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Note

Difference between free and ligand-bound folate-binding protein in cow's milk concerning affinity for chromatographic gels

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The occurrence of a folate-binding protein (FBP) in cow's milk has been established by several investigators[1–3]. Gel-chromatographic studies showed that FBP had a molecular weight similar to that of β-lactoglobulin (35,000), but, by using an ion-exchange chromatographic technique, it was possible to separate FBP from β-lactoglobulin[3–5].

As a part of studies on the mechanism of folate binding, we attempted to estimate the molecular size of FBP in cow's milk by determining the extent of folate binding in effluent fractions from the chromatography of FBP solutions on Sephadex G-100 gel; only a small part of the original folate-binding capacity (FBC) was recovered in the effluent. Large amounts of protein-bound [^{3}H]folate were, however, eluted after application of a [^{3}H]folate solution to the column. We tentatively interpret these findings as follows: FBP is retained in the column, owing to a high affinity for the gel matrix, but this affinity decreases markedly after binding of [^{3}H]folate, resulting in the appearance of [^{3}H]folate-FBP in the effluent. The work described here provides further evidence in support of this idea.

MATERIALS AND METHODS

Isolation of FBP

Whey protein was obtained from cow's milk by precipitation with ammonium sulphate as described by Armstrong *et al.*[6], and the ammonium sulphate was removed by dialysis against distilled water. The whey protein was then dialysed against 0.05 *M* imidazole buffer solution of pH 6.3 and applied to a DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) column (2 cm^2 × 35 cm), then elution was carried out at 5° with a linear gradient (0.03–0.3 *M*) of sodium chloride in the imidazole buffer solution[7]. FBP appeared in the effluent fraction collected just before the start of the salt gradient, and the FBP concentration was increased by dialysis of the effluent against polyethylene glycol of mol. wt. 20,000 (Fluka). The FBP solution was then dialysed against 0.2 *M* acetate buffer solution of pH 3.5 to remove endogenous folate. The final folate concentration was determined by microbiological assay[8] and constituted less than 1% (1 nmole/l of folate) of the FBC (160 nmole/l of folate). The FBP solution was finally dialysed against 0.17 *M* Tris buffer solution of pH 7.4, and aliquots of this stock solution were stored at −85°. Conventional protein assays were

insufficiently sensitive to permit measurement of the FBP concentration. The maximum FBC of the stock solution was determined in equilibrium dialysis experiments with increasing external concentrations of [^{3}H]folate. The FBC of various dilutions of the stock solution was directly proportional to the FBP concentration; we have therefore expressed the concentration of FBP in units of FBC.

Equilibrium dialysis

Equilibrium dialysis experiments were performed, at 37°, in 0.17 *M* Tris buffer solution of pH 7.4; the FBP solution was placed in a dialysis bag (1.00 ml), and [^{3}H]folate (Radiochemical Centre, Amersham, Great Britain) of specific activity 26–38 Ci/mmole was added to the external solution (200 ml). Equilibrium between protein-bound and free folate was achieved within 24 h. Radioactivity was counted in a Packard 2425 Tri-Carb liquid scintillation spectrometer, with Instagel (Packard) as scintillation fluid. Counting efficiencies were controlled by internal standardization with [^{3}H]toluene (Packard).

Gel chromatography

Two types of experiments were performed, the FBP being incubated (for 24 h at 5°) either with or without [^{3}H]folate before application to the column. In the first type of experiment, aliquots of the effluent fractions were taken for radioactivity measurements; in the second type, the FBC was determined in pooled effluent fractions subjected to equilibrium dialysis against increasing external concentrations of [^{3}H]folate.

Binding of FBP

Ultrogel AcA 34 (LKB Produkter, Bromma, Sweden) was washed five times in Tris buffer solution, weighed after removal of excess of buffer and added to FBP solutions (1 g of gel to 5 ml of solution). The FBP had been incubated (for 24 h at 5°) either with (*A*-series experiments) or without (*B*-series experiments) [^{3}H]folate before addition of the gel. Each mixture of gel and FBP was then incubated in a test-tube rotator for 24 h at 25°. Control experiments were performed in the same way, except that no gel was added. At the end of the incubation period, the gel was centrifuged down, and FBC was determined on the supernatant liquid. The amount of FBP bound to the gel sediment was calculated from the difference between the FBC measured in the control experiment and in that with the gel addition.

Gel sediment from the *B*-series experiments (see above) was washed three times in Tris buffer to remove any FBP that might have been trapped in the gel water, then re-suspended in Tris buffer (1 g of gel per 5 ml) containing excess of [^{3}H]folate, and subjected to the incubation procedure described above. At the end of the experiment, the FBC of the supernatant liquid was determined; from this value, the amount of FBP released from the sediment was calculated.

RESULTS

The results shown in Fig. 1 were obtained from two types of experiments. In one, FBP that had been incubated with [^{3}H]folate was applied to a Sephadex G-100 column and elution was carried out with imidazole buffer solution. The elution

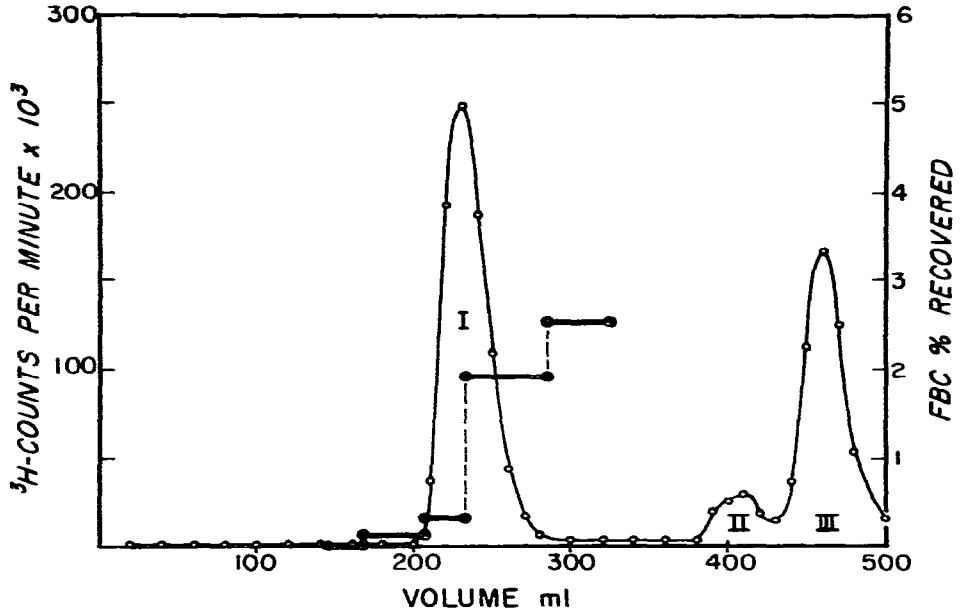

Fig. 1. Gel chromatography of FBP pre-incubated with (○) or without (●) [^{3}H]folate on a column (5.3 cm^2 × 85 cm) of Sephadex G-100, with 0.05 *M* imidazole solution of pH 6.3 as eluent (25 ml/h); 4-ml samples were applied and the column was operated at 5°. Blue dextran (Pharmacia), albumin (Behringwerke), β-lactoglobulin (Sigma) and $CoCl_2$ (Merck) were used for calibration (proteins were monitored by the UV absorption at 280 nm). Abscissa: volume (effluent fractions of 4.0 ml). Left ordinate: ^{3}H counts in effluent. Right ordinate: cumulated folate-binding capacity (FBC) in effluent as percentage of FBC of the sample.

profile (Fig. 1) has three peaks, a large one (I, at 230 ml) representing protein-bound [^{3}H]folate with a mol. wt. similar to that of β-lactoglobulin (35,000), and two small ones (II, at 410 ml; and III, at 460 ml) similar to those found in the elution profile of [^{3}H]folate (mol. wt. <1000). The smallest peak (II) may represent a minor impurity in the radioactive preparation.

In the second type of experiment, a solution of FBP was run through the column, and FBC was determined in the pooled effluent fractions (*cf.* Materials and methods). The steepest increase in the recovery of FBC was observed in the effluent fractions from 232 to 284 ml, where protein-bound [^{3}H]folate was localized. However, less than 3% of the FBC of the sample had been eluted after passage of 324 ml of eluent.

Fig. 2 shows the results of experiments similar to those leading to Fig. 1, but with Tris in place of imidazole as eluent. Peak I (at 186 ml) represents protein-bound [^{3}H]folate (mol. wt. 35,000), whereas peaks II (320 ml) and III (352 ml) are both attributable to [^{3}H]folate (mol. wt. <1000). The steepest increase in the recovery of FBC occurred in the effluent fraction from 172 to 212 ml. The fact that 16% of the FBC of the sample had been recovered after passage of 240 ml of eluent indicates that more FBP can be recovered when imidazole is replaced by Tris. The recoveries are comparable, as the amounts of FBP applied to both columns were the same (the FBC of both samples was 1.5 nmole of folate).

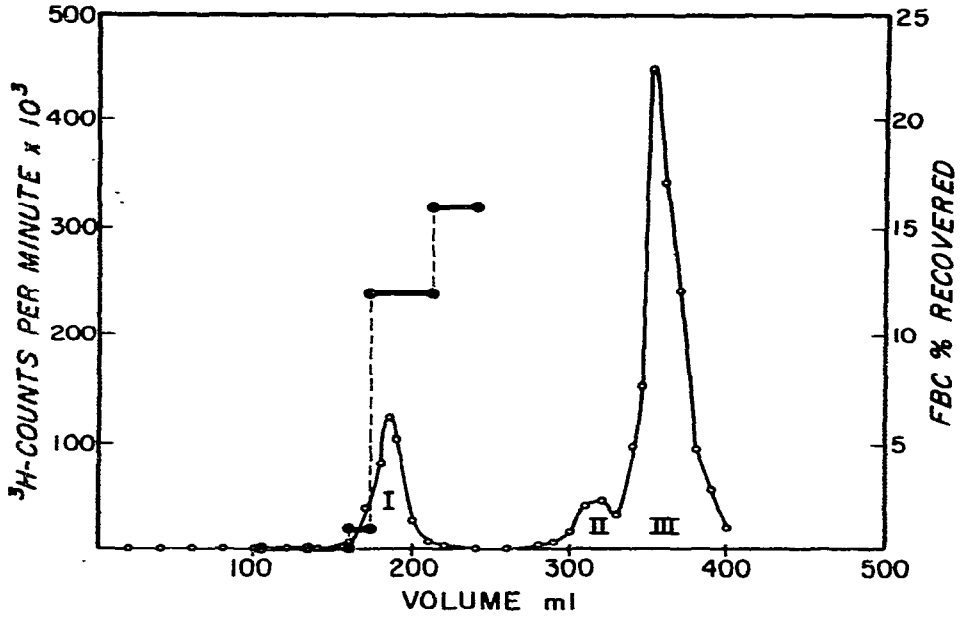

Fig. 2. Gel chromatography of FBP on a column (5.3 cm^2 × 70 cm) of Sephadex G-100, with 0.17 *M* Tris of pH 7.4 as eluent; other conditions and symbols as in Fig. 1.

The experiments leading to the profile shown in Fig. 1 were repeated with use of a column of Ultrogel AcA 34; the results are shown in Fig. 3.

When FBP pre-incubated with [^{3}H]folate was applied to the column, the elution pattern consisted of a large peak (I, at 272 ml) representing protein-bound [^{3}H]folate (mol. wt. 35,000) and a small peak (II, 360 ml) identified as [^{3}H]folate (*cf.* Fig. 4). When FBC was determined in pooled effluent fractions, the steepest increase in recovery of FBC occurred in the interval (260–300 ml) corresponding to localization of the protein-bound [^{3}H]folate. Only 5% of the FBC of the sample (3.5 nmole of folate) was recovered after passage of 300 ml of eluent.

Fig. 4 shows the elution profile of a [^{3}H]folate sample applied to the column just after the determination of FBC in pooled effluent fractions shown in Fig. 3. The two peaks (at 236 and 300 ml) are localized in the same effluent fractions as protein-bound [^{3}H]folate (*cf.* Fig. 3); the peak at 300 ml was eluted at 280 ml on re-chromatography.

The application of [^{3}H]folate was repeated after the column had been carefully rinsed to remove any trace of FBP (*cf.* legend to Fig. 4). Under these conditions, the elution profile of [^{3}H]folate was quite different, having only one large peak (360 ml) corresponding to a mol. wt. <1000.

Results from experiments on the binding of FBP to Ultrogel AcA 34 are shown in Fig. 5. At low FBP concentrations (FBC of 1–10 nmole of folate), there is virtually no binding of FBP, nor of the [^{3}H]folate–FBP complex, to the Ultrogel (no decrease of FBC in the presence of Ultrogel). At higher FBP concentrations (FBC of 80 and 160 nmole/l of folate), however, there is considerable binding of

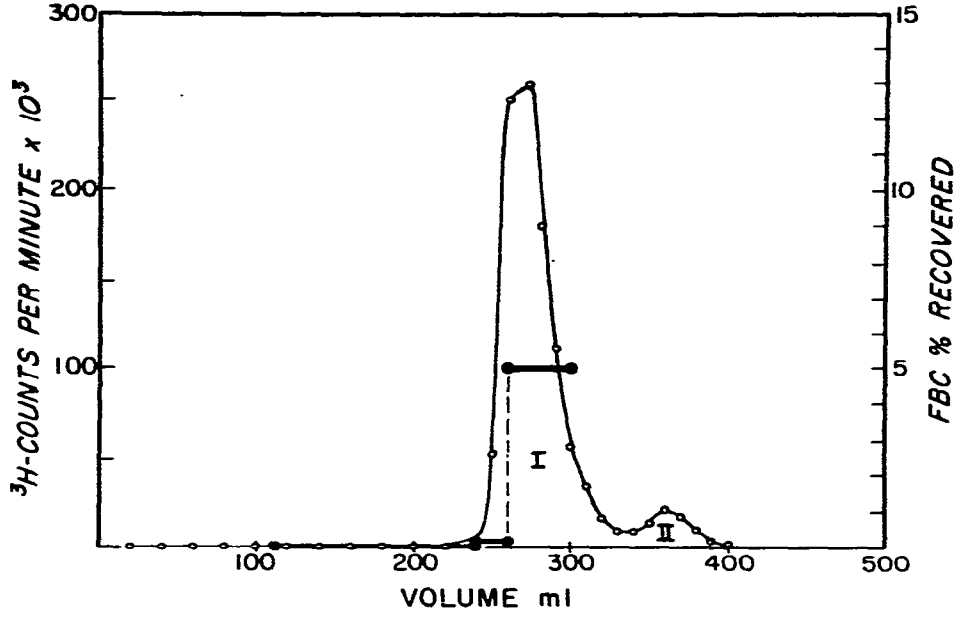

Fig. 3. Gel chromatography of FBP on a column (5.3 cm^2 × 67 cm) of Ultrogel AcA 34 (LKB), with 0.05 *M* imidazole as eluent (50 ml/h): other conditions, symbols and sample volume as in Fig. 1.

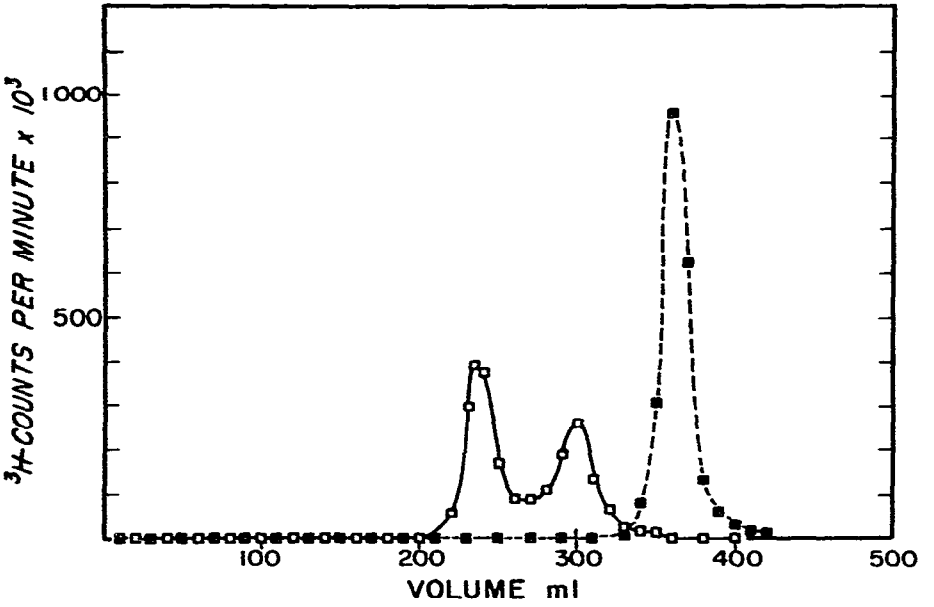

Fig. 4. Gel chromatography of FBP on Ultrogel AcA 34, with sample volume 4.0 ml and abscissa and ordinates as in Fig. 1. □, Elution profile of [^{3}H]folate applied to column just after chromatography of FBP not pre-incubated with [^{3}H]folate (*cf.* Fig. 3, ●); ■, elution profile of [^{3}H]folate applied after column had been rinsed with [^{3}H]folate solution and then with buffer solution for 24 h until no radioactivity was detected in the effluent.

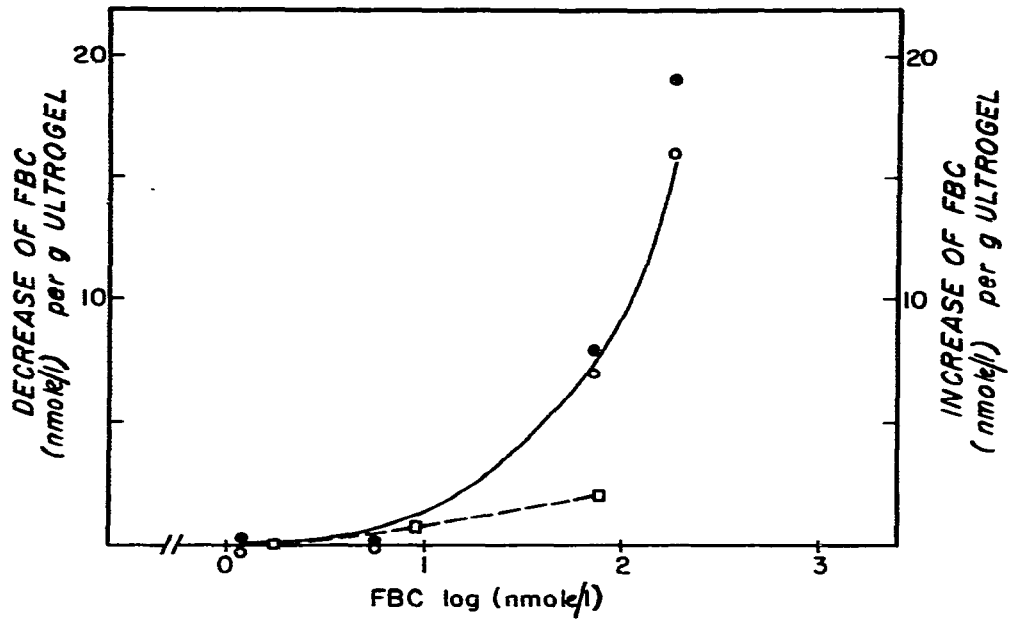

Fig. 5. Curves showing binding of FBP to Ultrogel AcA 34. Abscissa: FBC of FBP solutions (nmole/l of folate) on log scale). Left ordinate: decrease of FBC per g of Ultrogel added to FBP solutions pre-incubated with (□, *A*-series experiments) or without (○, *B*-series experiments) [^{3}H]folate. Right ordinate: increase of FBC after re-incubation of Ultrogel sediment from *B*-series experiments with [^{3}H]folate (●). Each symbol represents a single determination (for details, see Materials and methods).

FBP to the gel. This binding does not seem sufficient to saturate within the range of FBP concentrations studied. The tendency of the [^{3}H]folate–FBP complex to bind is far less pronounced. Exposure of gel sediments with bound FBP to [^{3}H]folate solutions gave rise to a quantitatively complete release of FBP. As expected, there was no release of FBP from gel sediments that had been incubated with low concentrations of FBP (no FBP being bound to the gel).

DISCUSSION

The experiments described show that FBP has a much higher affinity for certain chromatographic gels than has [^{3}H]folate–FBP. Binding of FBP to the gel is a reversible process, since the protein is released (probably in the form of a [^{3}H] folate FBP complex) following exposure of the gel to [^{3}H]folate. Further, binding of FBP to the gel does not, apparently, abolish the ability of this protein to bind folate. Binding of [^{3}H]folate to FBP may induce conformational changes in the binding sites on the FBP, resulting in a decreased affinity between this protein and the gel. It is tempting to suggest that this phenomenon may be utilized in developing a highly specific method for isolating FBP from milk or other biological fluids, and we are at present investigating this possibility.

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