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SEPARATION OF IODO COMPOUNDS IN SERUM BY CHROMATOGRAPHY ON SEPHADEX COLUMNS

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SUMMARY

The migration of iodothyronines through Sephadex columns is greatly influenced by the pH and ionic strength of the eluents. By controlling these variables, a method has been developed for separating the iodo compounds of serum into four discrete fractions: iodoprotein, iodide, triiodothyronine and thyroxine, during a single passage through a column of Sephadex G-25. The method is relatively rapid (less than 3 h per column run), and can be employed with large samples of serum. Recovery of labeled iodothyronines added to serum is essentially complete. Chromatography of purified labeled compounds showed less than 0.2% deiodination; no conversion of thyroxine to triiodothyronine during the procedure could be demonstrated.

INTRODUCTION

The behavior of aromatic amino acids on columns of highly crosslinked dextran gels, such as Sephadex G-25, may involve two types of interactions. First, aromatic compounds tend to be adsorbed to Sephadex[1-3], and they migrate more slowly than non-aromatic compounds of low molecular weight. This adsorption has been attributed to an interaction between the hydroxy-ether groups which crosslink the dextran chains[4] and substituent groups of the aromatic compound, such as the hydroxyl group of phenols or the halogen of halogenated phenols[5].

Secondly, the behavior of many charged molecules is strongly influenced by ionic strength and by pH. At very low ionic strength, negatively charged molecules tend to be excluded from gel grains, while positively charged molecules are adsorbed[1,3]. These effects have been attributed to the free terminal carboxyl groups of the gel acting as a cation exchanger, and they disappear at higher ionic strength. As for the influence of pH, basic compounds migrate more rapidly in acidic solvents, and acidic compounds more rapidly in basic solvents[2,3]; these effects, which may reflect the size of the layer of water of hydration[3], become less marked as the ionic strength is increased.

The common iodinated amino acids, monoiodotyrosine, diiodotyrosine, triiodothyronine (T3) and thyroxine (T4), as with other aromatic compounds, are adsorbed on Sephadex, and, as with other acidic compounds, their migration is

accelerated by alkaline eluents. At neutral or low pH, the iodotyrosines are retarded, diiodotyrosine more than monoiodotyrosine, and they can be separated[6-10], while iodothyronines are so strongly adsorbed that very large volumes are required to elute them. At high pH (*e.g.*, 0.015 *N* NaOH) the iodotyrosines are both eluted with inorganic iodide, followed by T3, then T4[7,10-13]; a good separation of T3 from T4 can be achieved in this manner.

Attempts have been made to separate iodo compounds in serum by chromatography on Sephadex columns. When serum is passed through a column at neutral pH, most of the T4 and T3 present remains firmly bound to serum proteins, and is eluted with them, so that separations cannot be achieved[14,15]. However, if serum is applied to a column equilibrated with base, T4 and T3 are removed from proteins and bound to the Sephadex[7,11,13,16]. Then, continued elution with base might be expected to result in elution of T3 ahead of T4, as occurs during chromatography at high pH of a protein-free solution containing T4 and T3. In practice, however, with serum as the starting material, it has proved difficult to obtain consistent separations of T4 from T3[7,11,13], and one group working on this problem has apparently turned to a more complex procedure for the analysis of thyroid hormones in serum[17].

We have explored further the behavior of T3 and T4 on Sephadex columns. By paying special attention to the role of ionic strength, a reliable method has been devised for separating T3 and T4 from each other and from other iodinated components of serum during a single passage of serum through a Sephadex column.

METHODS

T4 and T3 labeled with ^{125}I or ^{131}I were purchased from Abbott, and were transferred to dilute solutions of human serum albumin soon after receipt. Pooled human serum was obtained from clinical laboratory discards, care being taken to exclude turbid specimens. Sephadex G-25 fine was purchased from Pharmacia; it was allowed to swell in neutral saline solutions for 12–24 h, then was poured into columns 2.2 or 2.5 cm in diameter. The standard sample was 10 ml of a solution containing equal amounts of serum and of water or buffer, and T4 and T3 labeled with different iodine isotopes. It was discovered that the use of more concentrated serum solutions led to clogging of the column and/or erratic elution patterns of T4 and T3. The usual rate of elution was about 1.5 ml/min. An automatic fraction collector was employed to collect fractions eluted from the column.

Determinations of radioactivity were made in a dual-channel automatic gamma-ray spectrometer. When specimens contained both ^{125}I and ^{131}I, an appropriate correction was made for ^{131}I counts appearing in the ^{125}I channel. Recovery of radioactivity in individual column runs was determined by comparing total eluted counts with counts in an aliquot of the solution from which the sample was taken.

RESULTS

Adsorption of T4 and T3 from serum by Sephadex at high pH

Table I summarizes studies in which serum was applied to columns equilibrated at varying pHs. Complete adsorption of T4 and T3 by the gel occurred at a pH of 11.5, when the sample was at neutral pH, and at a pH of 11.0 when the sample pH

was also adjusted to 11.0. Note also that a partial separation of T4 and T3 could be achieved by pH manipulations, T3 being more readily split from its binding proteins. This phenomenon was not further explored, since it seemed unlikely that such separations would be sufficiently complete for analysis of the small amounts of T3 in normal serum.

TABLE I

BINDING OF T3 AND T4 FROM SERUM BY SEPHADEX

Size of column, 2.2 × 20 cm. Sample, 5 ml of serum + 5 ml of the indicated sample diluent to which [^{131}I]T4 and [^{125}I]T3 were added. Eluting solution, PBS containing 5% serum. Flow-rate, 1 ml/min; fraction size, 5 ml. In this system, T4 and T3, which were not eluted with the proteins of the sample, appeared later after the column pH had dropped to that of the PBS, and were associated with the proteins in the eluting solution. PBS = phosphate-buffered saline.

Equilibration solution	*Sample diluent*	*Proportion eluted with proteins* (%)	
		T4	*T3*
Glycine buffer, pH 10	PBS, pH 7.4	100	69
Dilute NaOH, pH 11	PBS, pH 7.4	41	2
Dilute NaOH, pH 11.5	PBS, pH 7.4	0	0
Glycine buffer, pH 10.5	Glycine buffer, pH 10.5	66	3
Dilute NaOH, pH 11	Dilute NaOH, pH 11	0	0

Elution of T4 and T3 bound to Sephadex

A wide variety of solutions were employed to elute T4 and T3 after they had been adsorbed from serum by Sephadex. With neutral buffers, very large volumes were required; when using phosphate-buffered (0.01 *M*) isotonic saline (PBS) of pH 7.4, about 7 column volumes of solution were required to elute one-half of the T3, and 8.5 column volumes to elute one-half of the T4. When solutions of NaOH, 0.015 *N* or greater, were used as eluent, the iodothyronines were eluted in much smaller volumes, and often were well separated, T3 being eluted ahead of T4 (Fig. 1a).

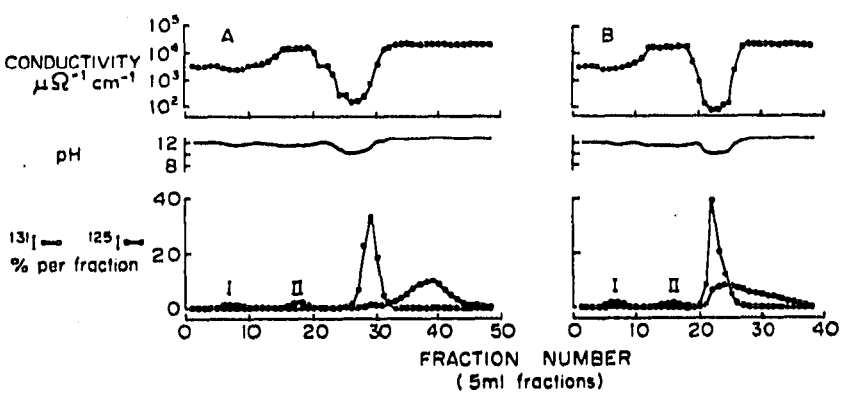

Fig. 1. Elution of iodothyronines from Sephadex with 0.1 *N* NaOH. (a) Column size, 2.5 × 15.7 cm. Equilibration solution, 0.015 *N* NaOH. Sample, [^{125}I]T3 and [^{131}I]T4 in 5 ml of pooled serum + 5 ml of PBS, pH 7.4. Eluents: (1) 20 ml PBS; (2) 0.1 *N* NaOH. Note conductivity drop to 150 $\mu\Omega^{-1}$ cm^{-1} with fraction 27. (b) Column size, 2.2 × 18.3 cm. Equilibration solution, sample and eluents same as in (a). Note conductivity drop to 75 $\mu\Omega^{-1}$ cm^{-1} with fraction 22. In this and subsequent figures, Peak I is near the column void volume and contains iodoprotein contaminating the labeled hormone preparations; Peak II is near the column total volume, and contains inorganic iodide, another contaminant.

However, under apparently identical conditions, the separation was sometimes poor; an example is shown in Fig. 1b. This behavior at first seemed quite capricious, but when the conductivity and pH of the eluted fractions were measured several features became apparent. As was earlier reported by GELOTTE[1], NaOH is retarded on Sephadex columns, migrating less rapidly than neutral salt solutions. Thus, when an NaOH solution follows a neutral salt solution, there will be a zone between the tail of the salt solution and the retarded NaOH front in which the ionic strength is extremely low. This can be seen in Fig. 1. First, because of retardation of NaOH, the lower pH initially associated with the sample is eluted well after the salts of the sample, which are migrating near inorganic iodide (Peak II). Hence, there is very little electrolyte present in the low pH zone, resulting in the low conductivity shown. In analyzing the results from several column runs, the degree of separation between T3 and T4 seemed to be related to the ionic strength in the low pH zone, as follows: when the conductivity fell below 100 $\mu\Omega^{-1}$ cm^{-1}, both T3 and T4 were found in the low pH zone, poorly separated (Fig. 1b); at higher conductivity, T3 was found in the low pH zone, fairly well separated from T4 (Fig. 1a); when the conductivity in the low pH zone was 200 $\mu\Omega^{-1}$ cm^{-1} or higher, T3 appeared after the pH had risen to about 12, and was almost completely separated from T4, similar to the chromatogram depicted in Fig. 3.

The migration of T4 and T3 in solutions of low ionic strength was then examined. As shown in Fig. 2, both the iodothyronines were readily eluted with deionized water, T4 migrating more rapidly than T3, *i.e.*, the reverse of the pattern seen with basic eluents. This may represent the phenomenon mentioned earlier, by which acidic compounds are excluded from gel grains, and more rapidly eluted, at very low ionic strength. Thyroxine, with its lower pH for hydroxyl ionization[18], could be more accelerated by such effects. In any event, a period of rapid migration of T4 while in a zone of low ionic strength seemed adequate to explain poor separations.

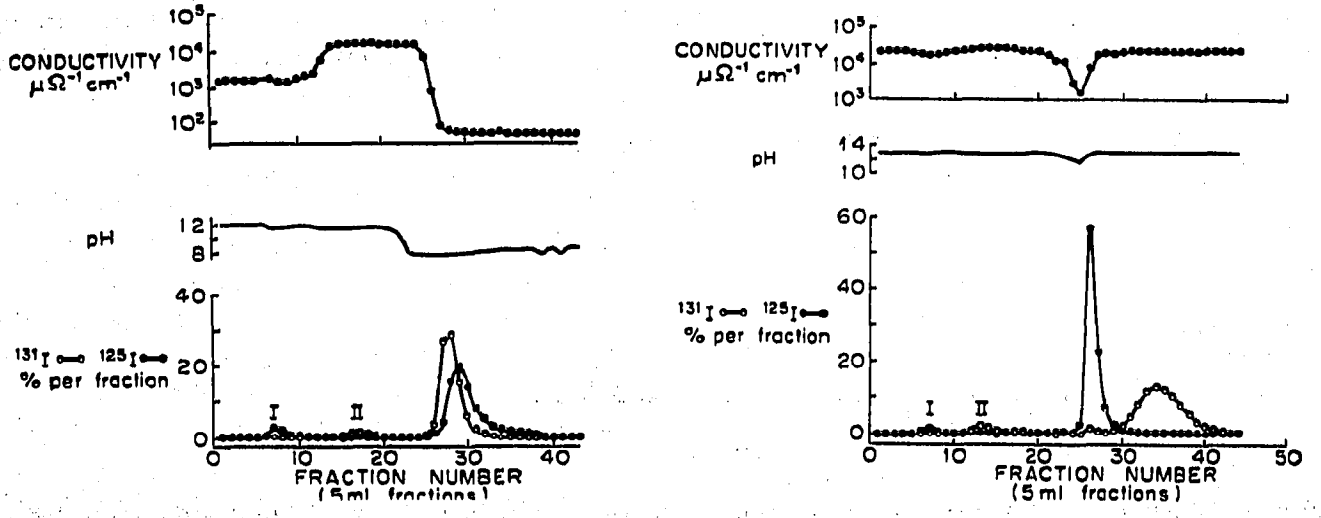

Fig. 2. Elution of iodothyronines from Sephadex with deionized water. Column size, 2.2 × 19.7 cm. Equilibration solution, as in Fig. 1. Eluents: (1) 50 ml of PBS, pH 7.4; (2) 225 ml of deionized water; (3) 5% serum in PBS (serum solution eluted no further radioactivity).

Fig. 3. Elution of iodothyronines from Sephadex with 0.1 *N* NaOH–0.005 *N* NaCl. Column size, 2.2 × 16.8 cm. Equilibration solution, 0.1 *N* NaOH–0.005 *N* NaCl. Sample, same as in Figs. 1 and 2. Eluents: (1) 20 ml of PBS; (2) 0.1 *N* NaOH–0.005 *N* NaCl.

To counteract this effect, small amounts of NaCl were added to the NaOH solutions used for elution, which led to reproducible separations of T3 from T4 (Fig. 3). Also, at NaOH concentrations of 0.005–0.01 *N*, the T3 regularly appeared at the point of pH increase, and was usually concentrated in one tube; *i.e.*, with a sample size of 10 ml, more than 50 % of added T3 was obtained in a 5-ml fraction. This presumably reflects the rapid migration of T3 through the high pH zone, and retardation when it advances into the low pH zone ahead of the NaOH, so that T3 becomes concentrated at the zone of pH change. Other examples of concentration of compounds at the interface between different eluting solutions were given by JANSON[3].

The migration of T3 and T4 through the column was further influenced by changes in pH and in ionic strength, high pH tending to accelerate, and high ionic strength tending to retard, the migration of both compounds (Fig. 4). Although the concentration of T3 at the zone of pH change no longer occurred when eluent solutions had a high NaCl concentration, good separations of T3 from T4 could still be obtained.

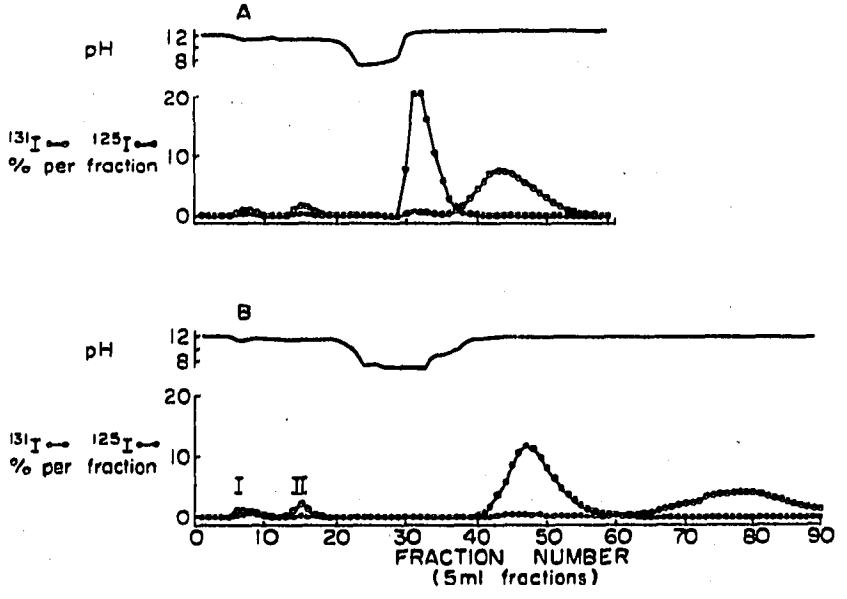

Fig. 4. Effect of NaOH concentration on elution of iodothyronines from Sephadex. Column size, 2.2 × 18.6 cm. Equilibration solution, 0.015 *N* NaOH. Sample, same as Figs. 1–3. In both columns, the sample was followed by 20 ml of PBS. Subsequent eluents were: (A) 0.1 *N* NaOH–0.1 *N* NaCl; (B) 0.015 *N* NaOH–0.1 *N* NaCl.

The exact composition of the solution with which the column was equilibrated prior to applying the sample did not seem to be critical, provided that it contained an NaOH concentration of 0.015 *N* or greater. The composition of the solution used to dilute the sample also seemed to be relatively unimportant; however, all these studies were conducted at sample pHs of 6–8.5. Finally, the practice of washing in the sample with an aliquot of PBS of pH 7.4, which was routinely followed in much of the exploratory work (*cf.* Figs. 1–4), later proved to have no effect on the T3–T4 separation. The final procedure adopted was as follows. The equilibration solution was 0.015 *N* NaOH–0.5 *N* NaCl; the sample diluent, 1 *N* NaCl–0.2 *M* phosphate buffer of pH 6.5; and the eluent, 0.1 *N* NaOH–0.005 *N* NaCl. With a

10-ml sample (5 ml of serum + 5 ml of sample diluent), good separations of T3 from T4 could be achieved with a column 65 ml in volume (2.2 × 17 cm) and a flow-rate of 1.7 ml/min. In a typical run, 200–250 ml were collected in 2–3 h. With shorter columns or more rapid flow-rates, overlap between T4 and T3 increased. Larger columns improved the separation, and on a much larger column, 2.5 × 30.5 cm (150 ml), it was possible to separate T3 from T4 in a sample of 20 ml (10 ml of serum + 10 ml of sample diluent). It seems likely that even larger samples could be accommodated by proportionate increases in column volume.

Degradation of T4 and T3 during chromatography

The stock solutions of labeled T3 and T4 always contained radioactive material eluting at the column void volume (Peak I in the figures) and at the column total volume (Peak II). Peak I behaved like iodoprotein and Peak II like iodide during electrophoresis or paper chromatography. However, when T3 purified by passage through the column was re-chromatographed, it contained very small amounts of iodoprotein (less than 0.2 %) or iodide (less than 0.1 %). Repeat column chromatography of fractions near the middle of the T4 peak showed more deiodination (about 2 %); no T_3 could be detected. Deiodination was reduced when propylthiouracil was added (final concentration, 1 m*M*) to all solutions used for purifying T4. Re-chromatography of this material revealed less than 0.2 % of inorganic iodide; neither iodoprotein nor T_3 was detectable (Fig. 5). It therefore seems advisable to add propylthiouracil when small amounts of deiodination might confuse results.

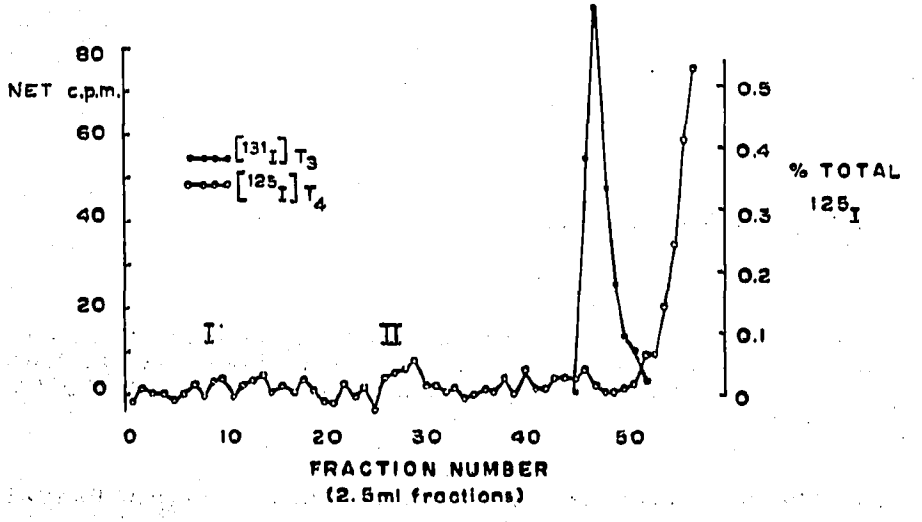

Fig. 5. Chromatography of purified [^{125}I]T4. Equilibration solution, 0.015 *N* NaOH–0.5 *N* NaCl. Sample, 5 ml of pooled serum + 5 ml of 1.0 *N* NaCl–0.2 *M* phosphate buffer, pH 6.5, containing [^{125}I]T4 (10,945 c.p.m.) purified by column chromatography and [^{131}I]T3 (266 c.p.m.). Eluent, 0.1 *N* NaOH–0.005 *N* NaCl. The values given for c.p.m. have been corrected for background and for ^{131}I counts appearing in the ^{125}I channel. Only the initial portion of the T4 peak, which extended through fraction 91, is shown. I and II indicate the position of a small [^{131}I] iodoprotein peak and a small [^{131}I]iodide peak, respectively, which contaminated the T3 preparation.

Recoveries

Pooling all results in which sera containing added labeled T4 or T3 were applied

to columns equilibrated with base and then eluted with basic solutions, the mean recoveries ± S.D. were:

$[^{125}I]T_3$ 99.1 ± 5.8 % (n = 51)
$[^{131}I]T_4$ 98.4 ± 6.3 % (n = 54)

Therefore, the mean recovery was not significantly different from 100 %, and it seems unlikely that an important proportion of either compound remains bound to the column.

Studies of sera from patients treated with ^{131}I

As a test of the efficiency of the column method, serum samples from patients who had recently undergone radioiodine treatment for hyperthyroidism were subjected to column chromatography, by employing the standard technique outlined above. A trace amount of $[^{125}I]T_3$ was added to the patient's serum to assist in identifying the $[^{131}I]T_3$ peak. A typical column run is depicted in Fig. 6, illustrating how an endogenous $[^{131}I]T_3$ peak was identified by comparison with the exogenous $[^{125}I]T_3$ peak. Serial results from the same patient are shown in Fig. 7; the pattern of appearance of T_3, T_4 and iodoprotein seemed compatible with previously documented patterns of thyroid secretion.

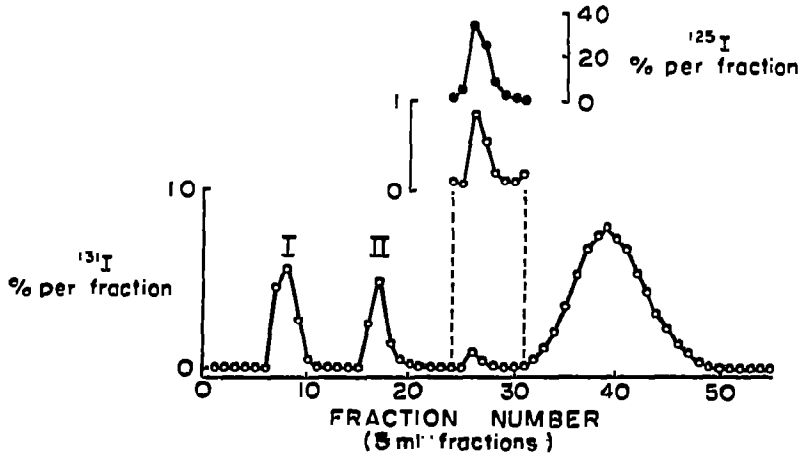

Fig. 6. Chromatography of serum from a patient treated with ^{131}I. The protocol is given in the text. The serum was drawn from a 65-year-old woman with thyrotoxicosis 8 days after she had received $Na^{131}I$ (6.7 mCi) $[^{125}I]T_3$ was added to serum before chromatography. Above the graph, the ^{131}I distribution in fractions 24–31 is shown on an expanded scale, together with the distribution of ^{125}I in the same fractions. The results suggest that $[^{131}I]T_3$ was present. Peaks I and II represent iodoprotein and iodide, respectively, although here the iodoprotein was an endogenous product of iodine metabolism.

DISCUSSION

The method described for separation of iodo compounds is relatively simple, free from artifacts and can be applied to large samples of serum. Compared with the many other methods that have been employed to analyse thyroid hormones in serum, the chief advantage of the present method is that the entire analysis requires only one column run, and the problems associated with solvent extractions and serial chromatographic procedures are avoided.

The inconsistent results of other investigators who have used Sephadex columns for this purpose may reflect two characteristics of the system. Firstly, some have applied whole serum to Sephadex columns; we noted that this seemed to overload the column and to result in poor separations. In fact, any concentration of serum higher than 50% compromised the resolving power of the procedure. Secondly, for consistent results it is important that NaOH solutions used as eluent contain small amounts of salt. As we have shown, the use of NaOH alone may result in a zone of very low ionic strength on the column, and in such zones T4 migrates more rapidly than T3.

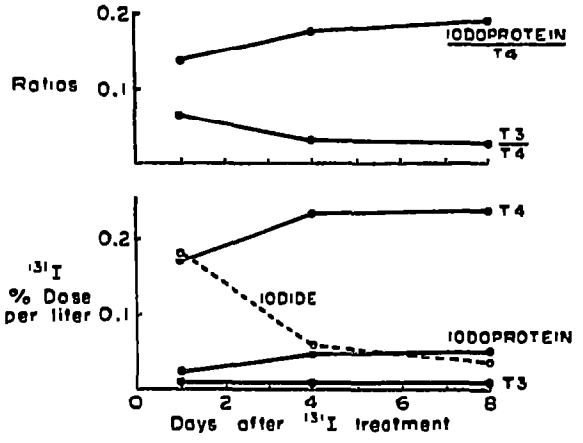

Fig. 7. Chromatography of serial specimens of serum following treatment with ^{131}I. Sera are from the patient described in Fig. 6. The lower panel shows the concentration of labeled T4, iodoprotein, iodide and T3, all expressed as percentage dose per liter of serum. The upper panel shows the iodoprotein:T4 and T3:T4 ratios.

This method is now being further applied to the study of labeled iodo compounds in serum following ^{131}I administration, and in studies of the rates of peripheral conversion of T4 to T3.

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NOTE ADDED IN PROOF

Another method for separation of T3 and T4 from serum on columns of Sephadex G-25 has recently been described[19], in which serum is diluted with an equal volume of 0.01 *N* NaOH prior to chromatography; 0.01 *N* NaOH is also used to suspend the Sephadex and as eluent. Dilution of the serum sample with base not only avoids the difficulties associated with chromatography of whole serum, but

also results in base being present throughout the elution, thus preventing the formation of a zone of low ionic strength. The only apparent disadvantage of this procedure is that it produces a rather broad T3 peak.

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