Dextran-Gel Filtration of Enzymes in the Presence of their High-Molecular-Weight Substrates

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1. Trypsin and ribonuclease were filtered through dextran gel (Sephadex G-100) columns in the absence and presence of their respective substrates. In the presence of their high-molecular-weight substrates the enzymes emerged earlier from the columns. This appeared to be due to the reversible formation of specific enzyme-substrate complexes. 2. The possibility of separation of an enzyme from other proteins with similar molecular weights was demonstrated with trypsin and cytochrome *c* in the presence of casein.

When proteins are filtered through a gel column their elution volumes are essentially a function of their molecular weights (Andrews, 1962; Wieland, Duesberg & Determann, 1963; Whitaker, 1963; Iwatsubo & Curdel, 1963; Andrews, 1964; Auricchio & Bruni, 1964). Thus proteins with widely differing molecular weights can usually be separated, whereas proteins with similar molecular weights emerge together.

During gel filtration of a mixture of proteins, one of which is an enzyme, if the column of gel is equilibrated with a solution containing a high-molecular-weight substrate of the enzyme then it is to be expected that reversible formation of the enzyme-substrate complex will decrease the elution volume of the enzyme. A specific separation of one enzyme from a mixture of proteins of similar molecular weight might thus be achieved. The purpose of the present paper is to demonstrate that by dextran-gel filtration a protein can be separated from other molecules of similar size if it forms a complex with a high-molecular-weight substance. This method of separation has been examined with trypsin and ribonuclease.

MATERIALS AND METHODS

Cytochrome *c* (from horse heart), type II, was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; ribonuclease, trypsin, malate dehydrogenase, lactate dehydrogenase, glutamate dehydrogenase and yeast soluble RNA were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; casein prepared according to Hammarsten was from E. Merck A.-G., Darmstadt, Germany; benzoyl-L-arginine ethyl ester hydrochloride was from Seravac Laboratories (Pty.) Ltd., Maidenhead, Berks. All other reagents were of analytical grade.

Sephadex G-100 (lot no. To2946; particle size 40–120 μ) (Pharmacia, Uppsala, Sweden) was suspended in a solution

of 6M-urea, stirred with a magnetic stirrer for about 1 hr. and exhaustively washed with water. The smallest particles were removed by decantation, and then a suspension of the gel was deaerated and packed into columns (1.5 cm. diam. $\times$ 79 cm.). Columns of gel were equilibrated with the desired buffer, which was allowed to pass through until the height of the gel bed remained constant. Proteins were dissolved in 1 ml. of the buffer used for equilibration, pipetted on to the top of the column and eluted with the same buffer. The same procedure was used with buffers containing 1% (w/v) of casein, 0.5% of soluble RNA or 0.02% of benzoyl-L-arginine ethyl ester hydrochloride.

Casein was brought into solution by boiling a 1% suspension in 35 mM-phosphate buffer, pH 7.6, prepared as described by Sørensen (1909), and containing NaCl (0.2M), cooling and filtering the solution, and adjusting its pH to 7.6 with 2N-NaOH. Before use a 0.5% solution of soluble RNA in 0.1M-sodium acetate buffer, pH 5.2, containing NaCl (0.2M), was dialysed against the same buffer for 4 days.

The effluent volume corresponding to maximum concentration of a solute (V_e) was estimated as described by Andrews (1964) to the nearest 1 ml. from an elution diagram by extrapolating both sides of the solute peak to an apex. All filtrations were done at 3°. The flow rates of the columns were approx. 10 ml./hr. In the absence of substrates the concentrations of the proteins in the effluent fractions were estimated from the extinction readings at 210 or 280 m μ according to the quantity of protein used. Cytochrome *c* was estimated at 408 m μ . The concentrations of ribonuclease (Anfinsen, Redfield, Choate, Page & Carroll, 1954), malate dehydrogenase (Mehler, Kornberg, Grisolia & Ochoa, 1948), trypsin (Kunitz, 1947; Schwert & Takenaka, 1955) and lactate dehydrogenase (Kornberg, 1955) were determined by the appropriate enzymic test.

RESULTS

Sephadex G-100 columns were equilibrated with 35 mM-phosphate buffer, pH 7.6, containing sodium chloride (0.2M) and were calibrated for molecular-weight determination with cytochrome *c* (mol.wt.

13000) (Paléus & Paul, 1963), ribonuclease (mol.wt. 13700) (Hirs, Moore & Stein, 1956), trypsin (mol.wt. 23800) (Cunningham, 1954), malate dehydrogenase (mol.wt. 70000) (Thorne & Kaplan, 1963) and lactate dehydrogenase (mol.wt. 117800) (Jaenicke, 1963). A calibration curve was obtained by plotting the elution volumes of the proteins against the logarithms of their respective molecular weights (Andrews, 1964).

The elution volume of trypsin was not affected when from 0.1 to 20 mg. was submitted to gel filtration. However, when the columns were equilibrated with and eluted with a 1% casein solution, the elution volumes were decreased and dependent on the amount of trypsin introduced into the column. The lower the trypsin concentration, the smaller was the elution volume (Table 1). When 0.1 mg. of trypsin was introduced into the column, the elution volume corresponded to mol.wt. 100000. There was no significant difference in the elution volumes of cytochrome *c*, ribonuclease and lactate dehydrogenase when submitted to gel filtration in the presence or absence of casein (Table 1). Neither was the elution volume of trypsin changed when it was filtered on a column equilibrated with the low-molecular-weight substrate benzoyl-L-arginine ethyl ester hydrochloride.

Similar experiments were performed with ribonuclease. The Sephadex G-100 columns were equilibrated with 0.1M-sodium acetate buffer, pH 5.2, containing sodium chloride (0.2M). The elution volume of 0.1 mg. of ribonuclease submitted to gel filtration in the presence of dialysed 0.5% soluble RNA solution was significantly smaller than in the absence of RNA. At a higher concentration of ribonuclease (1mg.) the elution volume was not significantly different in the presence or absence of

RNA. The elution volumes of malate and lactate dehydrogenase were not affected by soluble RNA, but the appearance of the cytochrome *c* from the column was accelerated (Table 2).

A mixture of cytochrome *c* (20mg.) and trypsin (0.1mg.) was submitted to gel filtration in the

Table 2. *Gel-filtration elution volumes of ribonuclease and other proteins in the absence and presence of soluble RNA*

The proteins were filtered through Sephadex G-100 columns (1.5 cm. $\times$ 79 cm.) equilibrated with 0.1M-sodium acetate buffer, pH 5.2, containing NaCl (0.2M), in the absence and presence of 0.5% of soluble RNA.

	Wt. used (mg.)	V_e in absence of RNA (ml.)	V_e in presence of RNA (ml.)
Cytochrome <i>c</i>	0.1	98	91
Malate dehydrogenase	0.1	61	61
Lactate dehydrogenase	0.1	50	52
Ribonuclease	0.1	96	86
Ribonuclease	1	96	94

Table 1. *Gel-filtration elution volumes of trypsin and other proteins in the absence and presence of casein*

The proteins were filtered through Sephadex G-100 columns (1.5 cm. $\times$ 79 cm.) equilibrated with 35 mM-phosphate buffer, pH 7.6, containing NaCl (0.2M), in the absence and presence of 1% (w/v) of casein.

Protein	Wt. used (mg.)	V_e in absence of casein (ml.)	V_e in presence of casein (ml.)
Cytochrome <i>c</i>	1	98	100
Ribonuclease	1	97	95
Lactate dehydrogenase	1	50	52
Trypsin	0.1	85	53
Trypsin	1	85	68
Trypsin	10	85	71
Trypsin	20	85	77
Trypsin	60	—	79

Fig. 1. Elution diagram of a mixture of 20 mg. of cytochrome *c* (O) and 0.1 mg. of trypsin (●) on a Sephadex G-100 column (1.5 cm. $\times$ 79 cm.) equilibrated with 35 mM-phosphate buffer, pH 7.6, containing NaCl (0.2M), in the absence (a) and in the presence (b) of 1% of casein.

presence and absence of 1% casein. In the absence of casein there was a slight separation (Fig. 1a), whereas in the presence of casein there was a complete separation of the proteins (Fig. 1b). After concentration by vacuum-dialysis at 3°, the trypsin was separated from casein by a second gel filtration in the absence of casein.

DISCUSSION

The results obtained by filtering trypsin at different concentrations through columns of dextran gel equilibrated with buffer plus casein show that, within certain limits, the elution volume of a protein can be varied. Under the conditions of our experiments the variation in the elution volume for trypsin does not seem to be due to an alteration in the mechanism of the gel-filtration process induced by the presence of casein. Proteins with lower molecular weights (cytochrome *c* and ribonuclease) and with a higher molecular weight (lactate dehydrogenase) than that of trypsin were eluted as expected from their molecular weights even in the presence of casein. It is improbable that the variation of the elution volume is due to a change in form of the enzyme in the presence of substrate, since a low-molecular-weight substrate, benzoyl-L-arginine ethyl ester hydrochloride, caused no change in the elution volume of trypsin. The possibility that casein can accelerate the elution of trypsin from the Sephadex column by neutralizing a charge complex between trypsin and gel existing in the absence of casein can also be excluded, because in the absence of casein the elution volume of trypsin was that expected on the basis of its molecular weight, whereas in the presence of casein the elution volumes corresponded to much higher molecular weights.

The concentration-dependence of the elution volume of trypsin in the presence of casein seems to be due to the reversible formation of an enzyme-substrate complex. The reversibility is clearly shown by the subsequent separation of trypsin from casein in a column equilibrated with buffer containing no casein.

The molecular weight of the substrate that binds to the enzyme is an important factor in determining the degree of reduction of the elution volume. A small substrate such as benzoyl-L-arginine ethyl ester hydrochloride does not significantly affect the elution volume of trypsin. On the other hand, the binding of the very large casein molecules greatly increases the size of the protein moiety being filtered, and causes a considerable reduction in the elution volume.

The variations in the elution volume of ribonuclease in the presence of soluble RNA were relatively small when compared with those of trypsin

in the presence of casein. This, however, was expected since the elution volume of the RNA corresponded to that of a protein with mol.wt. 17000 whereas the casein emerged at the void volume of the column (it had the same elution volume as 40mg. of glutamate dehydrogenase) and therefore existed in a form with mol.wt. greater than 300000 (Andrews, 1964).

The change in the elution volume of the basic cytochrome *c* in the presence of the acidic RNA shows that complexes other than the highly specific enzyme-substrate type may also be formed and alter the normal elution volume of a protein.

The separation of trypsin from cytochrome *c* in the presence of casein illustrates a possible application of the phenomenon that we have described. In favourable cases it might be possible to purify an enzyme from a crude preparation by only two steps: an initial gel-filtration step to separate the enzyme together with other proteins of the same size from the remainder of the proteins; then a second gel-filtration step in which a specific variation in the elution volume of the enzyme is induced, for instance by the presence of high-molecular-weight substrate, to separate it from other proteins of similar size.

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