction of T_4 from the filter paper, thus avoiding a time-consuming step in the procedure.

The mean plasma T_4 concentration calculated from the dot T_4, 12.6 μg/100 ml, is lower than the value of 17.2 μg/100 ml which we obtained from capillary plasma in 23 infants aged 3 days (1). Some of this difference could be a result of the truncation of the extreme upper range since about 7% of the values were 283 pg. Although the volume of plasma per dot is more constant than the volume of whole blood, published data from other laboratories are available only for the latter. A comparison of the computed T_4 concentrations using this data are presented in Table 6. The results of

Table 5. *Evaluation of neonatal samples where one or both initial results were <93 pg/dot*[1]

Category and subject	T_4 pg/dot									Cord TSH, μU/ml[2]
	Assay 1			Assay 2			Assay 3			
	1	2	Mean	1	2	Mean	1	2	Mean	
A. Initial and final value <93 pg										
1	91		91	50	45	48				11
2	61	82	72	95	[3]	95				9
3	60	90	75	70	50	60				18
4	85	95	90	80	70	75				17
5	75	85	80	45	55	50				25
6	80	88	84	95	105	100	75	85	80	11
7	65	70	68	205	70	138	70	[3]	70	23
B. Initial values <93 pg; final value >93 pg										
1	49	48	49	70	120	95	170	100	135	53
2	84	78	81	105	110	108	115	105	110	
3	77	86	82	115	115	115	110	125	118	
4	75	80	78	90	100	95	105	105	105	3
C. Initial values discrepant; final value <93										
1	95	35	65	55	70	63	80	95	88	18
2	115	75	95	65	75	70	75	65	70	29
D. Initial values discrepant; final value >93										
1	86	120	103	100	85	93				
2	144	39	92	90	160	125				
3	125	82	104	75	105	90				
4	149	82	116	80	110	95				
5	143	83	113	130	80	105				
6	101	72	87	125	170	148				
7	143	82	112	115	55	85	85	115	95	18
8	60	>283	>60	255	>283	>255				
9	87	152	120	150	85	118				
10	99	79	89	150	150	150				
11	75	110	92	125	140	133	155	105	130	
E. Initial values discrepant; final values discrepant										
1	81	206	144	60	125	93	150	40	95[3]	

[1] TSH: thyroid-stimulating hormone.
[2] By Dr. T. P. Foley, Jr.; normals <2.5–60 μU/ml.
[3] Quantity not sufficient for further repetition.

Table 6. *Comparison of whole blood T_4 concentrations determined from dried filter paper spots in various laboratories*

Laboratory	*n*	dot diameter, inches	T_4/sample, pg	Whole blood/ sample, μl	Recovery, %	Whole blood T_4, μg/100 ml
Dussault (2, 3) (Quebec)	36,000	1/2	1,610	40	72	5.6
O'Donnell (6) (Toronto)	1,300	1/2	2,250	31	100	7.3
Larsen (Pittsburgh)	983	1/8	189	3.5	95	5.8

Dussault (2, 3) and those reported here are virtually identical, but both are lower than those of O'Donnell *et al.* (6). The reasons for the latter discrepancy are not clear and may involve differences in the calculated whole blood per dot.

The results of determinations of T_4 in dried blood obtained by fingerstick from adults indicates that the method readily identifies samples with low plasma T_4 concentrations. If T_4 values are low in congenitally hypothyroid children, they should be recognized readily. In addition, the simplicity and ready availability of the method suggests that it might be incorporated into a routine postnatal screening program to identify infants with less severe hypothyroidism.

The application of this method on a larger scale is currently underway in collaboration with centers receiving larger numbers of samples than we are able to obtain locally. At the present time, a single technician can perform approximately 700 assays/week in duplicate including the determination of each individual result. Based on our results to date, we have adopted the following tentative plan for large scale screening. First, only single samples of each patient will be assayed. With one exception (Table 5, *group C, subject 2*), a cutoff point of 100 pg would not have resulted in misclassification of any of the infants tested. Rather than calculating each individual sample, we are currently determining the actual T_4 only in dots with T_4 content less than 132 pg (the third standard). We have also eliminated the two higher standards from the standard curve since these levels cannot be accurately quantitated at the antibody dilution optimal for detection of the smaller quantities of T_4. Specimens with T_4 values less than 100 will be repeated in duplicate and follow-up data obtained on all subjects with T_4 values lower than 2 SD below the mean (93 pg/dot). Any infants with T_4 values less than 3 SD below the mean (<45 pg/dot) will be recalled immediately for serum T_4 and TSH testing. Based on our present experience, this would amount to recall of the patient for a new specimen for T_4 determination in about 0.9% (9/983) of the population. With the present estimates of the frequency of congenital hypothyroidism, only 1 of 80—90 such infants would prove to be hypothyroid. However, until more precise data is available regarding the T_4 concentrations at 3 days in infants with congenital hypothyroidism, it appears advisible to accept this rather high rate of false positives for pilot study purposes.

T_4 values were obtained at age 4—6 weeks for *subjects* A_1, A_3 and A_5 and all were normal. Since two of these infants were premature, the low initial values may reflect hypoproteinemia. The normal results of the cord TSH values give us reasonable confidence that none of the remainder of the infants in question have primary hypothyroidism. The more aggressive follow-up approach outlined above is anticipated in conjunction with our studies of larger numbers of patients.

SUMMARY

A method has been described for immunoassay of T_4 using a 1/8-inch dot of filter paper containing dried capillary blood. The dots can be incubated directly in the antibody-tracer-sodium salicylate solution without prior extraction. Recovery to T_4 is 95% and corresponds to the T_4 contained in 1.5 μl plasma. There is an excellent correlation of dot T_4 values with plasma T_4 values measured directly in adults with low, normal, and elevated serum T_4 concentrations. The mean dot T_4 in 983 samples from 3-day-old infants was 189 $\pm$ 48 (SD) pg T_4/dot corresonding to a plasma concentration of 12.6 $\pm$ 3.2 μg T_4/100 ml. Although nine neonates were found to have dot T_4 values <93 pg/dot (plasma T_4 <6.2 μg/100 ml), none had elevated cord TSH values. This appears to be a feasible method for screening the neonate for congenital hypothyroidism and a larger pilot study to determine the appropriate dividing line between the normal and high risk population is currently underway.

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18. Requests for reprints should be addressed to: P. Reed Larsen, M.D., Thyroid Unit, Peter Bent Brigham Hospital, 721 Huntington Ave., Boston, Mass. 02115 (USA).
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