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The Separation of Iodinated Tyrosines and Thyroxine from Serum

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Anion-exchange resins have been used for the separation of the iodotyrosines and iodothyronines from serum proteins. Blanquet, Meyniel, Mounier, Stoll & Maraud (1957), Blanquet, Meyniel, Mounier & Tobias (1957) and Meyniel, Blanquet, Mounier & Estibotte (1958) used aqueous or aqueous ethanolic hydrochloric acid for the elution of iodinated compounds from Dowex 1 or 2 but the separations achieved were incomplete. Galton & Pitt-Rivers (1959) used sodium acetate buffers for elution of the iodotyrosines and 50% (v/v) acetic acid for the elution of thyroxine and tri-iodothyronine from Dowex 1 (X 2). Lissitzky & Lasry (1958) used aqueous formic acid for elution of all the organic iodo-compounds from Dowex 1 (X 8). They obtained good separation of the iodotyrosines, but, as they pointed out, the volumes of aqueous formic acid required to elute each compound were large, and formic acid is difficult to evaporate to dryness *in vacuo*.

A further problem connected with the analysis of serum on anion-exchange resins is concerned with the competitive binding of iodothyronines by the serum proteins and the active sites of the resin. As a result considerable amounts of thyroxine and tri-iodothyronine may escape from the columns unless unmanageably long ones are used.

This paper describes modifications of the technique of Galton & Pitt-Rivers (1959) whereby the iodotyrosines and iodothyronines are separated with different concentrations of aqueous acetic acid. It also describes the partial enzymic hydrolysis of

serum, which inactivates its thyroxine-binding properties and permits the loading of relatively large quantities on to a column of only 3 cm. $\times$ 1 cm.

MATERIALS AND METHODS

Resin. Since variable results were obtained with commercial grades of resins, analytical-grade Dowex resin A.G. 1 (chloride form; X 2; 200–400 mesh), manufactured by the Bio-Rad Corp., was used. In most experiments it was converted into the acetate form by washing with saturated sodium acetate solution until chloride-free; a few washings with 0.2M-sodium acetate gave equally good results. Excess of acetate was removed with deionized water, which was also used to prepare the reagents. Columns 3 cm. long and 1 cm. in diameter were used.

Serum. Human serum was used for most of the experiments, but rabbit or rat serum gave the same results.

^{131}I -Labelled compounds. ^{131}I -Labelled thyroxine and tri-iodothyronine were obtained from Abbott Laboratories, Oak Ridge. ^{131}I -Labelled mono- and di-iodotyrosine were prepared from hydrolysates of rat thyroid gland by paper-chromatographic separation in butan-1-ol equilibrated with 2N-acetic acid and elution with aq. N-NH₃ soln. at 3° (Pitt-Rivers, 1962). Fresh aqueous solutions of the labelled iodotyrosines were contaminated with about 5% of iodide, and the amount of iodide increased within 1 or 2 weeks to about 15%, even on storage in the cold. ^{131}I -Labelled 'thyroglobulin' was prepared by homogenizing two or three rat thyroid glands in deionized water (about 5 ml.) and filtering through Schleicher and Schüll paper no. 575. The rats were injected with 20–50 μC of ^{131}I 16 hr. before removal of the thyroid.

Eluents. Dilutions of aqueous acetic acid were used except for thyroglobulin, which was eluted with 0.2M-acetate buffer, pH 3.6 (sodium acetate, 0.5 ml.; acetic acid, 9.5 ml.). Each fraction consisted of 3 ml., since this was the greatest volume that could be counted in the well-type scintillation counter.

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RESULTS

Separation of mono- and di-iodotyrosine on resin columns. Mono- and di-iodotyrosine were put on the columns in 0.1–5 μ g. amounts in aqueous solution. Retention of both compounds by the resin was complete, and was unaffected by serum. All the monoiodotyrosine could be eluted with about 8 $\times$ 3 ml. fractions of 0.025 % acetic acid. No di-iodotyrosine was eluted from the resin by 28 fractions of this eluent, but it all came off the column when 10 fractions of 1 % (v/v) acetic acid were used.

Retention of thyroxine by the resin. When thyroxine (up to 0.1 μ g.) in aqueous solution was applied to the resin, it was quantitatively retained. In the presence of serum different amounts were lost in the protein-containing eluate, depending on the amount of serum applied and the rate of elution. The most satisfactory procedure was to apply the serum to the column in 0.5 ml. amounts and wait 40 min. to 1 hr. between applications for equilibrium to be achieved. When the total volume of serum so applied was 1 ml. the loss of thyroxine from the column was 1.5–2.0 %. When the volume of serum was raised to 3 ml., the loss of thyroxine from the column was about 10 %. Attempts were made to depress thyroxine-binding of serum by addition of tetrabromo- and tetrachloro-thyronines, but these were only moderately successful. Denaturation of the serum proteins with urea led to complete clogging of the columns.

Thyroxine-binding by serum was almost completely overcome by the partial hydrolysis of the serum with twice-crystallized papain activated with cysteine for 20–24 hr. (0.5–1 mg./ml. of serum). Serum (9.5 ml.) containing ^{131}I -labelled thyroxine was incubated at 37° with gentle shaking for 24 hr. with 5 mg. of papain. The digest was then put on to a 3 cm. $\times$ 1 cm. column in 0.6 ml. portions every 45 min. As each fraction came off the column it was made up to 3 ml. and counted in the well-type counter. No radioactivity was lost with any of the protein fractions but the thyroxine was quantitatively eluted with 50 % (v/v) acetic acid.

Thyroglobulin. Galton & Pitt-Rivers (1959) found that 0.2M-acetate buffer, pH 3.6, eluted thyroglobulin from Dowex 1 (X 2) columns without eluting monoiodotyrosine. However, with subsequent batches of Dowex 1 (X 2) tested in the present work, monoiodotyrosine was rapidly eluted by acetate buffer, pH 3.6.

^{131}I -Labelled thyroglobulin (0.1–1 mg.) was added to 1 ml. of serum. The mixture was placed on a

column which was eluted with water; 52 % of the radioactivity came off with the serum. The rest could not be eluted with dilute acetic acid but most of it was displaced by 0.2M-acetate buffer, pH 3.6. About 15 % of the thyroglobulin could not be eluted with any concentration of acetic acid or any acetate buffer. All the thyroglobulin was removed as follows: before use the resin was washed with 0.2M-acetate buffer, pH 5.6. The serum containing thyroglobulin was acidified to pH 4–5 with acetic acid, placed on the column and washed off with one 3 ml. fraction of acetate buffer, pH 3.6. This removed the thyroglobulin quantitatively but did not remove any labelled monoiodotyrosine added to a similarly treated column. However, monoiodotyrosine began to come off the resin with a second 3 ml. fraction of acetate buffer, pH 3.6. Complete separation of the two was achieved by removing the thyroglobulin as described above, washing the column with one 3 ml. fraction of water and then eluting the monoiodotyrosine with 0.025 % acetic acid.

Thyroxine and tri-iodothyronine. Attempts to separate these hormones by elution with graded concentrations of acetic acid were not successful.

SUMMARY

1. Elution of monoiodotyrosine and di-iodotyrosine from an anion-exchange resin with dilute solutions of acetic acid affords a means of separation whereby the components can easily be concentrated without danger of chemical degradation.

2. The competition for thyroxine between serum proteins and the resin, which may result in considerable losses of thyroxine in the protein eluate, has been overcome by partial hydrolysis of the proteins with crystalline papain. This permits the loading of short columns (3 cm. $\times$ 1 cm.) with considerable amounts of serum.

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