Journal of Chromatography, 170 (1979) 221–226
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CHROM. 11,491

Note

Simple determination of the folate binding capacity of proteins by chromatography on nitrocellulose and poly(vinyl chloride) membranes

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(Received September 25th, 1978)

The investigation of folic acid binding proteins (FABP) is important because of their role in folate transport in the organism and their use in the determination of folates by radioisotopic techniques. Methods devoted to the determination of the binding capacity of FABP are based on the adsorption of the free ^{3}H-labelled pteroylglutamic acid ([^{3}H]PGA) on charcoal coated with various substances. It has been shown that the standardization of the former method is difficult and that the results are significantly influenced by the presence of non-specific proteins that interact with charcoal[1–4].

In this work, we investigated whether membrane chromatography on nitrocellulose (NC)[5,6] and poly(vinyl chloride) (PVC)[7,8] ultrafilters could help to overcome and simplify this analytical problem. We have found that [^{3}H]PGA bound to FABP remained at the start during chromatography on both carriers, while free [^{3}H]PGA together with other low-molecular-weight substances migrated with the front[5–8]. We used this fact as a basis for a rapid method for the determination of FABP capacity.

MATERIAL AND METHODS

Isotonic Britton buffers[9] were used in all experiments. They were prepared by mixing appropriate volumes of the basic stock solution (0.2 *M* sodium hydroxide solution) and of the acidic stock solution containing equimolecular 0.0285 *M* concentrations of citric acid, barbital, boric acid and potassium dihydrogen orthophosphate. This buffer system covers the pH range 2.6–11.8. The pH values were checked with a PHM 64 pH meter (Radiometer, Copenhagen, Denmark). The [^{3}H]PGA was 3′,5′,9(*n*)-[^{3}H]pteroylglutamic acid, TRK-212, specific activity 15 Ci/mmole ($55.5 \cdot 10^{10}$ Bq), obtained from the Radiochemical Centre (Amersham, Great Britain). SLS-31 scintillation fluid (Spolana, Neratovice, Czechoslovakia) and an Intertechnique SL-40 scintillation spectrometer were used.

The NC membrane filter was Synpor 6, pore size 0.4 μm (VCHZ Synthesia, Uhříněves, Czechoslovakia). BDWP 10 000 and BCWP 10 000 PVC membranes were obtained from Millipore (Bedford, Mass., U.S.A.) and SM 12 804 pore size 0.9 μm)

and SM 12 806 (pore size 0.6 μm) membranes from Sartorius (Göttingen, G.F.R.). Before ascending chromatography was carried out, the NC membrane was washed in boiling water and then cut into 50 × 8 mm strips. PVC membrane strips of the same size were pre-treated with 40% ethanol. Both types of membranes were then placed on a suitable soaking material and washed with few drops of the buffer solution. The membranes should be handled carefully with flat forceps. A 1% aqueous solution of phenol red served as a flow marker. When necessary, the chromatograms were dried at 37° for 20 min and stained with Coomassie blue to detect protein spots.

FABP was isolated from cow's milk by the ammonium sulphate method used for the isolation of β-lactoglobulin[10,11]. The final precipitate was dissolved in a minimal amount of pH 6.3 buffer, which had previous been diluted 10-fold. This sample was dialysed for 24 h against the pH 4.0 buffer (diluted 10-fold)and then for 48 h against the pH 7.2 buffer (diluted 10-fold). The temperature was kept at 4° throughout the isolation procedure. No toluene was added. The final dialysed sample, usually containing less than 0.8% of proteins, was divided into several test-tubes and stored at −20° without losing its folate binding capacity for 2 months. Pig serum prepared from fresh blood from healthy pigs was stored at −20°. The concentration of proteins was determined as described by Lowry *et al.*[12].

Standard binding procedure and ascending membrane chromatography

The stock sample of FABP was thawed and diluted with pH 7.2 buffer to give the desired binding capacity (usually 30-fold). A 100-μl volume of the solution was incubated with 10 μl (380 pg) of [^{3}H]PGA in a test-tube at laboratory temperature for 15–30 min. A 10-μl volume of the incubated sample was applied to a transparent polyethylene foil (20 × 30 cm) at a point marked with a pencil on an underlying sheet of white paper. A small droplet (about 0.5 μl) of the flow marker was applied at the same position immediately before the sample. One end of the membrane strip was fixed between two strips (15 × 100 mm) of Whatman No. 1 filter-paper by means of paper-clips at the top of the holder (Fig. 2). The starting edge of the wet membrane was then contacted with the drop of the sample on the polyethylene foil.

After the sample had soaked in completely at the starting position, the holder with the membrane was lifted briefly, then 10 μl of the buffer were applied to the polyethylene foil and development of the chromatogram was continued. This procedure was repeated twice. Care must be taken to prevent the membranes from becoming dry (*e.g.*, by using a suitable box). Chromatography was stopped when the front marked by phenol red approached the filter-paper wick and all of the buffer had been soaked up (after about 20 min). The chromatogram was then cut 1 cm above the start into two parts: the part at the start containing [^{3}H]PGA bound to FABP and the front part containing free [^{3}H]PGA (see Fig. 1). The strips were dissolved directly in vessels containing 10 ml of scintillation fluid. The resulting ratio of bound to free [^{3}H]PGA was expressed as a percentage of the sum of the radioactivity applied to the chromatogram.

RESULTS AND DISCUSSION

Fig. 1 illustrates the chromatographic separation of free [^{3}H]PGA migrating with the front from [^{3}H]PGA bound to FABP, which is adsorbed strongly at the starting

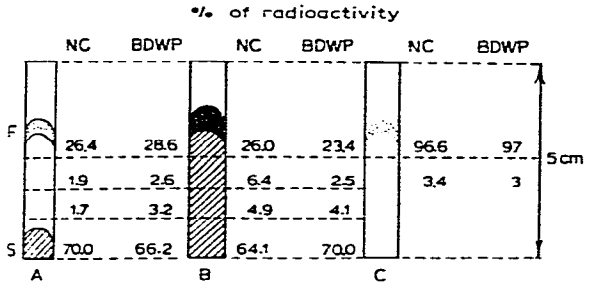

Fig. 1. Example of the chromatographic separation of free and bound [^{3}H]PGA. Strip A, 38 pg of [^{3}H]PGA and 3.0 μg of FABP from cow's milk; B, 38 pg of [^{3}H]PGA and 800 μg of pig serum proteins; C, 38 pg of [^{3}H]PGA. NC = Nitrocellulose membrane, Synpor 6; BDWP = poly(vinyl chloride) membrane, Millipore BDWP 10 000; S = start; F = front. The standard binding assay procedure was employed. Dark areas correspond to proteins, dotted areas to phenol red (indicator of the chromatographic front). The chromatograms were cut as indicated by the horizontal broken lines. The corresponding radioactivity is expressed as a percentage.

zone of NC and PVC membranes. The amount of radioactivity in the zones between the start and front was relatively small. When [^{3}H]PGA was chromatographed alone without FABP, almost all of the radioactivity was found in the front zone (strip C). The result represented by strip B confirmed that pig serum contained a significant fraction of FABP[13]. This FABP fraction was adsorbed on the membranes in the starting zone more strongly than other serum proteins, so that even a large excess of the non-specific proteins did not influence the binding assay procedure significantly. We have found that pig serum diluted 20-fold still bound 30% of the added [^{3}H]PGA. It was calculated that 1 ml of pig serum was able to bind 60 ng of [^{3}H]PGA. There was no significant difference between the results achieved with different types of membranes when working the same sample of partly purified FABP and [^{3}H]PGA. Most experiments were performed with Synpor 6 and BDWP membranes.

The type of holder with easily adjustable height (Fig. 2) was most suitable for work with the fine fragile membranes.

Fig. 3 shows the pH dependence of the binding capacity of FABP (average results of three different FABP batches)[4,14,15]. The concentrations of FABP were

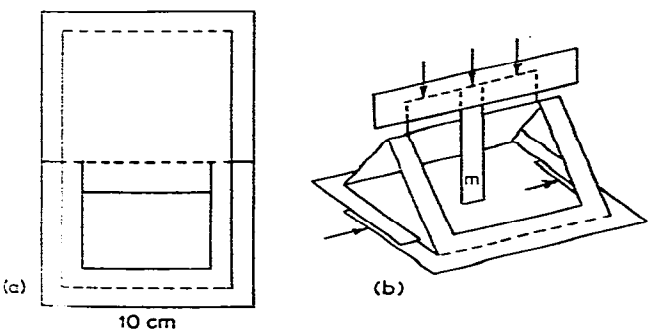

Fig. 2. Simple holder for ascending membrane chromatography. (a) Sheet of hard paper (0.2–0.6 mm thick) to be cut (full lines) and folded (broken lines); (b) the holder ready for use; arrows indicate where the paper clips have to be applied. m = Membrane strip.

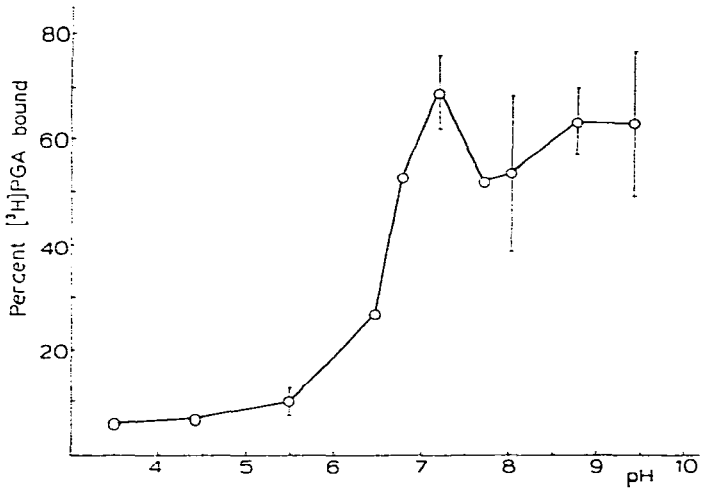

Fig. 3. pH dependence of [³H]PGA–FABP complex formation. The standard binding assay procedure was employed, except that the pH was adjusted to the desired value using the conventional buffer: 38 pg of [³H]PGA and FABP isolated from cow's milk were used.

adjusted to bind all the [³H]PGA added (see Fig. 5). At the standard condition (pH 7.2) the binding capacity was 69% (S.D. = ±7%, $n = 61$).

Fig. 4 shows that the formation of the [³H]PGA–FABP complex was completed after 15 min of incubation of the reagents under the given conditions. When FABP was adsorbed on the membrane and then [³H]PGA was run over it, complex formation was incomplete.

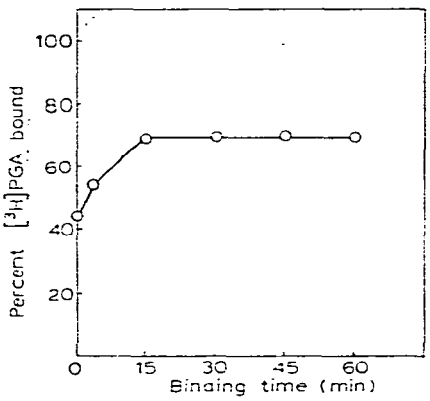

Fig. 4. Time dependence of [³H]PGA binding to FABP. The standard binding assay procedure was employed; 38 pg of [³H]PGA and 3.0 μg of FABP from cow's milk were used.

Fig. 5 shows that the binding capacity of FABP was a linear function of increasing binder concentration up to the region of the inflection point, where all [³H]PGA present was bound.

Fig. 6 illustrates that the sum of the activity (in counts per minute) applied to the chromatograms increased in proportion to the concentration of [³H]PGA curve

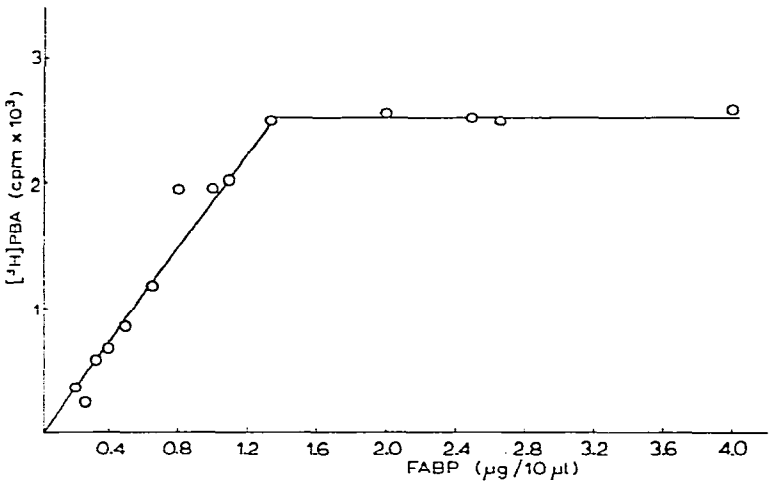

Fig. 5. Effect of increasing amount of FABP on the binding of [³H]PGA. The standard binding assay procedure was employed; 38 pg of [³H]PGA was used.

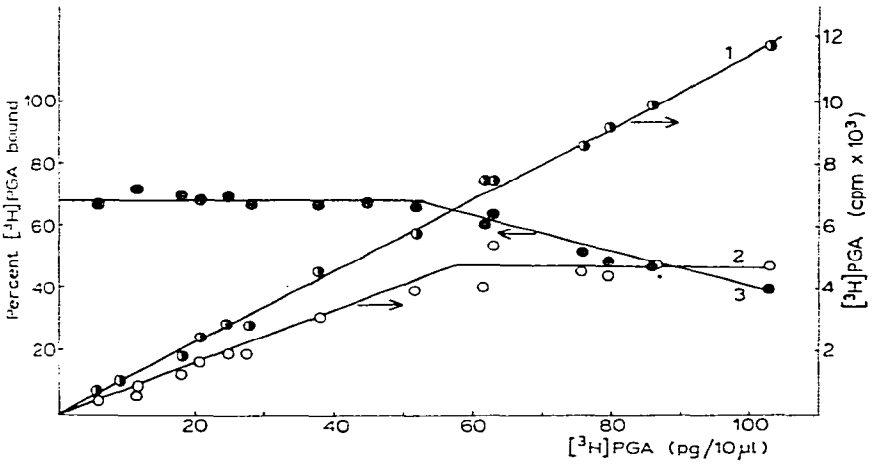

Fig. 6. Change in bound [³H]PGA as a function of the amount of [³H]PGA added to the incubation medium. 1 = Sum of cpm of bound and free [³H]PGA (see standard procedure and Fig. 1); 2 = cpm of bound [³H]PGA; 3 = bound [³H]PGA expressed as a percentage of total [³H]PGA added. A constant amount of FABP (1.66 μg) from cow's milk was used.

1). However, the activity corresponding solely to protein-bound [³H]PGA increased linearly with increasing [³H]PGA concentration only up to the region of the inflection point, where the binding capacity of FABP was saturated (curve 2). Curve 3 shows that 70% of the radioactivity of [³H]PGA added was bound even at its lowest concentrations, before the saturation of FABP. After having reached the inflection point, the relative percentage of bound [³H]PGA began to decrease. The relationship between the purity of [³H]PGA and the degree of its binding to FABP is under investigation.

ACKNOWLEDGEMENT

The authors are indebted to Mrs. E. Kotýnková for excellent technical assistance.

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