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AFFINITY CHROMATOGRAPHY OF MOUSE ALPHA-FETOPROTEIN (AFP) ON OESTRADIOL–SEPHAROSE ADSORBENTS—ISOLATION AND PROPERTIES

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SUMMARY

Affinity chromatography on oestradiol–sepharose adsorbents has enabled the isolation, in a single step and with a good yield, of alpha-fetoprotein (AFP-1) from mouse amniotic fluid. The mouse AFP not retained on the oestradiol adsorbent was subsequently purified by conventional specific immunoadsorption (AFP-2 preparation). The physico-chemical, immunological and oestradiol-binding properties of both preparations have been studied. The molecular weight and the sedimentation coefficient of AFP-1 and AFP-2 were estimated to be 72,000 daltons and 4.5 *s*. Fresh AFP-1 and AFP-2 were found homogeneous by electrophoretic and immunological criteria. The association constants for oestradiol-17β of AFP-1 and AFP-2 were in the same range, 0.5 and 0.6 $\times$ $10^8 M^{-1}$ respectively, while the molecular ratio of oestradiol to protein binding was twofold higher for AFP-1 than for AFP-2. Freezing and thawing of AFP-1 resulted in molecular heterogeneity probably due to intermolecular association of AFP monomers.

INTRODUCTION

Alpha-fetoprotein (AFP), a fetospecific serum globulin, is synthetized by the liver and secreted into the serum during fetal and early post-natal life [1]. The reappearance of AFP in the serum of adults bearing primary hepatoma, permits one to consider this protein as a tumour-associated fetal antigen [2]. Previous work has demonstrated that both rat and mouse AFP possess a high binding affinity for oestrogens [3–5]. Recently, it has been suggested that two populations of AFP molecules are present with different affinity for oestrogens [6–8] in rat and mouse amniotic fluid.

In this paper we describe the isolation of mouse AFP from amniotic fluid: (a) by affinity chromatography on oestradiol–sepharose adsorbents and (b) by specific immunoadsorption. Two distinct preparations of mouse AFP were obtained and their physico-chemical, immunological and oestradiol-binding properties studied.

MATERIALS AND METHODS

Animals. Swiss mouse amniotic fluids (MAF) were obtained by puncturing the amniotic sacs of 18-day-old embryos. The fluids were pooled, centrifuged at 4,500 *g*, treated with dextran-coated charcoal to remove endogenous steroids, dialysed overnight against 2 l. of 0.25 M KCl, pH 7.4, and then stored at −20°C.

Biochemicals. [^{3}H]-oestradiol-17β (45 Ci/mmol) and [^{14}C]-oestrone (56 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, England) and unlabelled oestradiol-17β and oestrone from Roussel–Uclaf (Paris). Agarose was obtained from l'Industrie Biologique Française (Genevilliers, France). AH-Sepharose-4B was from Pharmacia (Uppsala, Sweden). 1-Ethyl-3(3-dimethylaminopropyl)-carbodiimide-HCl was from Serva (Heidelberg, Germany). 17β-Oestradiol 17-hemisuccinate was from Steraloids (Pawling, U.S.A.).

The sulfhydryl reagent, 5,5 dithiobis(2-nitrobenzoic acid), was obtained from Sigma (St. Louis, U.S.A.). All other reagents were of analytical grade.

Electrophoresis. Electrophoretic separations were performed in acrylamide–agarose gel slabs [9]. The composition of the gels was 0.8% agarose and either 5 or 11% acrylamide monomer in Tris–glycine buffer at pH 8.7. After the electrophoretic run, protein bands were stained with Coomasie blue [9]. Electrophoretic patterns were recorded in a Veron scanning densitometer.

Preparative electrophoresis was carried out on the same gel using an Apelab apparatus (Apelab, France), with 24 $\times$ 12 $\times$ 0.6 cm slabs. A single reservoir (20 $\times$ 1 $\times$ 0.6 cm) was cut into the gel and filled with 8 ml of MAF containing about 8 mg of AFP. The electrophoretic run was performed at 8 V/cm for 5 hours. After electrophoresis, the AFP band was localized by staining with Coomassie blue and then cut off from the slab and divided into 8 equal portions. Each portion was homogenized with 1 ml of phosphate buffered saline in a Polytron and stored at −20°C until used.

Immunochemicals. All antisera were prepared in rabbits. For antisera to adult mouse serum (AMS) and to mouse amniotic fluid (MAF), the rabbits were

injected once subcutaneously, with 0.5 ml of AMS or MAF supplemented with 1 ml of Freund's complete adjuvant, followed one month later by an intramuscular injection of 0.5 ml of AMS or MAF diluted 1/10 with buffered saline. The next day the rabbits received 0.3 ml of AMS or MAF intravenously and two days later 0.5 ml by the same route. Ten days later the rabbits were punctured and their blood collected.

Antiserum to mouse AFP was obtained by immunizing rabbits with mouse AFP purified by preparative electrophoresis in acrylamide-agarose gel. Two ml of the homogenized gel containing about 1 mg of mouse AFP were mixed with 1 ml of Freund's complete adjuvant and injected as indicated above. This was followed one month later by a new subcutaneous injection of the same quantity of AFP mixed with 1 ml of 1% aqueous aluminium alum. Ten days later the rabbits were punctured and their blood collected. The antiserum was rendered specific for AFP by adsorption on an insolubilized adult mouse serum (AMS) prepared according to the procedure of Avrameas and Ternynck[10].

Immunodiffusion technique. Immunoelectrophoretic analysis according to the method of Grabar and Williams[11] and quantification of AFP by the electroimmunodiffusion method of Laurell[12] were made in 1.2% agarose gels. Crossed antibody electrophoresis was done by the combination of an electrophoretic run in acrylamide agarose gel (5% acrylamide monomer) and an electroimmunodiffusion run in 1.2% agarose plates containing antiserum specific to mouse AFP.

Immunoadsorption. Rabbit antibodies to whole adult mouse serum (AMS) were isolated by adsorption and elution from insolubilized AMS according to the method of Avrameas and Ternynck[10]. The isolated antibodies were then coupled to glutaraldehyde-activated agarose beads (Biogel) as described by Ternynck and Avrameas[13].

Oestradiol binding. The oestradiol-binding activity of mouse AFP was studied as follows:

(a) Immunoautoradiographic assay according to the technique described previously [4].

(b) Density gradient centrifugation sucrose gradients (5–20%) were prepared in 0.1 M Tris–0.0015 M EDTA buffer at pH 7.5. Samples (0.2 ml) of mixtures of AFP and [^{3}H]-oestradiol-17β were layered on the top of the gradients (4.5 ml) and centrifuged for 7 h at 40,000 rev./min in an L360 Spinco ultracentrifuge (SW 50.1 rotor). Fractions of 0.1 ml each were collected and added to 5 ml of Unisolve-1 (Koch–Light Laboratories, London, England) determined in an Intertechnique SL-40, liquid scintillation counter.

(c) Equilibrium dialysis. One-ml aliquots of MAF diluted 1/100 in 0.15 M NaCl or 1 ml of an AFP solution (10–50 μg/ml) were dialysed in dialysis tubing for 24 h at +4°C against 15 ml of 10 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA and [^{3}H]-oestradiol-17β (180 to 5.62 pM) and unlabelled oestradiol-17β (30 to 0.94 nM). Fractions of 0.2 ml each were collected and added to 5 ml of Unisolve-1. The radioactivity was counted as above.

Oestradiol adsorbent. The oestradiol–sepharose adsorbent for affinity chromatography was prepared according to the technique of Cuatrecases *et al.*[14] with minor modifications. Briefly, 5 g of AH-sepharose-4B were washed thoroughly in several changes of 50% aqueous dioxan. To the beads suspended in 20 ml of the same solution were added 10 ml of 50% aqueous dioxan containing 20 ml of 17β-oestradiol-hemisuccinate and 1.2 g of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide-HCl. The mixture was shaken by mild rotation overnight at room temperature. After coupling, the beads were placed in a sintered glass funnel and washed successively with 250 ml of each of the following solutions: 50% aqueous dioxan, 50% methanol, distilled water, and 0.25 M KCl, pH 7.4. The beads were then suspended in 20 ml of the last solution and stored at +4°C.

Molecular weights were evaluated by the electrophoretic method of Weber and Osborn[15] using 5% acrylamide agarose gels in Tris–glycine buffer containing 0.1% sodium dodecyl sulfate (SDS). Human IgG, myoglobin, ovalbumin and bovine lactoferrin were used as reference standards.

Thiol group

Sulfhydryl and disulfide groups in AFP preparations were determined with 5,5-dithiobis(2-nitrobenzoic acid) [16, 17].

Amino acid analysis of the 6 N HCl hydrolysates of AFP were carried out in a Technicon Autoanalyzer II.

Protein determination. The biuret method [18] was employed with human serum albumin as the protein standard.

RESULTS

Isolation of mouse AFP by affinity chromatography

The suspension of oestradiol–sepharose beads (see Materials and Methods) was centrifuged at 4000 *g* for 10 min. The supernatant was discarded and the packed beads were resuspended in 40 ml of MAF solution, placed in a stoppered plastic flask and mildly agitated by rotation overnight at +4°C. Unless otherwise indicated, subsequent operations were done at room temperature. After incubation the suspension was centrifuged as before. The supernatant was removed and the beads placed in a 200 ml sintered glass funnel and washed by suction with 200 ml aliquots of 0.5 M KCl until the optical density of the filtrates dropped to less than 0.01. The filtrates (MAF-1) were concentrated to about 20 ml by ultrafiltration in an Amicon cell with a PM10 Diaflo membrane and stored at +4°C.

The AFP bound to the washed beads was desorbed by elution with 50 ml of a saturated solution of oestrone in 0.5 M KCl containing 15% dioxan. The washed beads were suspended in this solution and

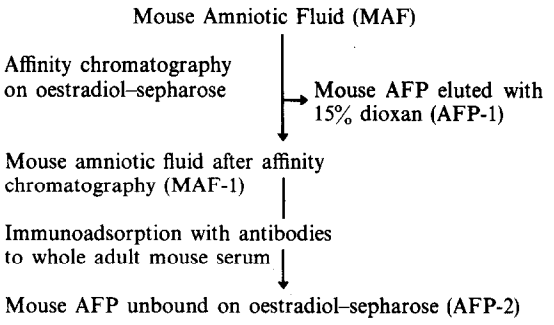

Fig. 1. Schema of the isolation procedure for mouse AFP.

the mixture was maintained with mild rotation for 2 h and then centrifuged as indicated above. The supernatant containing the eluted AFP was decanted, dialysed overnight at +4°C against phosphate buffered saline and concentrated by ultrafiltration through a PM10 Diaflo membrane in an Amicon cell. The isolated AFP preparation (AFP-1) was stored at −20°C.

Isolation of mouse AFP by immunoadsorption

The mouse AFP not bound to the oestradiol–sepharose beads and recovered in the MAF-1 solution (see above) was isolated by immunoadsorption with isolubilized antibodies to whole adult mouse serum proteins (AMS) according to the procedure described previously for rat AFP [5].

This AFP preparation (AFP-2) was also stored at −20°C. The schematic representation of the isolation procedure and the analytical data obtained are shown in Fig. 1 and Table 1, respectively.

Purity control

After electrophoresis in 5% acrylamide agarose gels. Fresh preparations of both AFP-1 and AFP-2 gave a single broad band with a mobility slightly slower than serum albumin (Fig. 2). A unique arc of precipitation was revealed after immunoelectrophoretic analysis of the same preparation against anti-MAF antiserum (Fig. 3A).

Table 2. Binding of oestradiol-17β by native and purified preparations of mouse alpha-fetoprotein (AFP)

	$K_a(M^{-1})$	n_1^*
MAF	0.40×10^8	0.64
AFP-1	0.50×10^8	0.60
AFP-2	0.60×10^8	0.30

* Calculated on the basis of a MW of 72,000 daltons.

Physico-chemical and chemical characterization

One single band was observed after electrophoresis in SDS acrylamide–agarose gels of either AFP-1 or AFP-2. The molecular weight of both preparations as estimated by this method was 72,000 daltons. The sedimentation coefficient was calculated after sucrose gradient ultracentrifugation; a unique value close to 4.5 *s* was obtained for both preparations.

The amino acid composition of the AFP-1 preparation is shown in Table 3 together with data reported by Watabe for mouse AFP isolated by an immunoadsorption procedure [19]. There is excellent agreement between both sets of figures. The high proportion of thiol groups in mouse AFP is noteworthy and was confirmed with our preparation (AFP-1) after the biochemical determination of total sulfhydryl and disulfide groups. One single free sulfhydryl and ten disulfide bridges were found per molecule of AFP.

Oestradiol binding properties

After incubation with [^{3}H]-oestradiol-17β, the binding activity of AFP-1 and AFP-2 was demonstrated by immunoautoradiographic assay (Fig. 3B). The apparent association constants and the molar concentration of binding sites for the interaction of oestradiol-17β with AFP-1 and AFP-2 were determined by equilibrium dialysis. The association constant of whole mouse amniotic fluid (MAF) was obtained by the same method. The results are compared in Table 2. From Scatchard plots of experimental data, a single class of binding sites was estimated

Table 1. Comparison of mouse alpha-fetoprotein (AFP) isolated by affinity chromatography on oestradiol–sepharose and by immunoadsorption with antibodies to whole adult mouse serum

Isolation procedure	Total protein (mg)	Mouse AFP (mg)	Yield
A. Affinity chromatography			
Mouse amniotic fluid (MAF) 40 ml	98	39	
MAF after chromatographic assay (MAF-1) (20 ml)	53.3	7.78	
Mouse AFP eluted in 15% dioxan (AFP-1)	—	10.75	28%*
B. Immunoadsorption			
MAF-1 (15 ml)	39.0	5.83	
Mouse AFP yield after immunoadsorption	—	3.20	55%†

* Percentage of AFP-1 recovered from the initial MAF sample.
† Percentage of AFP-2 recovered from 15 ml of MAF-1 sample.

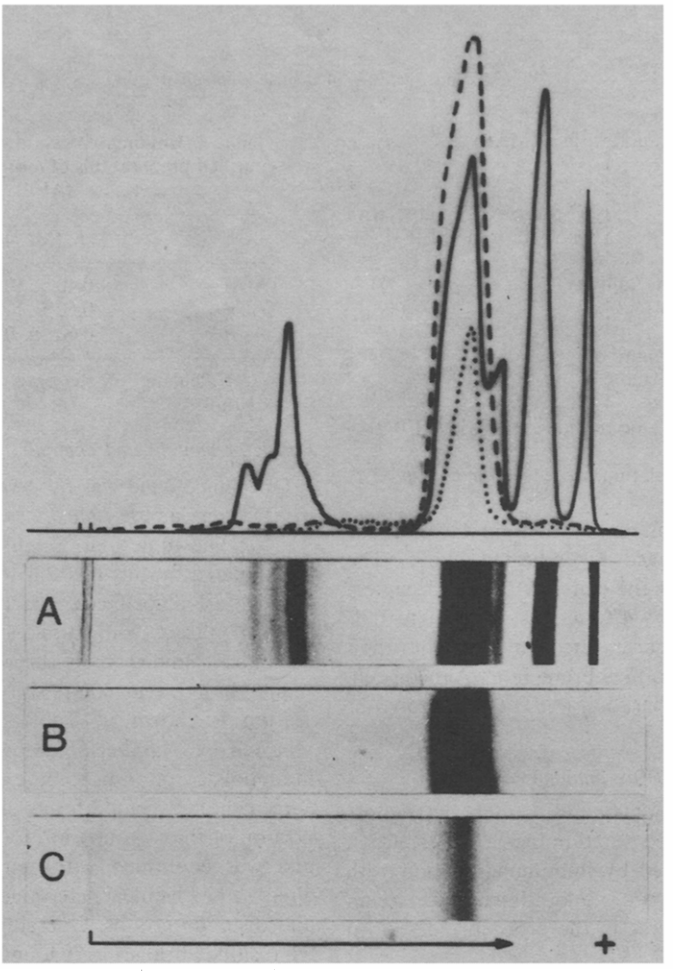

Fig. 2. Electrophoresis in 5% acrylamide-agarose gels of (A) mouse amniotic fluid (MAF), (B) fresh mouse alpha-fetoprotein (AFP-1), (C) fresh mouse AFP-2. The densitometric scanning of the 3 electrophoretic patterns of MAF (——), AFP-1 (– – –) and AFP-2 (· · · · · ·) is seen at the top of the figure.

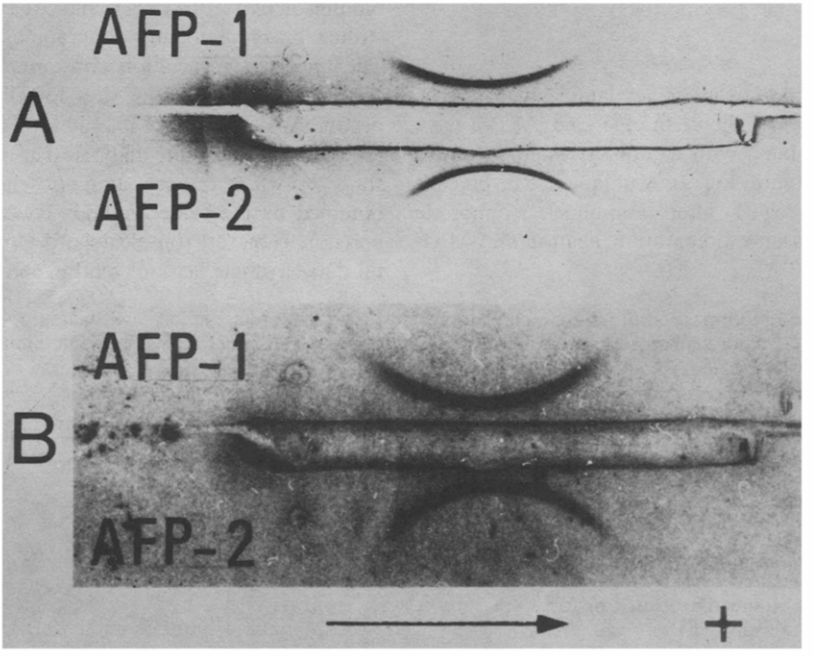

Fig. 3. Oestrone-binding ability of AFP-1 and AFP-2. Aliquots of both preparations of AFP were subjected to immunoelectrophoresis using a rabbit anti-mouse amniotic fluid. After development of immune precipitates (24 h), the plate was washed in buffered saline for 24 h, air dried under filter paper and incubated with [^{14}C]-oestrone (0.2 μCi/ml) for 2 h at laboratory temperature. The plate was then washed in the same buffer for 24 h, dried in air and put in contact (3 weeks) with a Kodak Kodirex film. (A) Protein staining (Amido Black) of the immunoelectrophoretic patterns of AFP-1 and AFP-2. (B) Autoradiographs of the same samples.

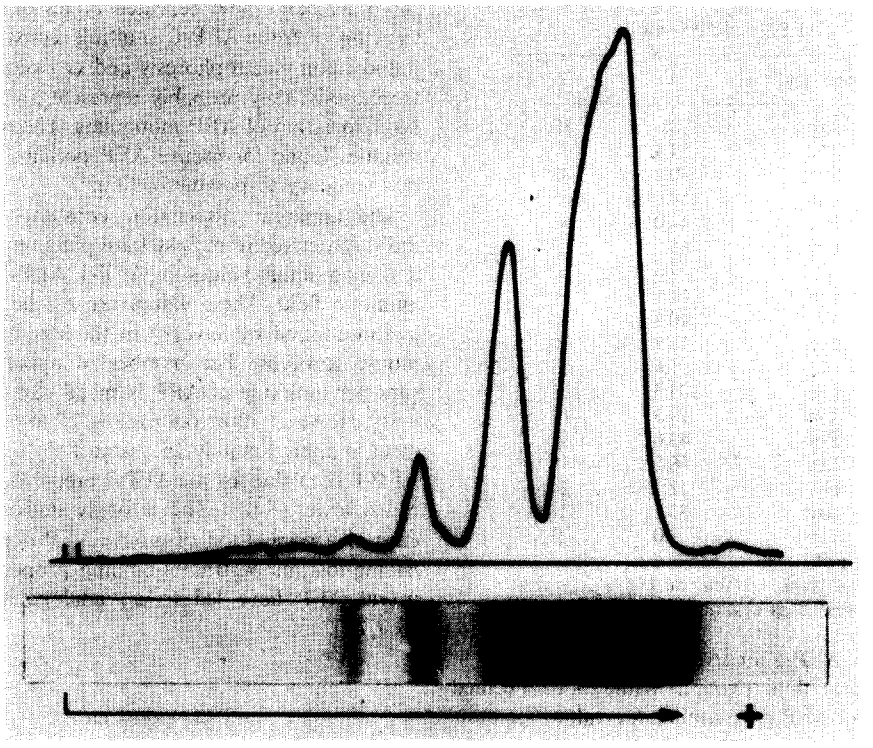

Fig. 4. Electrophoresis in 5% acrylamide-agarose gels of AFP-1 after several cycles of freezing and thawing. The densitometric scanning of this electrophoretic pattern is seen at the top of the figure.

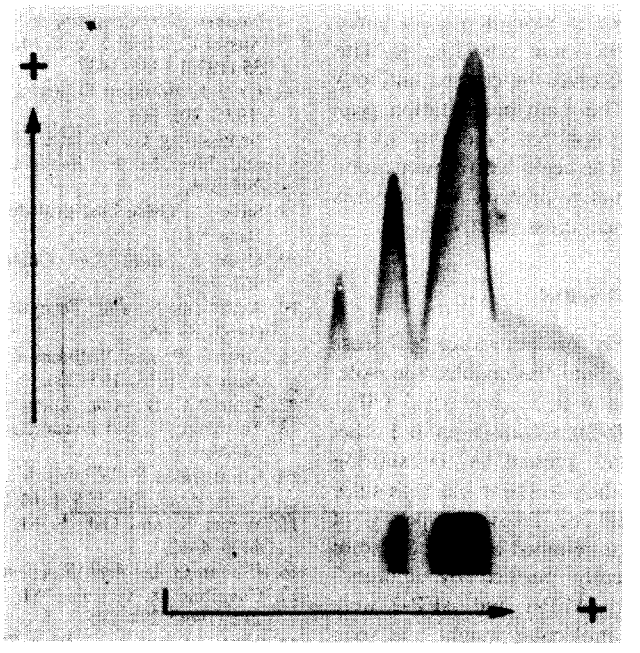

Fig. 5. Crossed antibody electrophoresis. An aliquot of AFP-1 was subjected to electrophoresis in 5% acrylamide-agarose gel. After the electrophoretic run, a longitudinal 4-mm wide strip was stained for proteins with Coomassie blue. Another 4-mm wide strip was placed in a trough of a 1.2% agarose gel containing antiserum to mouse AFP and was allowed to run for 4 h. The agarose plate was then washed in buffered saline, dried under filter paper and stained for protein with Coomassie blue. Top: crossed antibody immunodiffusion pattern. Bottom: acrylamide agarose electrophoretic pattern.

Table 3. Amino acid composition of mouse AFP. (mol/1000 mol)

Amino acid	1	2
Asp	78.6	80
Thr	54.4	49
Ser	80.2	83
Glu	154.0	165
Pro	44.0	48
Gly	46.5	50
Ala	77.3	77
Cys	61.5	30*
Val	40.3	42
Met	18.9	20
Ile	51.6	53
Leu	103.5	104
Tyr	19.3	19
Phe	43.6	41
Lys	68.5	78
His	21.7	24
Arg	33.1	34
Trp	3.0	3

(1) This work, (2) Watabe H.[19].
* Expressed as cystine.

in either the AFP-1 or AFP-2 preparation or in mouse amniotic fluid (MAF). While both purified preparations of AFP gave similar K_A values for oestradiol-17β, the molar ratio (oestradiol/AFP) was twofold higher for AFP-1 than for AFP-2.

Molecular heterogeneity. Fresh preparations of AFP-1 and AFP-2 showed a single broad protein band after electrophoresis in both 5 and 11% acrylamide–agarose gels. However, repeated cycles of thawing and freezing of the AFP-1 preparation resulted in the appearance of several protein bands after electrophoresis in the same gels (Fig. 4). This heterogeneity was also observed by crossed antibody electrophoresis (Fig. 5). The main precipitation peak corresponds to the electrophoretic mobility of the native fresh preparation. The peaks with slower mobility are polymers of the native protein. All the peaks showed high immunological cross-reactivity.

DISCUSSION

Affinity chromatography on oestradiol–sepharose beads of mouse amniotic fluid has enabled the isolation, in a single run and with a good yield (30%), of AFP (AFP-1) free of albumin, transferrin and other minor protein constituents present in the starting material. The capacity of the adsorbent was high since 0.5 0.6 mg of AFP could be recovered per g of swollen beads. The AFP unretained by the oestradiol adsorbent was subsequently purified by immunoadsorption (AFP-2). Both AFP-1 and AFP-2 were found to have the same molecular weight and sedimentation coefficient and the same electrophoretic mobility as native AFP (Fig. 1). However, the asymmetric profile of the densitometric patterns of native AFP and of the AFP-1 preparation suggest some electrophoretic microheterogeneity, a property already reported for human [20] and rat [5, 21] AFP. On the other hand, after repeated cycles of freezing and thawing of fresh AFP-1, multiple constituents were found upon electrophoresis and cross-antibody electrophoresis: they probably represent an intermolecular association of AFP monomers. The many cystein residues found for mouse AFP perhaps account for this tendency to polymerization.

The apparent association constants for oestradiol-17β derived from Scatchard plots of experimental data give similar values for AFP-1, AFP-2 and mouse amniotic fluid. These values are of the same order as those found by Savu[8] in the sera of 18-day-old mouse embryos. The number of apparent binding sites per molecule of AFP is in all cases lower than unity. However, if the oestradiol-17β to protein molar ratio is approximately the same (0.6) for MAF and AFP-1, the value for the AFP-2 preparation is significantly lower ($\sim$0.3). This strongly suggests the existence of a population of mouse AFP deprived of, or having lost, its oestradiol-binding properties. Studies in rat AFP have led to an analogous conclusion [6, 7, 22].

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