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Note

Gel filtration of thyroxine-binding proteins
Identification of rat plasma protein fractions separated on Sephadex G-200

The separation of proteins in rat plasma by gel filtration on Sephadex G-200 has been extensively studied by DOLEŽALOVÁ *et al.*[1], who identified most of the main fractions of the serum proteins. A comparative study of the rat plasma and milk proteins on Sephadex G-200 was carried out by LAURELL AND MORGAN[2].

On the other hand, the rat plasma and liver proteins were studied from the point of view of the specific thyroxine-binding proteins by HAMADA *et al.*[3] on Sephadex G-100 only. A systematic study of the fractionation of thyroxine-binding proteins of rat plasma on various types of Sephadex gels was described by HOCMAN[4].

The aim of the present paper is to describe a separation of radiothyroxine-labelled rat plasma proteins into four groups, their characterization by polyacrylamide gel electrophoresis and the establishment of their thyroxine-binding abilities.

Experimental

Freeze-dried pooled blood plasma from normal white rats was dissolved in distilled water to form a 10% solution, which was kept frozen at −10 to −15°. Before the experiment 0.5 μCi of I^{125}-labelled thyroxine (RT4) (Radiochemical Centre, Amersham, Great Britain) was added to 0.5 ml of melted plasma solution and incubated for about 1 h at + 4° in order to bind thyroxine to its carrier proteins.

A chromatographic column (length 95 cm, I.D. 1 cm) of Sephadex G-200 (Pharmacia, Uppsala, Sweden) was prepared[5] in Tris–HCl buffer (pH 7.4, 0.01 *M*), and 0.5 ml of the RT4-labelled plasma was applied to the top of the column and eluted with the same buffer. Fractions of 1 ml of the eluate were collected on a fraction collector (Fractiomat, Budapest, Hungary). This separation procedure was carried out in a cold room (+ 4°), where the fractions obtained were stored until required for further processing.

The radioactivity of each fraction was measured in a Model 1185 automatic gamma-counter (Nuclear-Chicago, Chicago, Ill., U.S.A.) and the protein contents of the same fractions were determined from absorbance measurements made at 280 nm in a VSU 1 spectrophotometer (Zeiss, Jena, G.D.R.).

The ratio of the counts per minute of radioactivity (c.p.m.) divided by the relative protein concentration (1000 × $Ex_{280\ nm}$) was calculated. This ratio gives a relative measure of specific radioactivity and therefore a measure of the amount of thyroxine bound to a unit of protein.

According to the peaks of radioactivity and absorbance, some of the fractions were combined and freeze-dried. The radioactivity of each such peak was expressed as a percentage of the total radioactivity from all of the peaks. As the radioactivity represents the added radiothyroxine, its percentage value gives a measure of the amount of bound thyroxine present in a particular fraction (Fig. 1).

The separated and freeze-dried protein fractions obtained by the above procedure were further characterized by electrophoresis on polyacrylamide gel by the method of DAVIS and ORNSTEIN[6]. A 10-mg amount of each freeze-dried sample containing some of the protein fraction collected (Fig. 1, collected fractions indicated by bars) was dissolved in 0.5 ml of 40 % sucrose solution and incubated at 37° with 0.5 μCi of RT4 for 30 min. A 0.1-ml volume of this mixture with 0.1 ml of acrylamide was applied to the top of a small polyacrylamide gel column, polymerized and electrophoresis was carried out in a 7 % gel in Tris–glycine buffer (pH 8.6) at + 4°. The separation was completed in 60–90 min, the current being 2.5 mA for each sample. Each protein sample was separated by electrophoresis in at least two parallel runs. After the electrophoresis, one of the columns was dyed with a 1 % solution of Amido Black 10 B in 7 % acetic acid for 30 min and then bleached three times in 7 % acetic acid. The other column was left unstained, and was then cut into 1-mm wide slices and the radioactivity of each such slice was measured as above.

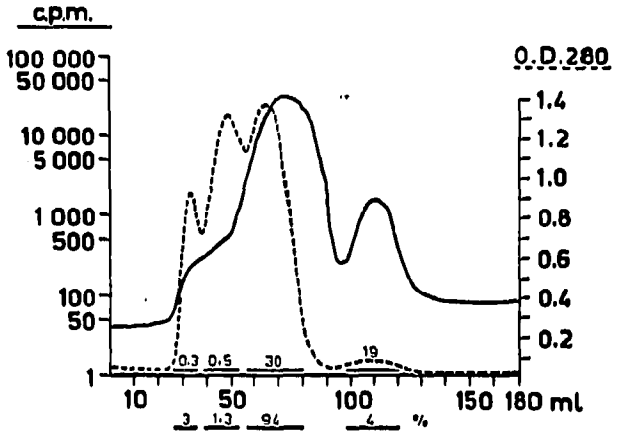

Fig. 1. Separation of radiothyroxine-labelled rat plasma on Sephadex G-200. Full line, radioactivity (c.p.m.); broken line, absorbance at 280 nm. Bars indicate collected fractions; numbers above the bar represent the average relative specific radioactivity, and below the bar they represent the percentage of radioactivity in each collected fraction.

Results and discussion

As indicated in Fig. 1, according to the absorbance at 280 nm, four fractions of rat plasma were collected after gel filtration on Sephadex G-200. The electrophoretic separation of the proteins of the first peak (containing 3 % of added radiothyroxine) is shown in Fig. 2. The lower part of the figure shows the gel column, stained to indicate the presence of proteins, and above the gel is the graphical distribution of radioactivity (a relative measure of the content of added radiothyroxine) in corresponding parts of the gel. The electrophoretic separation indicates the presence of high-molecular-weight globulin fractions. As the sample contained only 3 % of radiothyroxine, as shown by the results of gel filtration, this fraction probably does not contain significant amounts of thyroxine-binding proteins.

The distribution of proteins and the radioactivity of the second fraction of proteins (containing 1.3 % of radiothyroxine, Fig. 1) is shown in Fig. 3. As neither this

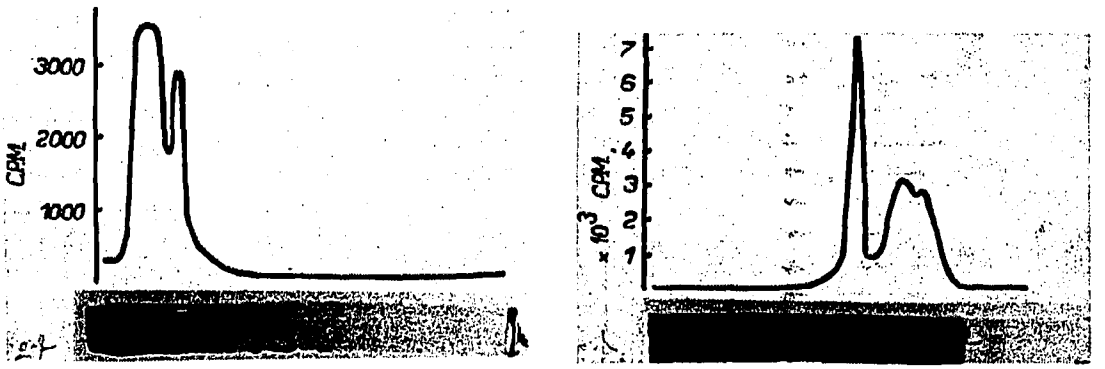

Fig. 2. Separation of proteins from the first peak (containing 3% of radiothyroxine, see Fig. 1) by electrophoresis on polyacrylamide gel. Lower part, the gel stained for the identification of proteins by Amido Black 10B. Upper part, distribution of radioactivity of radiothyroxine in the corresponding parts of the above protein fraction. The anode is to the right.

Fig. 3. Separation of proteins from the second peak (containing 1.3% of radiothyroxine, see Fig. 1) by electrophoresis on polyacrylamide gel. Details as in Fig. 2.

nor the previous fraction contains a significant amount of radiothyroxine, as indicated by gel filtration, only negligible amounts of specific thyroxine-binding proteins can be present in this fraction. The distribution of radioactivity in the upper parts of Figs. 2 and 3 is due only to the binding of RT_4 to the proteins present in the sample, because, owing to its aromatic structure, thyroxine is easily bound to almost any compound.

In the proteins of the third peak (Fig. 4), there was a considerable amount of thyroxine (94 %). This peak probably contains albumin and other thyroxine-binding proteins of rat plasma.

The fourth, final peak contains only negligible amounts of proteins, according to the relatively low absorbance at 280 nm, but contains the bulk of low-molecular-weight compounds of rat plasma, which were not excluded from the Sephadex G-200. This fraction will probably be contaminated by radioactive iodide, which in small amounts is usually present in all radiothyroxine preparations. In spite of the presence

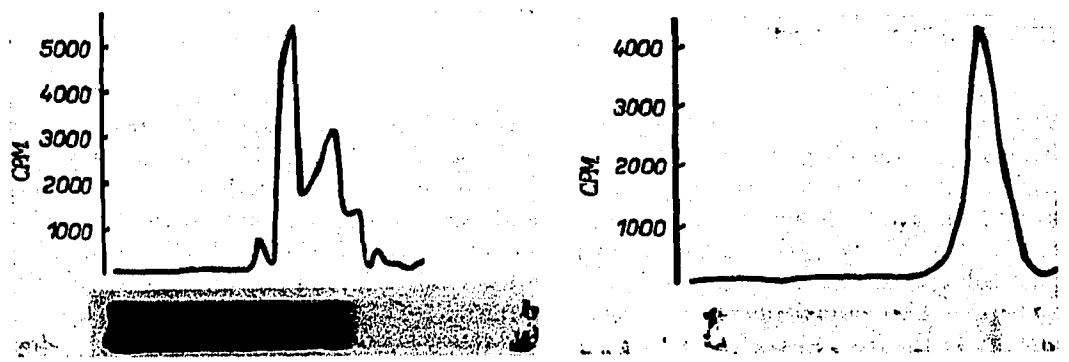

Fig. 4. Separation of proteins from the third peak (containing 94% of radiothyroxine, see Fig. 1) by electrophoresis on polyacrylamide gel. Details as in Fig. 2.

Fig. 5. Separation of proteins from the fourth peak (containing 4% of radiothyroxine, see Fig. 1) by electrophoresis on polyacrylamide gel. Details as in Fig. 2.

of this contaminating iodide, there is probably some radiothyroxine present in this fraction. As the concentration of proteins is low, the specific radioactivity, and therefore the amount of thyroxine per unit amount of protein, is high. STERLING *et al.*[7] showed that the molecular weight of the human thyroxine-binding globulin is approximately 35,000. The possibility exists of the presence of a similar low-molecular-weight protein in rat plasma, in fraction four (containing 4 % of radiothyroxine, Fig. 1). The electrophoresis on polyacrylamide gel, stained to indicate the presence of proteins, reveals a single band for this fraction of proteins, where the radioactivity is also located (Fig. 5). This result seems to indicate the presence of a comparatively well separated fraction of low-molecular-weight thyroxine-binding proteins of rat plasma in this fraction.

This fourth peak, containing 4 % of radiothyroxine, probably does not contain free, unbound radiothyroxine. As shown in previous work[8], part of the radiothyroxine added to the sample of rat plasma before the separation on Sephadex remains free. However, this unbound, free radiothyroxine becomes completely bound to the material of the Sephadex column[9] during the gel filtration and can be desorbed from the column only by prolonged washing[10]. In our experiments, the originally free radiothyroxine, now bound to the Sephadex material, appeared by prolonged washing long after the fourth peak in Fig. 1.

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