Zone Electrophoresis in Filter Paper of Serum I^{131} After Radioiodide Administration.* (19930)

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Recent observations in our laboratory(1) have shown that the serum of patients who have received large doses of radioiodide may contain variable quantities of an iodinated substance which is insoluble in butanol and from which thyroxine, diiodotyrosine, and iodide are liberated by alkaline hydrolysis. The data suggested that this substance might be thyroglobulin, or polypeptides resulting from breakdown of thyroglobulin, and the appearance of this material in the blood was related to the amount of radiation delivered to the normal or neoplastic thyroid tissue. Tong *et al.*(2) have made similar observations in rats. Several recent reports have described the behavior of the serum I^{131}, obtained after administration or radioiodide to patients, during zone electrophoresis of the serum in filter paper(3-5). The major portion of the serum organic I^{131} was found in a single area, having a mobility comparable to that of the alpha-globulins, although there was some variation in these reports as to whether the mobility was that of the $alpha_1$-globulins, the $alpha_2$-globulins, or intermediate between the two. A smaller portion of the radioactivity appeared in the albumin area. The patients used in these electrophoretic studies had received from 5 to 100 millicuries of radioiodide prior to the time the serum was examined. Insufficient data (*e.g.*, amount of I^{131} retained by the thyroid tissue, and estimated bulk of thyroid tissue) were given to permit evaluation of the destructive effect of the I^{131} doses. Although it is not likely that the amounts of I^{131} used in the patients with Graves' disease (5-20 millicuries) would have caused release of "non-thyroxine iodine" into the circulation, it is distinctly possible that the larger doses might have done so.

The following studies were undertaken to determine the electrophoretic mobility of the "non-thyroxine iodine" in serum following large doses of I^{131}.

Methods. Radioiodide (I^{131}) was obtained from Oak Ridge and administered orally, with 10 micrograms or less of carrier iodide, to 5 patients. Details with regard to diagnosis, amount of I^{131} administered, and estimated radiation to thyroid tissue are given in Table I. Measurement of I^{131} in the urine and calculation of the approximate radiation delivered to thyroid tissue during the second 24 hours were carried out as previously described(1). Serum was obtained from 4 to 9 days after the I^{131} was administered. In one patient (Case 1) serum was obtained 2 minutes after intravenous administration of 60 micrograms of I^{131}-labeled l-thyroxine.§ The serum was extracted with n-butanol, directly in some instances(1), and after precipitation of the serum proteins with trichloroacetic acid in others(2). When essentially all of the serum I^{131} is thyroxine, approximately 70 to 85% of the I^{131} is butanol-soluble by the direct method, and 90 to 100% is butanol-soluble by the trichloroacetic acid method. *Chromatographic analysis* was car-

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§ l-Thyroxine, obtained through the courtesy of Dr. John Mote of Smith, Kline & French Laboratories, Philadelphia, Pa., was labeled with I^{131} by Dr. G. Gleason of Abbott Laboratories, North Chicago, Ill. Its identity as thyroxine and its purity were confirmed in our laboratory by chromatographic analysis in the butanol-amyl alcohol-ammonia system, in 80 per cent phenol in water, and in butanol-acetic acid-H_2O (75:10:15)(6).

TABLE I. Summary of Data on 6 Patients and Serum Radioiodine Studies.

Case No.	Clinical data*	I^{131} dose (millicuries)	Approx. radiation to thyroid tissue in 2nd 24 hr (rep β)	Serum sample (days after dose)	BuOH solubility (% of total)	Chromatography (% of total in thyroxine band)	Fractional precipitation (% of total soluble in 1.75 M PO_4 buffer)	Iodide (% of total not precipitated with protein by 2.98 M PO_4 buffer)
1.	Athyreotic Angina pectoris (i.v. l-thyroxine)	0.5 (l-thyroxine)	—	(2 min.)	71† 96‡	93	—	—
2.	Graves'disease	20	5340	5	89‡	94	71	4
3.	Euthyroid Hypertensive heart disease Impaired renal function	9	5150	4	76‡	—	72	25
4.	Athyreotic Functioning thyroid carcinoma with bone metastases	150	3870	7	61†	80	74	3
5.	Athyreotic Functioning thyroid carcinoma with skull and soft tissue metastases	180	16500	6	38†	32	53	15
6.	Athyreotic Functioning thyroid carcinoma with bone and soft tissue metastases	207	15500	9	29‡	15	38	5

* Case 1 received a thyroidectomizing dose of I^{131} (50 millicuries) 6 mo prior to this study. Case 4 had had a total thyroidectomy followed by 2 large doses of I^{131} (>100 millicuries). The last dose was given 10 mo prior to this study. Case 5 received 247 millicuries of I^{131} 7 mo prior to this study. Case 6 received 175 millicuries of I^{131} 5 mo prior to this study.

† Direct method of extraction.

‡ Trichloroacetic acid method of extraction.

ried out by direct application of untreated serum to a narrow filter paper strip (Fisher E & A qualitative paper) and development in a system containing 70 parts n-butanol and 30 parts primary amyl alcohol, equilibrated with 2N ammonium hydroxide.[1] Approximately 50 μg of unlabeled thyroxine was streaked at the origin prior to application of the serum. Analysis for radioactivity and stable thyroxine were carried out as previously described(1). *Zone electrophoresis* in filter paper was performed by the methods of Kunkel and Tiselius(7) with slight modifications. The upper glass plate was raised about 0.5 cm above the paper and the sides were sealed with masking tape. The paper was pressed flat on the lower glass plate with a roller. The electrode vessels contained 1M KCl and were connected to the buffer vessels with agar-KCl bridges. Barbital buffer at pH 8.6, ionic strength 0.1, was used. Whatman 3 MM filter paper was cut into 3 cm by 30 cm strips, and two or, occasionally, three parallel strips were used for each run. Approximately 0.02 cc serum were applied to each strip. The potential across the platinum electrodes was 90 to 100 volts in 4 instances, and was 75 volts in Case 1 and 200 volts in Case 4. The current varied from 4 to 6 milliamperes among the individual runs and was maintained for 14 to 25 hours at room

[1] 70 cc n-butanol, 30 cc primary amyl alcohol and 100 cc 2N ammonium hydroxide were mixed and allowed to separate. The aqueous layer was placed in the bottom of a 15 cm x 45 cm covered glass cylinder. The alcohol layer was placed in a smaller vessel which fitted inside and on the bottom of the cylinder. After several hours were allowed for equilibration, the paper was suspended so that its end dipped into the alcohol mixture.

temperature. After staining of the paper by the technic of Durrum(8) (as modified by Kunkel and Tiselius) radioautographs were prepared by exposure to an x-ray film with a thin sheet of paper interposed, and or the paper strip was cut into 0.5 cm segments and radioactivity measured with a thin mica window Geiger-Mueller tube. Since the strips were stained and washed prior to analysis for radioactivity, only protein-bound I^{131} was measured. The overall length of the serum patterns varied from 12 to 20 cm. In one instance (Case 6), electrophoresis was continued for 72 hours (length, 27.5 cm) in order to obtain more discrete protein bands.

Results. The butanol solubility and chromatographic analysis of the serum I^{131} recorded in Table I, indicate that in 4 instances (Cases 1, 2, 3, 4) most of the serum I^{131} (80% or more) behaved like thyroxine. In the remaining 2 patients (Cases 5, 6), a large proportion of the serum I^{131} (more than 60%) was insoluble in butanol and remained at the origin on the chromatographic strips. Representative chromatographic patterns are shown in Fig. 1.

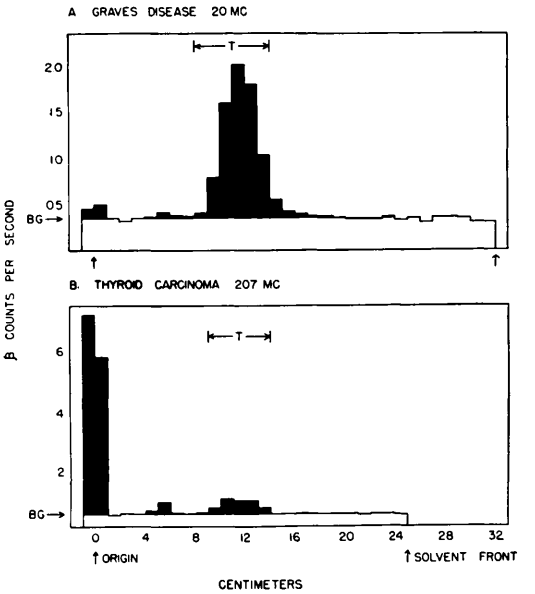

FIG. 1. Chromatographic analysis of untreated serum, butanol-amyl alcohol-ammonia system. A. Case #2. B. Case #6. The blackened area represents radioactivity in excess of background (BG). "T" indicates the position of stable thyroxine. The unlabeled band at approximately Rf 0.2 is in the position of inorganic iodide.

Zone electrophoretic analysis of these serums, however, revealed similar patterns in all instances. Most of the radioiodine (approximately 85%) appeared in a single band which had a mobility intermediate between that of the $alpha_1$- and $alpha_2$-globulins, but overlapped both of these bands. The remainder of the radioiodine had a mobility similar to that of the serum albumin. Under the conditions used in these experiments, there was no demonstrable difference between the mobility of the butanol-soluble and the butanol-insoluble serum I^{131}, except that in one patient (Case 6) there was relatively more of the serum I^{131} (approximately 25%) in the albumin region. Representative electrophoretic patterns are shown in Fig. 2.

In order to test the possibility that these two forms of the serum I^{131} might be associated with a single protein, an attempt was made to separate them by fractional precipitation of the serum proteins. A modification of the method of Derrien, utilizing increasing concentrations of phosphate buffer, pH 6.5, was used(9-11). In Table I, the proportion of the serum I^{131} which was soluble at a phosphate buffer concentration of 1.75 Molar[¶] is recorded. It may be seen that when the serum I^{131} was largely butanol-soluble, more than 70% of the radioiodine remained in solution in 1.75 Molar phosphate buffer (Cases 2, 3, 4); whereas relatively smaller amounts of radioiodine were soluble at this salt concentration when butanol-insoluble I^{131} was present in the serum (Cases 5, 6). The greater the proportion of butanol-insoluble I^{131} in the serum, the more I^{131} was recovered in the phosphate buffer precipitate. When the protein precipitated by 1.75 Molar phosphate buffer in Case 6 was redissolved in distilled water and subjected to zone electrophoresis, the I^{131} was found to have the same mobility as in whole serum, although little if any of the alpha globulins of the serum was present.

[¶] 1.75 Molar phosphate buffer concentration was attained by adding an equal volume of a stock solution (an equimolecular mixture of K_2HPO_4 and KH_2PO_4, 3.5 Molar), to serum diluted with water so that the final serum dilution was 1:30. This was, therefore, a 50% solution of the stock solution.

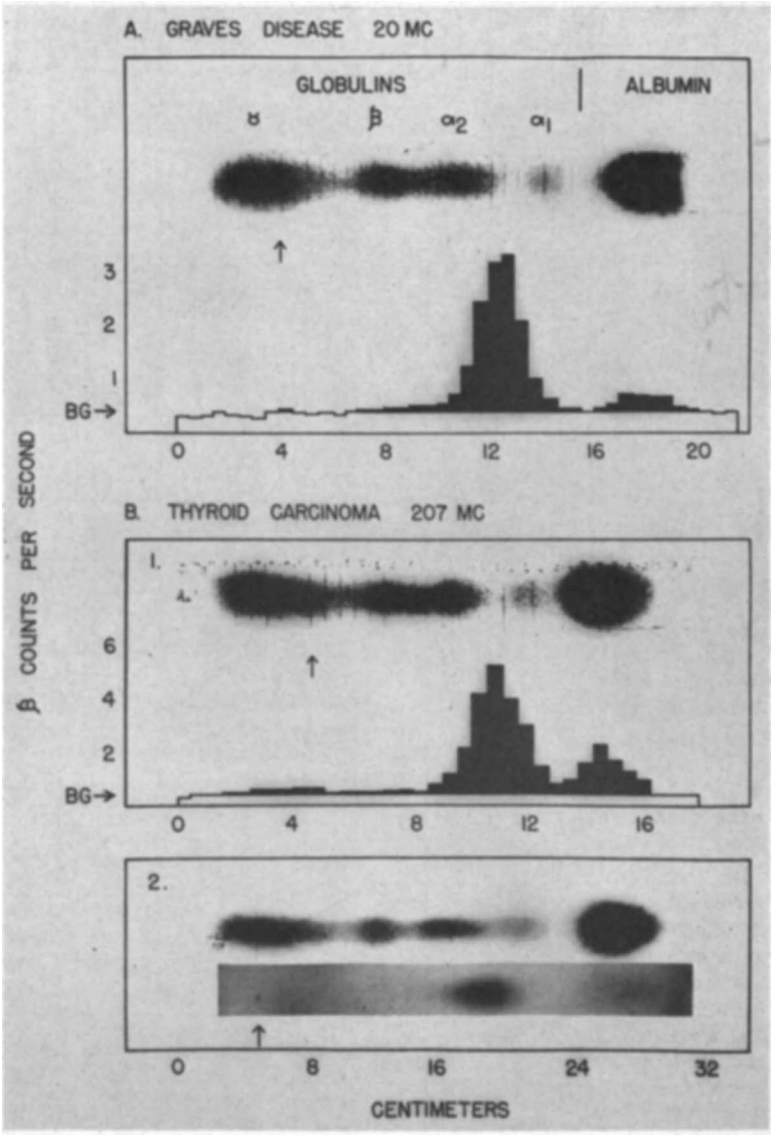

FIG. 2 Photographs of electrophoretic patterns of the serum proteins from Case #2 (A), and Case #6 (B) are compared with the location of radioactivity determined by counting 0.5 cm segments of the strips (A, B1) and by radioautography(B2). The arrows indicate the positions at which the serum samples were placed on the paper. The blackened areas in graphs A and B1 represent radioactivity in excess of background (BG). The small amount of radioactivity apparent in the gamma globulin area in B1 was found in no other instance. The radioautograph in B2 is intended to demonstrate the location of the major band of radioactivity in the alpha globulin region. The gray areas on the remainder of the radioautograph are due to technical problems in its reproduction and not to radioactivity, thus obscuring the band in the albumin region.

This method of fractional precipitation of the serum proteins also enabled estimation of the radioiodide content of the serum by determination of the I^{131} which was not pre-

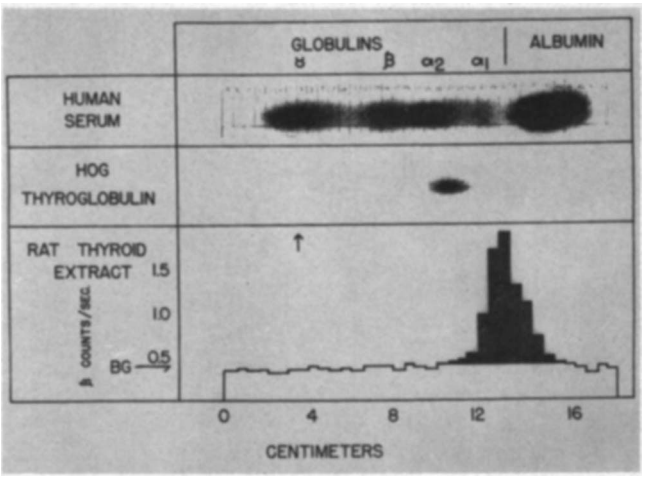

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FIG. 3. Photographs of electrophoretic patterns of: A. Normal human serum containing rat thyroid extract, 1 part per 100, and B. Purified hog thyroglobulin. The graph (C) represents the location of the radioactivity in A due to the rat thyroid extract, as determined by counting 0.5 cm segments of the strip. The blackened area in C represents counts above background (BG). In B, a small amount of protein tailing from the origin (arrow) to the major band is not visible in photograph.

cipitated with the serum proteins by 2.98 Molar phosphate buffer(11).** These values are recorded in Table I.

In order to determine whether thyroglobulin might have an electrophoretic mobility comparable to that of the serum I^{131}, thyroglobulin from two animal species was studied as follows:

1. Purified hog thyroglobulin†† was obtained in lyophylized form, containing approximately 25% NaCl. A 1% solution in distilled water was prepared and immediately subjected to zone electrophoresis. Almost all of the protein moved in a single band with a mobility intermediate between $alpha_1$- and $alpha_2$-globulin, as demonstrated by a parallel run of normal human serum (Fig. 3)

2. A crude extract was prepared from the thyroid gland of a rat, which had received 60 microcuries of radioiodide intraperitoneally 48 hours earlier, by grinding the thyroid with 0.5 cc of isotonic saline at 0°C and allowing the larger particles to sediment. Within 15 minutes after the animal was killed, an 0.01 cc aliquot of the extract was subjected to zone electrophoretic analysis. Parallel to this, was a run containing 0.02 cc of a mixture made by adding 0.05 cc of the thyroid extract to 5 cc of normal human serum. The results indicated that the I^{131} in the rat thyroid extract had a mobility intermediate between that of the $alpha_1$-globulin and the albumin in the serum. The mobility of the I^{131} in the thyroid extract-serum mixture is shown in Fig. 3. In the absence of serum, approximately 60% of the I^{131} had an identical mobility, the remainder tailing from this band to the point of origin.

Discussion. These results coincide with the findings of others with regard to the zone electrophoretic pattern of the normal serum I^{131}. It appears that thyroxine is attached to a protein (or proteins) which moves with the alpha-globulins at pH 8.6 but which differs from those making up the bulk of the $alpha_1$-

** 85% by volume of the phosphate buffer stock solution.

†† Purified hog thyroglobulin was obtained through the courtesy of Dr. Robert Kroc, Chilcott Laboratories, Morris Plains, N. J. (Lot No. 91921). It was prepared from hog thyroid extract by precipitation with ammonium sulfate at about pH 6. Ultracentrifuge analysis in the laboratory of Dr. Mary L. Peterman, Sloan-Kettering Institute, showed it to be 75% homogeneous with a sedimentation constant of 17.2 S in a 1% solution.

and $alpha_2$-globulins. Presumably, this protein is present in quite small amounts, and it has a peculiar affinity for thyroxine, since it contains practically all of the thyroxine added to normal serum *in vitro*(3) or administered to a patient by intravenous injection. It is of interest that no difference in electrophoretic mobility was detected between the serum I^{131} in a patient with a normal thyroid gland, a patient with Graves' disease, and a patient with functioning thyroid carcinoma and no normal thyroid tissue.

It is clear, however, that the behavior of serum iodine during electrophoresis at pH 8.6 cannot be used as evidence concerning the identity of the normally circulating thyroid hormone, since the normal, butanol-soluble serum I^{131}, and the butanol-insoluble serum I^{131}, ("non-thyroxine I^{131}") that follows thyroid tissue destruction with radioiodine, have essentially the same mobility. It is possible that under conditions of pH and ionic strength other than those used in this study, a differentiation might be obtained.

The serum I^{131} which appears in the albumin band may be an artifact since there is some evidence that serum albumin is capable of binding thyroxine *in vitro*(12), and it is possible that, during handling of the serum, some of the thyroxine may be released from its specific protein. The finding of relatively more I^{131} in the albumin band in Case 6 may, however, be of significance.

The ability to differentiate between the butanol-soluble serum I^{131} and the butanol-insoluble serum I^{131} by fractional precipitation with phosphate buffer is evidence that the normal "thyroxine-binding protein" in the serum is not identical with a protein released from irradiated thyroid tissue (*e.g.*, that it might be non-iodinated thyroglobulin). This finding also indicates that the radiation-induced "non-thyroxine iodine" in the serum is probably not attached to the normal "thyroxine-binding protein."

Considerations presented elsewhere(1,2) suggest that the "non-thyroxine iodine" may be thyroglobulin. While the identity in the mobility in zone electrophoresis of "non-thyroxine I^{131}" and purified hog thyroglobulin is consistent with this view, it cannot be considered proof, especially in view of the foregoing discussion. The difference in mobility between the purified hog thyroglobulin and the rat thyroid extract may be due to a species difference or to an effect of other substances in the crude thyroid extract. It may be noted that studies with purified hog and beef thyroglobulin in classical boundary electrophoresis systems(13,14) also indicate that this substance has a mobility of the order of that of the alpha globulins of human serum. This is true at pH 7.7 as well as at pH 8.3.

It is still possible, nevertheless, that some or all of the thyroglobulin released from the thyroid tissue may have been broken down into smaller components.

Summary and conclusions. 1. Serum from 6 patients who had received radioiodide or I^{131}-labeled thyroxine was studied by zone electrophoresis in filter paper, at pH 8.6. Most of the I^{131} had a mobility intermediate between the $alpha_1$- and $alpha_2$-globulins, and a small proportion had the mobility of albumin. No differences were detected between thyroxine added to serum (*in vivo*) and the thyroid hormone secreted by a normal thyroid gland, by a Graves' disease thyroid gland, and by a functioning thyroid carcinoma. 2. Two patients had received very large doses of radioiodide and their serum contained large amounts of butanol-insoluble I^{131}, whereas in the other patients the serum I^{131} was largely butanol soluble, and behaved like thyroxine on paper chromatography. These two forms of serum I^{131} had similar mobility on zone electrophoresis, but could be differentiated by fractional precipitation of the serum proteins with phosphate buffer. The radioiodine, therefore, was probably attached, in each instance, to a different protein, and the identity in their mobilities was fortuitous. 3. Zone electrophoresis of purified hog thyroglobulin, and of I^{131}-labeled rat thyroglobulin in a crude thyroid extract, revealed that these substances also had a mobility in the range of the alpha globulins of human serum. This finding is compatible with the previous suggestion that the butanol-insoluble I^{131} ("non-thyroxine I^{131}") may be thyroglobulin, released into the circulation from damaged thyroid tissue.

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Availability of Vitamin E in the Newborn Infant.* (19931)

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The erythrocytes of rats deficient in vit. E may be hemolyzed by dialuric acid(1,2) *in vivo* and *in vitro*. They are also susceptible to the hemolyzing effect of dilute solutions of hydrogen peroxide(3). Vit. E given *in vivo* or added to suspensions of red cells *in vitro* prevents the hemolysis by dialuric acid as well as by hydrogen peroxide.

The hemolytic action of hydrogen peroxide differs in several respects from that of dialuric acid. Hemolysis of cells deficient in vit. E is complete in 15 to 30 minutes with dialuric acid, while with hydrogen peroxide hemolysis progresses only slowly and even after prolonged incubation is usually less marked than with dialuric acid. Moreover, the red blood cells of newborn infants show increased susceptibility to hydrogen peroxide and at the same time complete resistance to dialuric acid. In adults hemolysis of red blood cells by hydrogen peroxide rarely exceeds 5%(3).

The question arose whether the hemolysis in the blood of newborn infants by hydrogen peroxide might be related to vitamin E deficiency and whether addition of vit. E either in the test tube to a suspension of red blood cells, or through ingestion by the newborn infant, or, prenatally, by the pregnant mother, would restore the normal resistance of the erythrocytes toward hydrogen peroxide.

Experimental. Observations were made on normal newborn infants in the Nursery of the Hospital of the University of Pennsylvania. Normal adults served as negative controls while vit. E deficient rats were used as positive controls. *Blood* was collected in 0.9% NaCl-1% sodium citrate solution (about 15 drops of blood in 2 ml) in a 15 ml centrifuge tube. The suspension was centrifuged, the supernatant fluid withdrawn, and the cells made up in a 2.5% suspension (estimating the volume of cells from the graduations on the tube) with a mixture of equal parts of normal saline and phosphate buffer, pH 7.4.† After incubation at 37°C for one hour the tubes were again centrifuged, the supernatant fluid withdrawn, and a 5% suspension of the cells in normal saline made.

* Preliminary report given at Meeting of the Am. Pediat. Soc., Atlantic City 1951, see *Am. J. Dis. Child.*, 1951, v82, 237.

† Phosphate buffer, pH 7.4:

	ml
Potassium monophosphate, 0.2 M	25.0
Sodium hydroxide, 0.2 M	19.7
Water to make	100.0