Filtration Chromatography on Substituted Sephadex in the Purification of the Hypothalamic Hormone TRF (TSH-Releasing Factor).* (30502)

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We have previously reported on the use of ion exchange chromatography on carboxymethylcellulose for partial purification of hypothalamic TRF(1). In this early note, however, and in a subsequent one(2) we drew attention to the low capacity of the exchanger for retaining TRF and also to the somewhat erratic behavior of the method due to heterogeneity of the various batches of carboxymethylcellulose. With the need of processing large quantities of the crude TRF concentrate for further purification and isolation (see 3), it was decided to investigate the substituted polydextran gel, carboxymethyl-Sephadex, as an ion exchanger in view of its reported high capacity.

This note will describe the method finally adopted for a routine purification of TRF by gel chromatography on CM-Sephadex. It will be shown that the method can be programmed for quantities of the hypothalamic extract ranging from a few hundred milligrams to very large preparative columns taking up to 25 g of the extract, *i.e.*, in one single pass the TRF concentrate from 15 to 18,000 hypothalamic fragments. We wish also to report several observations made in using carboxymethyl-Sephadex, as few publications have appeared so far on the use of this substituted dextran polymer.

Materials and methods. CM-Sephadex C-50 medium grade, 100-270 mesh particle size, (lot no. TO 1600) received from the manufacturer in the Na form, was freed of Na by successive washings with 0.001M and 0.1M pyridine acetate (pH 5.0) and 2N acetic acid; it was finally equilibrated in 0.001M pyridine acetate, pH 5.0 (molarity of the pyridine is designated). The C-50 cross linkage rather than C-25 was chosen so that the small TRF molecule would have no restriction of travel through the gel other than that due to the ion exchange process(3). After various preliminary experiments, stepwise chromatography was selected, the second buffer being 0.09M pyridine acetate, pH 4.0, followed by 2N acetic acid.

Various size columns were used: for small quantities of the TRF concentrate (from 200 mg to 1.0 g) a column 3.8 × 15 cm was packed with *ca* 8 g of the exchanger. The preparative column was 15 × 75 cm; the maximal bed height that was compatible with a flow rate ≧ 100 ml/hour was 33 cm, or *ca* 250 g of the dry exchanger (see below). Charges of 10 g to 25 g of the TRF concentrate were applied on this column, the buffer changes being similar to what we have described above for the smaller column.

Regeneration of the gel: gel was removed from the column and added to a large excess of 1% pyridine in water (10 volumes); in less than 12 hours with constant stirring the slurry was thus adjusted to pH 4.6 to 5.0. After a few hours settling, the liquid phase was removed with suction and the gel was equilibrated by repeated washings with 0.001M pyridine acetate pH 5.0; this completed the regeneration procedure.

TRF concentrate: The material used for purification on CM-Sephadex was the crude TRF concentrate prepared by gel filtration on Sephadex G-25 of the 2N acetic acid extract of sheep hypothalamus, as described earlier(4,3). In terms of specific activity (units of biological activity/mg dry weight) this material contained 1 U/mg (see 5).

The bioassay for TSH-releasing activity was the *in vivo* method described earlier by Yamazaki *et al*(6) or a recent modification of it which increases its sensitivity approximately 5-fold (see 7). The presence of arginine-vasopressin was assessed *vs* the USP

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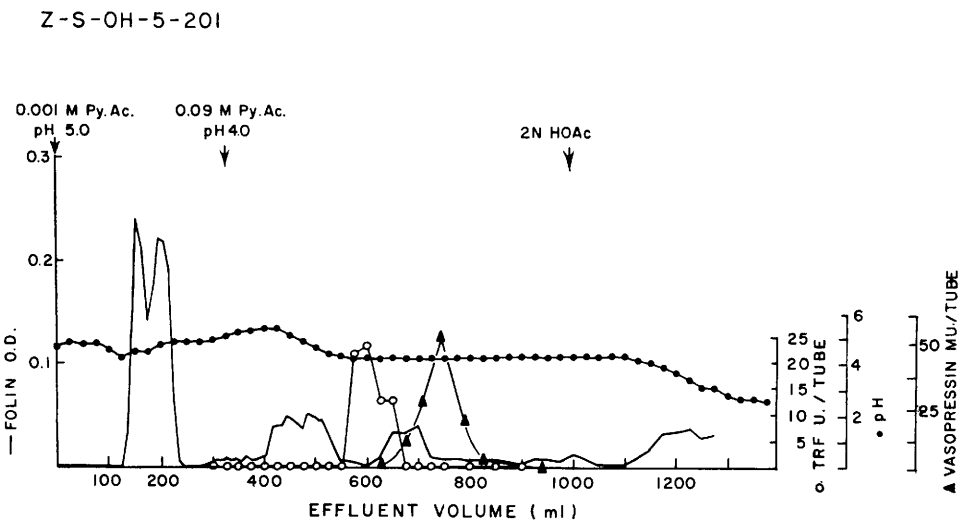

FIG. 1. Filtration chromatography of 374 mg TRF concentrate on a CM-Sephadex C-50 column, 3.8 × 15 cm; flow rate 10 ml/hr; 5 ml fractions. Stepwise buffer changes indicated by notations on arrow.

Posterior Lobe Reference Standard in the pithed rat with direct recording of blood pressure through the carotid artery. Melanocyte stimulating activity (MSH) was measured by the *in vitro* method of Shizume *et al* as described in(8).

Results and discussion. Considerable difficulties were encountered in trying to obtain a reasonable flow rate with C-50 equilibrated in 0.001M pyridine acetate when packing columns of 1.0 to 1.2 cm diameter. It was then decided to increase the diameter to *ca* 4.0 cm; a flow rate of 15.0 to 20.0 ml/hour was thus easily obtained for gel bed height of *ca* 15 cm and with a pressure head of *ca* 20 cm water. Fig. 1 describes results obtained in the gel chromatography of 374 mg of the TRF concentrate; the biological activity relating to TRF was strongly retained on the exchanger equilibrated with 0.001M pyridine acetate pH 5.0 and was eluted quantitatively upon application of the 0.09M buffer (374 TRF units applied, 450 TRF units recovered).

Fig. 2 shows the results obtained in the chromatography on a preparative scale of 21.0 g of the TRF concentrate on a column 15 cm diameter with a gel bed height of 33 cm, flow rate 140 ml/hour. Here again, the biological activity is sharply eluted upon application of the 0.09M buffer. The recovery of biological activity is excellent (21000 units applied, 25000 units recovered). The specific activity of the lyophilized TRF fraction is *ca* 10 U/mg, thus showing a purification factor of approximately 10× following chromatography on the substituted Sephadex. In every experiment with C-50 Sephadex, we have consistently found a recovery of biological activity (TRF units applied *vs* TRF units recovered) somewhat above 100%; this may well

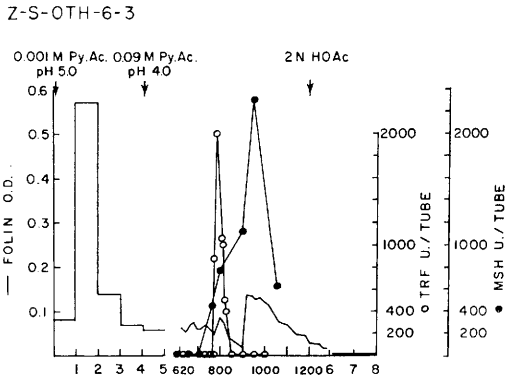

FIG. 2. Filtration chromatography of 21.0 g TRF concentrate on a CM-Sephadex C-50 column, 15 × 33 cm; flow rate 140 ml/hr. Fractions 1-5, collection in flasks, total volume 12.380 liters. 620 *et seq.* represent tube numbers (20 ml fractions); the number of the tubes was calculated from the volume of fractions 1-5, which corresponded to collecting 619 tubes, 20 ml each. Fractions 6-8, collection in flask, 6 liters. Total volume of effluent collected 22.020 liters.

be within the limits of confidence of the simple bracketed bioassays used. This could also be explained by the separation during chromatography of some inhibitor of TRF, present in the starting material. The TRF activity appears to be fairly well separated from the peaks of pressor- and melanocyte stimulating-activity; the MSH and pressor activities detected by the bioassays correspond to the small contaminations by arginine-vasopressin and α-MSH, to be found in the TRF concentrate prepared by filtration on G-25(3,4).

Purification of TRF by filtration chromatography as in the conditions described here has also been used recently to replace the IRC-50 stage after countercurrent distribution as reported in(3).

Several observations were made during these experiments with the substituted carboxymethyl-Sephadex which may be worth reporting. With 1.0 cm i.d. columns, the flow rate would fall to $\leq$ 0.10 ml/hour as soon as the gel bed height reached 5.0 cm; good flow rates (over 15 ml/hour) were obtained when the diameter was increased to 3.8 cm for gel beds up to 30 cm height. With the preparative column 15 cm i.d., the flow rate decreased exponentially with bed heights greater than 33 cm. This bed height was thus considered as the upper limit at which the flow obtained would be 100-150 ml/hour; the column was usually run with a constant level buffer head of 75 cm/water. As expected, there was a striking contraction of the hydrated gel when the buffer with the high molarity was introduced and still further contraction upon applying 2N acetic acid. As the gel bed contracted, the flow rate was not impaired; if anything, it had a tendency to increase. When equilibrated in the 0.001M pyridine acetate buffer, the C-50 Sephadex packs so loosely that it is difficult to apply the sample without some evidence of channeling, even with a 2 cm top lay of Sephadex G-25. This behavior, however, has not given any difficulty with the resolution of the peaks eluted subsequently. *Aging of CM-Sephadex:* It was found that after 4 or 5 regenerations the batch of C-50 Sephadex so treated would no longer be amenable to correct packing, the matrix behaving as if its grains, when in suspension, were of a superfine size; the net result being a complete arrest of the flow for as little as 1 cm height of hydrated gel.

Summary. Purification of the TRF concentrate prepared by gel filtration of acetic acid extracts of hypothalamic tissues on Sephadex G-25 can be achieved following gel chromatography on carboxymethyl-Sephadex C-50 equilibrated in 0.001M pyridine acetate. The TRF activity is quantitatively eluted upon stepwise application of 0.09M pyridine acetate buffer. The method described can be used with small columns (4 $\times$ 15 cm) with charges $\leq$ 1 g TRF concentrate and also with large size preparative columns (15 $\times$ 33 cm) with charges up to 25 g of the TRF concentrate.

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