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Evidence for an intrinsic factor bound to rat alpha$_1$ fetoprotein. Fluorescence investigation

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Summary. An endogenous chromophore regularly associated with rat alpha$_1$ fetoprotein was detected by a systematic study of the fluorescence spectra of the protein purified by immunoabsorption. This fluorescent ligand, of low molecular weight, was dissociated from rat alpha$_1$ fetoprotein by exhaustive dialysis of the protein at low concentration. A phase partition method with aqueous preparations of rat alpha$_1$ fetoprotein allowed extraction of this chromophore into n-butanol and determination of its spectrofluorimetric characteristics : an excitation maximum at 305 nm and an emission maximum at 365 nm. We have also shown that this endogenous chromophore competed with estradiol-17β for the same binding sites in rat alpha$_1$ fetoprotein. In conclusion, the implication of a specific transport role of this protein for small ligands in fetal development is discussed.

Introduction.

Alpha$_1$ fetoprotein (AFP) is a fetal plasma protein produced in large quantities by the fetal liver and yolk sac (Bergstrand and Czar, 1956 ; Abelev, 1974). The biological role of AFP during fetal development is still unknown. In order to determine this role, its binding with a number of substances has been studied. The parameters for AFP binding with extrinsic biological molecules (bilirubin : Berde *et al.*, 1979 ; Ruoslahti, 1979 ; deoxycholate : Ruoslahti, 1979 ; retinoic acid : Ruoslahti, 1979 ; aflatoxin : Evrain *et al.*, 1978), synthetic molecules (parinaric acid : Berde *et al.*, 1979) and anilinonaphtalene sulfonate (Grigorova *et al.*, 1980) are very similar to those found for serum albumin. The similarities and structural analogies between AFP and serum albumin (Ruoslahti and Terry, 1976 ; Grigorova *et al.*, 1977) suggest that AFP could play the same transport role for small molecules in the fetus as albumin does in the adult, and that these binding properties must correspond to a biological function (Parmelee *et al.*, 1978). Evidence for endogenous ligand(s) bound to native AFP is of interest since a more specific biological role could then be ascribed to AFP binding. It has been noticed in our laboratory that pure rat AFP preparations, obtained by an immunoabsorption method (Cittanova *et al.*, 1974) and dialyzed for different lengths

of time, showed variations in their estradiol-17β binding properties and also spectral changes related to dialysis times. This paper reports some experiments demonstrating the existence of a fluorescent ligand regularly found associated with rat AFP.

Material and methods.

Rat AFP preparations. — The rat AFP used was prepared by an immunoabsorption method (Cittanova *et al.*, 1974) which permitted a high degree of purification. Protein concentrations were determined by the method of Lowry (Lowry *et al.*, 1951).

Equilibrium dialysis. — The binding parameters of rat AFP with estradiol-17β were determined by the method of Meyer and Schellman (1961) : dialysis cells, with a total volume of 200 μl, were separated into two equal compartments by a Visking membrane (18/32, Union Carbide Corp.). Constant amounts of binding protein in 80 μl 0.15 M phosphate buffer, pH 7.4, containing 50 mg/l of gelatin, were dialyzed against estradiol-17β (10^{-9} to 10^{-6} M) (Roussel UCLAF) with 2×10^5 dpm (^{3}H) estradiol-17β (2, 4, 6, 7 (^{3}H) estradiol-17β — 95 Ci/mmole — 99 p. 100 pure from the Radiochemical Centre, Amersham, England) in an equal volume of the same buffer. Equilibrium was reached after gentle shaking for 20 h at 4 °C. The radioactivity in 50-μl samples from each compartment was then determined.

Spectroscopy. — All the reagents were grade-A and spectrofluorimetrically pure. L-tryptophan was obtained from Calbiochem. L-tyrosine, used as an internal standard for the spectrofluorimetric assay, was purchased from Sigma. The experiments were carried out in phosphate buffer of low ionic strength (4 mM, pH 7.4). Fluorimetry was carried out with a FICA model 55 000 MK II differential absolute spectrofluorimeter. Quantum yields were measured by the method of Chen (Chen *et al.*, 1969) whereby the unknown quantum yield of the chromophore is obtained by comparison with that of a standard fluorochrome (here, L-tryptophan in aqueous solution at pH 6.0) (quantum yield = 0.12 ; Chen *et al.*, 1969).

Extraction of the chromophore. — The chromophore was extracted by gently stirring 4-ml AFP solutions (0.15 mg/ml) with 4 ml of n-butanol (Merck) for 30 min at 4 °C. After centrifugation, the organic phase was removed.

Chromatography. — Thin-layer chromatography was carried out with 20 × 20 cm Whatman PLK5 preparative thin-layer plates. Sulphuric acid (8 p. 100) in 50 p. 100 ethanol was used for staining.

Results.

Binding parameters of rat AFP with estradiol-17β. — Three pure rat AFP samples (I, II and III) at the same concentration (2-3 mg/ml) were dialyzed for different times against water adjusted to pH 7.4 with ammonia. Sample I was dialyzed for 72 hrs, sample II for 24 hrs and sample III for 12 hrs. Equilibrium dialysis studies of binding

with estradiol-17β were carried out with the three samples using a protein concentration of 5×10^{-8} M (rat AFP molecular weight : 70 000). The Bjerrum plots (Weber, 1975) of the binding results (fig. 1) show a fractional number of estradiol-17β binding sites. Slight variations in the dissociation constant were found for the three samples : 4, 6 and 8×10^{-8} M for samples I, II and III, respectively, and they decreased with increasing dialysis time, suggesting the interference of a dialyzable substance at the estradiol-17β binding site.

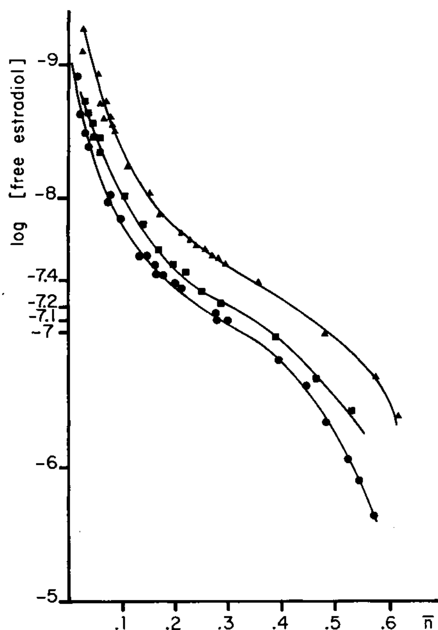

FIG. 1. — *Bjerrum plots of estradiol-17β binding by 3 rat AFP samples dialyzed respectively : 72 hrs* (sample I -▲-▲), *24 hrs* (sample II -■-■) *and 12 hrs* (sample III -●-●). $\bar{n}$ represents moles estradiol-17β bound per mole protein. The dissociation constants for the three samples of rat AFP are, respectively, 4,6 and 8×10^{-8} M for samples I, II and III. The number of binding sites is 0.6 in all cases. Results are the means of 4-5 experiments.

Spectroscopic evidence for the existence of a chromophore associated with pure rat AFP. — Spectrophotometric and spectrofluorimetric studies were carried out with rat AFP samples I and II (dialyzed for 72 and 24 hrs, respectively). Figure 2 shows the ultraviolet spectra of these samples. Two molar extinction coefficients at 278 nm were obtained (4.55×10^4 and 5.20×10^4 M^{-1} cm^{-1} for samples I and II, respectively). However, the overall shape of the spectra was identical.

Spectral differences between the two samples were also found by spectrofluorimetry : rat AFP had a maximal fluorescence emission at 330 nm when excited at 280 nm. The emission spectra of the two samples differed in intensity and there was

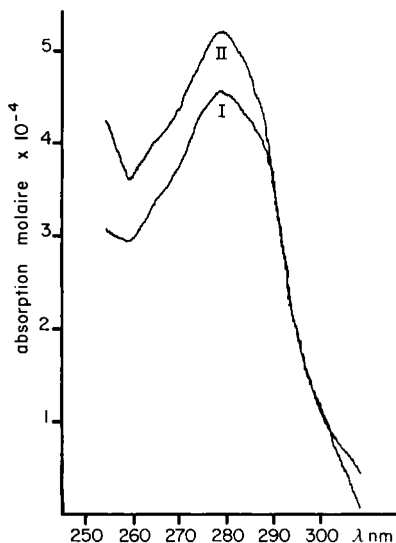

FIG. 2. — *Molar absorptivity of AFP samples I* (curve 1) *and II* (curve 2) in 4 mM phosphate buffer pH=7.4. The molar extinction coefficients at 278 nm are 4.55×10^4 M^{-1} cm^{-1} and 5.20×10^4 M^{-1} cm^{-1} for samples I and II, respectively.

an isobestic point at 370 nm (fig. 3). This suggested the presence of a fluorophore associated with the AFP and interfering with the protein fluorescence emission. If such a fluorophore existed, a second emission spectrum, different from that of the protein,

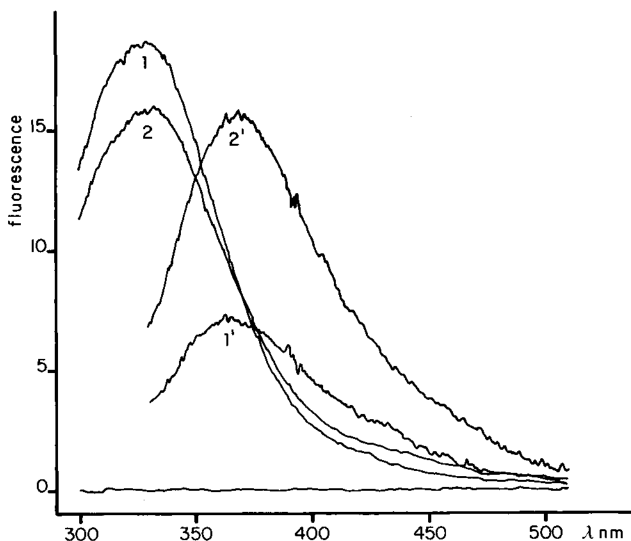

FIG. 3. — *Fluorescence spectra of pure AFP samples I and II* which are at the same optical density (OD = 0.100 at 278 nm). Spectra 1 and 2 represent the fluorescence emission spectra of the protein for samples I and II when the samples are excited at 280 nm. Spectra 1′ and 2′ represent the fluorescence emission spectra of the chromophore for samples I and II when the samples are excited at 310 nm. In the latter case, fluorimetric recording sensitivity is ten times higher than the sensitivity used for recording the protein spectra.

should be measurable. We attempted to record the emission spectra for excitation at wavelengths higher than that of protein excitation maximum, i.e. every 5 nm between 285 and 340 nm. An emission spectrum with a maximum at 370 nm was obtained in this way for rat AFP samples I and II excited at 310 nm (fig. 3). This was further evidence that a chromophore might be associated with the protein. Furthermore, figure 3 shows that fluorescence intensity at 370 nm, i.e. chromophore concentration, was higher after 24-hr dialysis (sample II) than after 72-hr dialysis (sample I). This figure also shows that a greater amount of the presumed chromophore was bound by the protein in sample II, giving rise to quenching of the protein's own fluorescence.

Removal of the endogenous chromophore from rat AFP. — Exhaustive dialysis of rat AFP samples I and II resulted in the disappearance of fluorescent emission at 370 nm. Further, the fluorescence intensity of the protein increased (*i.e.*, quenching decreased). This was more evidence that the crhomophore was dialyzable (*i.e.* of low molecular weight). We also removed the chromophore by treating the rat AFP samples (1 mg/ml) with a mixture of Norit-A charcoal (3.3 mg/ml) and dextran (0.33 mg/ml).

It was possible to extract the chromophore into an organic medium by phase partition with n-butanol. The excitation and emission fluorescence spectra of the butanolic fraction were recorded after this extraction (fig. 4). The excitation and emission maxima of the chromophore were 305 and 365 nm, respectively, in n-butanol ; they were slightly displaced towards the blue relative to the maximum in aqueous solution or associated with AFP. The quantum yield of the chromophore in n-butanol was calculated as 0.08.

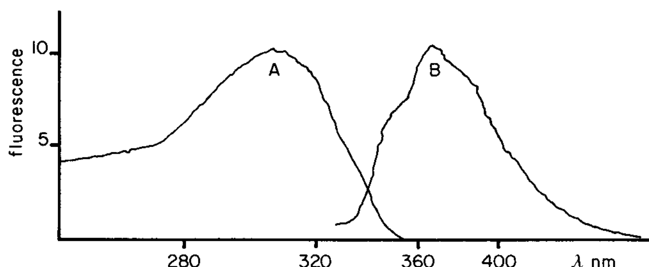

FIG. 4. — *Fluorescence excitation* (A) *and emission* (B) *spectra of the chromophore of rat AFP extracted in n-butanol.* The excitation and emission maxima are 305 and 365 nm, respectively.

Chromatographic study. — In order to compare the chromophore described here with the mixture of fatty acids isolated from fetal rat serum by Benassayag *et al.* (1977, 1979), we used the same method of organic extraction of the fetal rat serum. The organic extracts obtained were submitted to thin-layer chromatography using benzene : methanol (95:5) as the developing solvent. The stained chromatogram showed several spots, including one at R_f 0.60 which corresponded to the fatty acid mixture described. A fresh chromatogram was cut into nine bands of equal width between R_f 0.1 and 1. Each band was eluted with ethyl acetate : ethanol (1:1). The eluate from each band was then tested by spectrofluorimetry.

The intrinsic factor of chromophoric origin described here was found in the eluate corresponding to the R_f 0.85 $\pm$ 0.05 zone, and was obviously different from the compounds isolated by Benassayag *et al.* as no fluorescence was found in the eluates corresponding to the R_f 0.60 $\pm$ 0.05 zone.

Conclusion and discussion.

Because of its high estrogen affinity, rat AFP up to now has been considered as a steroid transporter (Nunez *et al.*, 1976). However, the following results suggest that this is not the exclusive role of rat AFP. In effect, recent studies in our laboratory have shown that its role can be extended to the transport of several exogenous ligands (Evrain *et al.*, 1978 ; Grigorova *et al.*, 1980) in the same way as adult rat albumin. With respect to the transport of endogenous ligands, several authors have shown the existence of polyunsaturated fatty acids physiologically associated with AFP (Parmelee *et al.*, 1978) or existing in fetal sera (Benassayag *et al.*, 1977) and having a high affinity binding site on AFP (Carleton Hsia *et al.*, 1980).

In this paper, we have demonstrated the existence of a low molecular weight fluorescent ligand of rat AFP, regularly associated with the purified protein. This ligand, of unknown chemical structure, differs from steroids and fatty acids, its spectrofluorimetric characteristics rather indicating an indolic structure. Indolic compounds are important from a nutritional point of view (essential amino acids) and because of the role of pineal indols (*e.g.*, melatonin) in the reproductive function. Thus, it would seem plausible that high-affinity AFP binding of an indolic derivative could play a specific role in fetal development, or at least at a particular stage of fetal development.

In conclusion, the evidence for several endogenous ligands physiologically associated with AFP suggests that the role of AFP transport could be extended to compounds other than hormones. It is possible that these various transport capabilities could be related to the existence of several AFP subspecies.

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Résumé. L'étude systématique des spectres de fluorescence de l'$alpha_1$ fœtoprotéine de rat purifiée par une méthode d'immunoabsorption, a permis de détecter la présence d'un chromophore endogène régulièrement associé à la protéine ; la dissociation de ce ligand fluorescent de faible poids moléculaire de l'$alpha_1$ fœtoprotéine de rat, a été obtenue après dialyse exhaustive de la protéine à faible concentration. Une méthode de partage de phase des préparations aqueuses d'$alpha_1$ fœtoprotéine de rat a permis d'obtenir ce chromophore dans le butanol et de déterminer ses caractéristiques spectrofluorimétriques : le maximum d'excitation de ce chromophore est à 305 nm et son maximum d'émission à 365 nm. Il a été également observé que ce chromophore endogène entre en compétition avec l'œstradiol-17β pour les mêmes sites de fixation sur l'$alpha_1$ fœtoprotéine de rat. En conclusion, le transport spécifique de petits ligands impliqués dans le développement fœtal, est proposé comme rôle de l'AFP.

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