mitochondrial incorporation. This, however, is not so (Table 5), since the cell sap derived from liver tumour was at least as effective as that derived from normal liver for the incorporation of amino acids into normal liver mitochondria. In contrast the tumour pH 5 fraction was considerably less effective than a similar fraction from normal liver in this respect. It thus appears that there are factors present in the cell sap which are not concerned with the incorporation of amino acids into microsomes, but which promote incorporation into mitochondrial protein.

SUMMARY

1. After the incubation of microsomes and pH 5 fraction of liver and liver tumour with [^{14}C]leucine, the radioactivity of the tumour-microsomal protein is lower than that of liver. When liver microsomes were incubated with tumour pH 5 fraction, instead of liver pH 5 fraction, a lower extent of incorporation resulted.

2. The adenosine triphosphate-dependent incorporation of leucine and lysine into the ribonucleic acid of pH 5 fraction of liver tumour was lower than into that of normal liver.

3. The adenosine triphosphate-dependent incorporation of [^{14}C]leucine into preparations of liver and tumour mitochondria was studied. Incorporation into the mitochondrial protein of both tissues took place in the absence and presence of cell sap, but was higher in its presence.

4. Under all experimental conditions the amino acid incorporation into tumour mitochondria was lower than into liver mitochondria. The stimulating action of tumour cell sap, however, on liver mitochondria was as great as that of liver cell sap. The tumour pH 5 fraction, on the other hand, was less effective than that of liver in stimulating mitochondrial incorporation.

5. Ribonuclease stimulates the incorporation into liver-mitochondrial protein in the absence of cell sap, but reduces the incorporation stimulated by cell sap.

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A Quantitative Method for the Separation of Thyroid Hormones and Related Compounds from Serum and Tissues with an Anion-Exchange Resin*

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The use of paper chromatography and radioautography in the study of the metabolism of ^{131}I-labelled thyroid hormone is well known. Some concentration of the iodinated compounds present in biological material is usually necessary and the most widely used technique is that of extraction with butanol. However, the amount of material that can be extracted in this way is limited; this method is therefore not suitable when the amount of radioactivity is small, as chromatographic analysis of the extracted compounds is virtually impossible.

Blanquet, Dunn & Tobias (1955) have employed the anion-exchange resin Dowex-2 for the separation of organic and inorganic iodide present in hydrolysed thyroid gland or blood plasma; the

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resin was used in the hydroxyl form. Thyroxine, monoiodotyrosine and di-iodotyrosine were eluted in that order with 0·2N-hydrochloric acid; inorganic iodide remained on the resin throughout. Dowex-1 was equally effective and the two resins have been used in a study of thyroid function in the rat after administration of ^{131}I (Blanquet, Meyniel, Mounier, Stoll & Maraud, 1957). A 95% recovery of the iodinated compounds was effected by elution with 0·2N-hydrochloric acid containing 3·7% of ethanol (Meyniel, Blanquet, Mournier & Estibotte, 1958). However, relatively large volumes of hydrochloric acid were required to elute the iodinated amino acids.

Ingbar, Dowling & Freinkel (1957) used Dowex-1 equilibrated with acetate buffer at pH 4·5 for the purification of thyroxine-binding proteins. They found that thyroxine was dissociated from the plasma proteins by the resin; as the pH was reduced the thyroxine remained on the resin while all detectable amounts of protein were eluted, but the thyroxine could be quantitatively removed in concentrated solution by a further reduction in the pH of the eluent. The capacity of the resin for thyroxine was such that it could be concentrated from large volumes of serum. Further, as the solution of thyroxine obtained in this manner was free from protein or lipid material, it was suitable for paper chromatography.

The use of Dowex-1 was investigated for the separation, concentration and quantitative estimation of the thyroid hormones and related compounds from serum and tissues for chromatographic analysis. Preliminary experiments showed that thyroxine, which was absorbed by the resin at pH 4·5, could not be eluted with 0·1N-, 0·2N-, N- or 2N-hydrochloric acid, even after prolonged washing. It was, however, displaced with aqueous 40% (v/v) acetic acid at pH 1·6. Acetic acid solutions and acetate buffers were therefore used throughout this work.

METHODS AND MATERIALS

All experiments were carried out with a glass column of 1 cm. diam., containing a sintered-glass filter and the Dowex-1 resin: Dowex-1 R 1, X 2, 200–400 mesh, quaternary ammonium in chloride form.

Acetate buffers, pH 3·2–5·6, were made up from 0·2M-acetic acid and 0·2M-sodium acetate stock solutions; acetic acid was suitably diluted to give solutions of pH 1·4–3·0. The pH of all solutions was determined with a Cambridge pH meter.

Experiments were performed with serum to which had been added either thyroxine or tri-iodothyronine synthetically labelled with ^{131}I in the 3′ or 3′:5′ positions. The thyroxine and tri-iodothyronine samples were contaminated with iodide amounting to 12 and 15% respectively of the total ^{131}I in the samples.

A mixture of ^{131}I-labelled mono- and di-iodotyrosine was prepared from a mouse killed 4 hr. after injection of ^{131}I (10 μc). The thyroid gland was removed and was hydrolysed with trypsin in borate buffer at pH 8·6 for 24 hr. The hydrolysate was shown by chromatographic analysis to contain monoiodotyrosine, di-iodotyrosine, iodide and another iodinated fraction which remained at the origin of the chromatogram and was thought to be unhydrolysed or partially hydrolysed thyroglobulin.

The serum or thyroid hydrolysate containing one or more ^{131}I-labelled compounds was passed through the resin column (approx. 3 cm. high), which was equilibrated with buffer at pH 5·6. The resin was washed with the same buffer and then solutions of progressively decreasing pH were passed through the column, the solution being changed only when no further radioactivity could be eluted. Finally acetic acid, sometimes followed by 3N-NaBr to elute the iodide, was passed through the column. The eluate was collected from the column in 3 ml. serial fractions. The total radioactivity in the original samples and in the fractions eluted from the column was estimated in a Well-type scintillation counter. When necessary, a sample was counted in a total volume of 3 ml.

In order to determine whether a compound was eluted quantitatively from the column, one-dimensional chromatographic analysis of the iodinated compounds present in the original sample and in the fractions of eluate from the column was carried out. The solvent systems butan-1-ol–dioxan–aq. 2N-NH_3 soln. (4:1:5) and butan-1-ol–2N-acetic acid (1:1) were used throughout. Synthetic mono- and di-iodotyrosine, thyroxine and tri-iodothyronine were employed as markers and were stained by the method of Gross & Leblond (1951). Carrier iodide was stained with 0·1% of palladium chloride. The chromatograms were counted with an automatic recording ratemeter and the area under each peak was measured with a planimeter; the area is directly proportional to the radiation emitted from the corresponding part of the chromatogram. These measurements enable the distribution of the total radioactivity among the different compounds present on a chromatogram to be calculated. As the total radioactivity in the original sample and in the separate fractions from the column was also determined it was possible to trace quantitatively the fate of each compound.

When the radioactivity of a protein-containing fraction was very low, the solution was first exhaustively extracted with butan-1-ol and the concentrated extracts were analysed. Protein-free solutions were evaporated to dryness under reduced pressure and the residues were dissolved in methanol–aq. NH_3 soln. (sp.gr. 0·880) (3:1). Chromatographic analysis of the fraction eluted with NaBr was seldom possible since the NaBr was precipitated on concentration.

In a further experiment the kidneys of a normal mouse were removed, 0·9% NaCl soln. was added (6 ml.) and the tissues were homogenized with sand in a pestle and mortar and centrifuged. [^{131}I]Thyroxine was added to the extract in 0·9% NaCl soln. and the resulting solution was subjected to analysis on a column of Dowex-1.

RESULTS

Thyroxine. The results are shown in Table 1. As the serum passed through the column, 100% of the radioactivity present in the serum was absorbed.

Radioactivity amounting to 84% of that in the original sample was discharged from the column with 40–50% acetic acid (pH 1·4–1·6) and was identified by chromatographic analysis as thyroxine. The remaining activity was eluted with NaBr. The concentration of radioactivity in this fraction was too low to permit chromatographic analysis, but it was assumed to consist of iodide since 16% of the radioactivity in the original sample was in the form of inorganic iodide which had not been detected in the fraction eluted from the column at pH 1·4.

Tri-iodothyronine. The results are shown in Table 2. All the serum radioactivity was absorbed by the resin. (In all experiments, at least 96% of the radioactivity was absorbed.) Radioactivity (84·5%) was discharged with 30–50% acetic acid (pH 1·8–1·4) and identified by chromatographic analysis as tri-iodothyronine. The remaining 15% was removed with 3N-NaBr and was sufficiently active to permit chromatographic analysis; it consisted entirely of iodide and presumably represented the iodide present to the extent of 15% of total radioactivity in the original serum.

Monoiodotyrosine, di-iodotyrosine and thyroglobulin. The thyroid hydrolysate containing these compounds and iodide was analysed on the column. The results are shown in Table 3. Chromatographic analysis of the three principal fractions eluted from the column indicated that separation was complete. The distribution of the total radioactivity among the three main components of the hydrolysate was calculated from chromatographic analysis and column analysis (Table 3) and the results obtained were compared (Table 4). The monoiodotyrosine/di-iodotyrosine ratio determined by chromatography was 2·7; after separation on the resin it was 2·8. It was concluded that mono- and di-iodotyrosine were absorbed by Dowex-1 and quantitatively eluted with 1·15 and 10% acetic acid respectively.

Sodium chloride solution extract of kidney homogenate containing thyroxine. [^{131}I]Thyroxine (1 μc of ^{131}I) was added to an extract of kidney homogenate in 0·9% NaCl soln. The solution was poured on the top of a Dowex-1 column; it passed down the column at first but gradually the rate of flow diminished and finally stopped. However, under pressure (equal to 20 cm. water) the solution slowly resumed its passage through the resin. Under these conditions 96% of the radioactivity was absorbed. Although most of the tissue proteins passed unhindered through the column, prolonged washing

Table 1. *Separation of thyroxine from serum with Dowex-1*

Fraction	pH or solvent	Counts/min.	^{131}I-labelled compounds detected by chromatography
Serum + thyroxine	7·4	15 468	Thyroxine, iodide
1*	5·6	654	Thyroxine, iodide
2–9	5·6–1·8	—	—
10	1·67	641	Thyroxine
11	1·4	5 744	
12	1·4	5 959	
13	1·4	589	
14–20	Undiluted acetic acid	—	—
21–30	3N-NaBr	1 900	—
Total eluted material		15 487	

* Fraction containing most of original protein.

Table 2. *Separation of tri-iodothyronine from serum with Dowex-1*

Fraction	pH or solvent	Counts/min.	^{131}I-labelled compounds detected by chromatography
Serum + tri-iodothyronine	7·4	751 960	Tri-iodothyronine, iodide
1*	5·6	1 803	Tri-iodothyronine, iodide?
2*	5·6	1 716	
3–11	5·6–2·0	0–50	—
12	1·8	681	Tri-iodothyronine
13	1·67	440 310	
14	1·4	164 640	
15	1·4	29 500	
16	1·4	500	
17–25	Undiluted acetic acid	—	—
26–40	3N-NaBr	112 860	Iodide
Total eluted material		752 060	

* These fractions contained most of the original protein.

Table 3. *Separation of iodinated amino acids in a thyroid hydrolysate with Dowex-1*

Fraction	pH or solvent	Counts/min.	^{131}I-labelled compounds detected by chromatography
Thyroid hydrolysate	8·6	30 420	Thyroglobulin, monoiodotyrosine, di-iodotyrosine, thyroxine, iodide
1–5	5·6	—	
6–10	3·6	800	Thyroglobulin
11–16	3·4–3·2	—	—
17–24	3·0	20 447	Monoiodotyrosine
25–35	2·8–2·4	—	—
36–40	2·2	7 371	Di-iodotyrosine
41–100	1·6–1·4	300	Thyroxine?
101–120	3N-NaBr	1 102	Iodide
Total eluted material		30 020	

Table 4. *Percentage distribution of total ^{131}I among the different iodinated compounds in the thyroid hydrolysate as determined by two methods*

Compound	Paper-chromatographic analysis	Column analysis
Thyroglobulin	5	3
Monoiodotyrosine	65	67
Di-iodotyrosine	24	24
Other compounds	6	5

with buffer at pH 3·6 was necessary before the eluates were free from any detectable amount of protein. Thyroxine remained on the column throughout and was quantitatively eluted with 50 % acetic acid. It was observed that as the flow rate of the protein solution through the column was increased by raising the pressure, the efficiency of the resin in extracting the radioactive thyroxine was reduced.

DISCUSSION

It is evident from these experiments that thyroxine, tri-iodothyronine, mono- and di-iodotyrosine and thyroglobulin can be separated and concentrated from serum and tissues by absorption on Dowex-1. This technique has several advantages over that of butanol extraction. The iodinated compounds are separated from the proteins and lipid materials of the tissue; they are eluted with a volatile solvent which can easily be removed. The residue, on treatment with methanol–ammonia, gives a non-viscous extract which can be cleanly applied to filter paper for chromatographic analysis.

The capacity of the resin for thyroxine and its analogues is sufficient to permit their removal from much greater amounts of material than can be employed for butanol extraction. The technique therefore makes possible the study of thyroid-hormone metabolism in the tissues of animals given quite small doses of ^{131}I.

Inorganic and organic iodide present in a tissue can be accurately estimated by using Dowex-1. The protein-bound ^{131}I present in serum 24 hr. after a mouse had received 10 μc of ^{131}I was determined (*a*) by chromatographic analysis, (*b*) by column analysis and (*c*) by trichloroacetic acid precipitation. Chromatographic and column analyses gave comparable results, whereas a considerably higher value for protein-bound ^{131}I was obtained by trichloroacetic acid precipitation unless a lengthy procedure of washing, re-dissolving in alkali and re-precipitation was adopted.

Dowex-1 can therefore be used to advantage in the study of quantitative aspects of thyroid-hormone metabolism in various tissues and body fluids.

SUMMARY

1. The thyroid hormones and related compounds can be separated from biological material by absorption on the ion-exchange resin Dowex-1 equilibrated with acetic acid–sodium acetate buffer at pH 5·6.

2. The iodinated compounds are quantitatively eluted when the pH is reduced with acetic acid solutions of increasing concentration.

3. Thyroglobulin, monoiodotyrosine and di-iodotyrosine are eluted at pH 3·6, 3·0 and 2·2 respectively and thyroxine and tri-iodothyronine are eluted at pH 1·4. Iodide remains on the resin throughout.

4. Dowex-1 can therefore be used to concentrate the thyroid hormones in biological material for chromatographic analysis.

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