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Fetal and adult albumins are indistinguishable by immunological and physicochemical criteria

(neonatal medicine/bilirubin binding/immunochemistry/protein structure/spectroscopy)

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ABSTRACT **The existence of a functionally immature fetal albumin has been postulated to explain the reduced ability of newborn plasma to bind bilirubin and various drugs. In support of this, cord and adult albumin, isolated by a simple salting-out technique, were reported to differ in electrophoretic and chromatographic properties and in their resistance to alkali and proteolytic enzymes. However, the interpretation of these findings has since been questioned. To resolve this controversy, we have purified to homogeneity human serum albumins from pooled umbilical cord and adult donor plasma. The two albumins were compared and found to be indistinguishable by polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate, as well as by immunoelectrophoresis and double immunodiffusion using specific antibodies against both albumins. Furthermore, the amino acid compositions, the amino-terminal sequence (Asp-Ala-His-Lys-Ser-Glu-Val-Ala-), the carboxy terminus (Leu), and the peptide fingerprints were identical in the two albumins. No significant differences were found by circular dichroism in the ultraviolet (200–350 nm). Binding studies with bilirubin showed association constants of $3.7 \pm 0.7 \times 10^7\ M^{-1}$ for cord and $2.9 \pm 0.3 \times 10^7\ M^{-1}$ for adult albumin, respectively. The circular dichroic spectra of 1:1 bilirubin·albumin complexes showed considerable variation between the batches but were not significantly different. The only difference was found in the fluorescence spectra of the bilirubin·albumin complexes, in which complexes with adult albumin showed only 75% of the relative fluorescence exhibited with cord albumin. The combined results nevertheless strongly indicate that fetal and adult albumins are very similar, if not identical.**

Human serum albumin is an almost universal carrier for a host of metabolites and drugs (1). As such it plays a central role in the regulation of their activity or toxicity. This is of particular significance in neonatal medicine because newborns, with their immature detoxifying mechanisms (for review see ref. 2), are highly susceptible to various toxic agents. A well-known example is bilirubin encephalopathy resulting from neonatal jaundice.

During clinical studies it was observed that newborn serum has a lower ability to bind bilirubin and drugs than adult serum does (refs. 3 and 4 and references cited therein). To explain these observations, the existence of a fetal type of albumin, analogous to hemoglobin F, was postulated. In support of this, cord and adult albumin, isolated by a simple salting-out technique, were reported to differ in electrophoretic and chromatographic properties and to vary in resistance to alkali and proteolytic enzymes (5, 6). However, authors of subsequent papers questioned the existence of a functionally immature albumin and attributed the reduced binding of newborn serum to competitive binding by increased concentrations of certain metabolites such as arachidonic acid and hematin (7–11).

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To resolve this controversy, we purified cord and adult albumins to homogeneity and compared their biochemical, immunological, and bilirubin-binding properties. Combined results from the present study strongly indicate that a fetal type of albumin does not exist.

MATERIALS AND METHODS

Purification of Albumins. Pooled human umbilical cord and adult plasma were stored at −20°C. After thawing, cryoprecipitates were removed by centrifugation and the albumin was isolated by affinity chromatography on Cibacron blue FG3-A coupled to Sepharose 4B as described by Travis *et al.* (12). α_1-Lipoprotein was removed by treatment with colloidal SiO_2 (Aerosil 200, Degusa, Frankfurt, Germany) (13) and defatting was performed with charcoal (14). The preparations were then filtered through Millipore filters (0.45-μm pore diameter), dialyzed exhaustively against distilled water, and stored at −20°C until use.

Polyacrylamide Gel Electrophoresis. This was performed in 7.5% slab gels, with and without sodium dodecyl sulfate ($NaDodSO_4$), according to Laemmli (15).

Immunological Methods. Antibodies to the purified albumins were raised in guinea pigs as described (16). Immunoelectrophoresis was by standard procedure and double immunodiffusion was according to Ouchterlony (17).

Amino Acid Analyses, Amino-Terminal Sequences, Carboxy-Termini, and Peptide Maps. Amino acid analyses were performed on a Durrum D-500 analyzer after sample hydrolysis in 6 M HCl under reduced pressure at 110°C for 22 hr; prior to hydrolysis the proteins were oxidized with performic acid according to Hirs (18). Automatic protein sequencing was performed with a Beckman sequencer model 890B (updated) using the dimethylbenzylamine buffer system (19) and the Beckman peptide program (111374). The phenylthiohydantoin amino acid derivatives were identified directly by gas chromatography (19) and indirectly by amino acid analyses of the free acids after $HCl/SnCl_2$ hydrolysis (20). For carboxy-terminal analyses the native proteins were dissolved in 0.2 M *N*-ethyl morpholine acetate, pH 8.5, at 37°C; carboxypeptidase A was added to a final enzyme-to-substrate ratio of 1.0% (mol per mol); and aliquots were removed at timed intervals. Digestions were terminated by lyophilization and addition of sodium citrate buffer, pH 2.2 (18). Peptide maps of the oxidized proteins were obtained by two-dimensional paper electrophoresis (21) of the fragments produced by enzymatic digestion with *Staphylococcus aureus* protease (40 mg of enzyme per g of albumin) in 0.05 M ammonium bicarbonate, at pH 7.8, for 18 hr at 37°C; the peptide patterns were visualized by staining with ninhydrin.

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

Circular Dichroism. Spectra of albumin were obtained with a Cary 61 spectropolarimeter at protein concentrations of 0.23–0.31 μM (200–250 nm) and 23–31 μM (260–350 nm) in 0.15 M NaCl buffered with 0.01 M sodium phosphate, pH 7.4. Spectra of bilirubin·albumin complexes (1:1 molar ratio, 17 μM in water) were taken as described (22).

Fluorescence Spectra. Measurements were made on bilirubin·albumin complexes (1:1 molar ratio, 17 μM) in water, pH 7.4, after previous incubation at pH 8.5 for 1 hr, with a Perkin–Elmer spectrofluorimeter, model MPF-2A (22).

Association Constants of Bilirubin·Albumin Complexes. Association constants were determined by the peroxidase method as described by Brodersen and Funding (23) except that ethyl hydroperoxide (24) was used instead of hydrogen peroxide.

RESULTS

Homogeneity of the Isolated Albumins. The above isolation procedure yielded albumin preparations that were found to be homogeneous by polyacrylamide gel electrophoresis with and without $NaDodSO_4$ (Fig. 1), immunoelectrophoresis (Fig. 2A), and double immunodiffusion (Fig. 3). This was confirmed by analyses of amino acid composition (Table 1), amino-terminal sequence (eight residues, see below), and carboxy terminus (leucine), all of which agreed with the known primary structure of adult albumin (25, 26).

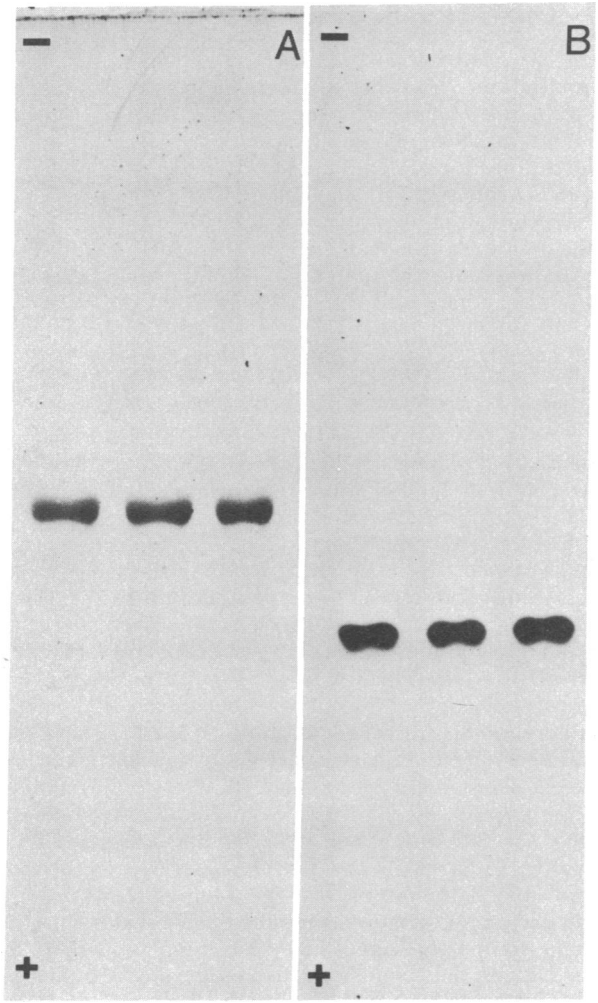

FIG. 1. Polyacrylamide gel electrophoresis at pH 8.8 in the presence (*A*) and absence (*B*) of $NaDodSO_4$. Left tracks, purified adult albumin; middle and right tracks, purified cord albumin (two batches).

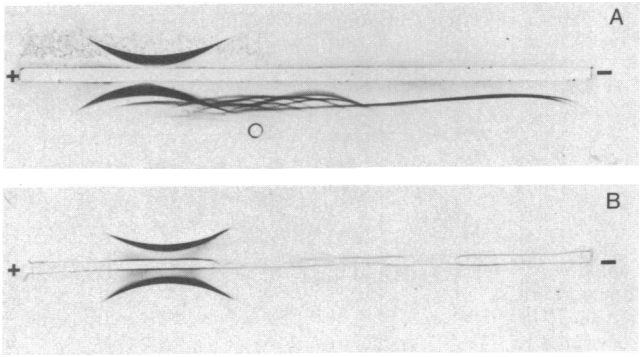

FIG. 2. Immunoelectrophoresis of purified cord and adult albumins with multi-specific (*A*) and specific (*B*) antisera. (*A*) Slit, horse antiserum against whole human serum; upper well, purified cord albumin; lower well, whole adult human serum. The same pattern was obtained when cord albumin was replaced by purified adult albumin (not shown). (*B*) Slit, guinea pig antiserum against purified cord albumin; upper well, purified cord albumin; lower well, purified adult albumin. The same pattern was obtained when the guinea pig antiserum was directed against purified adult albumin (not shown).

Polyacrylamide Gel Electrophoresis. In the presence of $NaDodSO_4$ both cord and adult albumin traveled as a single band with an apparent molecular weight of 69,000 (Fig. 1*A*), a value that approximates the known molecular weight of 66,500 (25). That both proteins had not only the same size but also the same charge was indicated by identical mobilities in polyacrylamide gels in the absence of $NaDodSO_4$.

Immunoelectrophoresis and Double Immunodiffusion. Immunoelectrophoreses of cord and adult albumins showed single symmetrical arcs of precipitation with specific antisera against each protein (Fig. 2*B*). Antigenic identity was further demonstrated by double immunodiffusion (Fig. 3).

Amino Acid Analyses, Amino-Terminal Sequences, Carboxy Termini, and Peptide Maps. As shown in Table 1, the amino acid compositions of cord and adult albumins were essentially identical. Moreover, the values were in good agreement with the known amino acid composition of adult albumin derived from the primary structure (25, 26). Automatic Edman degradation yielded identical sequences for the first eight amino acid residues (Asp-Ala-His-Lys-Ser-Glu-Val-Ala-) of both albumins. After 1 hr of exposure of both proteins to carboxypeptidase A the only amino acid detected was Leu (0.4–0.5 mol per mol of protein). These results are identical with those for

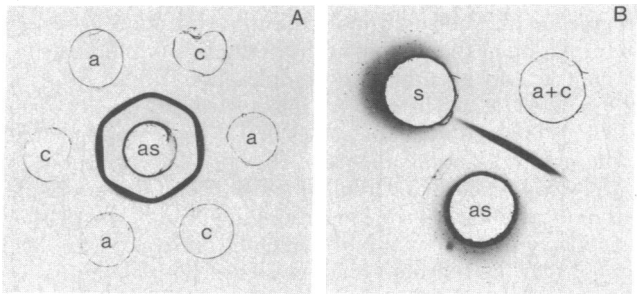

FIG. 3. Double immunodiffusion of purified cord and adult albumins against specific guinea pig antisera. Wells a, purified adult albumin; wells c, purified cord albumin; wells as, guinea pig antiserum against purified cord albumin; well s, normal guinea pig serum. The same patterns were obtained when the guinea pig antiserum was directed against purified adult albumin (not shown).

Table 1. Amino acid compositions of purified cord and adult albumins

Amino acid	Albumin	
	Cord	Adult*
Lys	58.4	58.4 (59)
His	15.5	15.3 (16)
Arg	23.4	23.2 (24)
Asx	52.3	52.1 (53)
Thr	28.5	28.2 (28)
Ser	21.7	22.2 (24)
Glx	81.9	81.1 (82)
Pro	24.3	24.7 (24)
Gly	12.5	12.5 (12)
Ala	61.0	60.9 (62)
Cys	33.0	33.8 (35)
Val	38.6	38.2 (41)
Met	6.2	6.3 (6)
Ile	7.6	7.7 (8)
Leu	61.4	61.2 (61)
Tyr	15.8	15.9 (18)
Phe	31.5	31.5 (31)
Trp	ND	ND (1)

* The values in parentheses are from ref. 25, with two corrections at positions 501 and 550 according to Walker (26), and represent the actual number of residues in the primary sequence of adult albumin. ND, not determined.

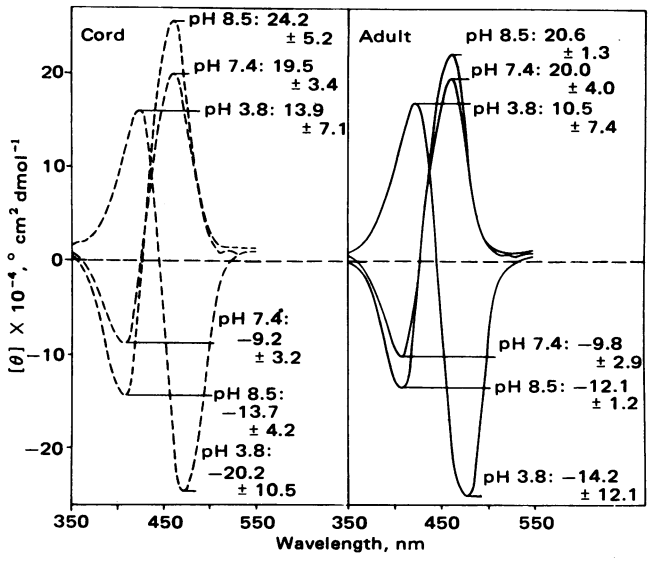

FIG. 4. Circular dichroic spectra of 1:1 complexes of bilirubin with one batch each of purified cord (---) or adult (– –) albumin. The spectra shown are selected for physiological relevance (pH 7.4) and for greatest ellipticities (pH 3.8 and 8.5). Mean ellipticities ± SD at the extrema from six cord and two adult albumin batches are listed.

the amino- and carboxy-terminal sequences of human albumin (25, 27). Thus, it appears that both proteins possess, at least at the extremities, identical primary structures.

Peptide maps produced by enzymatic cleavage of the proteins by *S. aureus* protease were identical (data not shown), further suggesting similarity. Because this method does not differentiate between substitutions in which the electrophoretic properties of the peptide(s) are conserved, the elucidation of the complete primary structure would be required to unequivocally prove total sequence identity.

Protein Circular Dichroism. Information on protein conformation was sought by circular dichroism in the ultraviolet. Two batches of adult albumin had circular dichroic spectra that in the far ultraviolet (200–250 nm) were within the range of spectra exhibited by five batches of cord albumin (data not shown). Because the ellipticity between 200 and 250 nm is especially informative on secondary structure content, the results are compatible with the suggestion that the two proteins contain similar, if not identical, proportions of α-helix, β-pleated sheet, and random coil.

Bilirubin-Binding Properties. Bilirubin, upon forming a complex with albumin, exhibits strong ellipticities in the visible region of the spectrum (28). This reflects an asymmetric structure of the bilirubin molecule imposed upon it by the conformation of the protein at the high-affinity binding site. At neutral and alkaline pH, complexed bilirubin forms a right-handed helix. This converts into a left-handed helix when the pH is lowered to acidic values (29). Fig. 4 reveals that at acidic, neutral, and alkaline pH, the spectra of bilirubin in complexes with cord and adult albumin are indistinguishable within experimental error, suggesting identity of the pH-dependent conformations of bilirubin in the two complexes. This implies that the bilirubin-binding sites of both albumins are probably identical.

Bilirubin in aqueous solution does not fluoresce appreciably. However, the formation of complexes with albumin is accompanied by the enhancement of bilirubin fluorescence (22, 30). This is thought to result from hydrophobic interaction and immobilization at the binding site. Therefore, the measurement of fluorescence spectra may be used to compare bilirubin binding sites. The fluorescence spectrum obtained with fetal albumin showed a single, essentially symmetrical, bell-shaped peak with its maximum occurring at 520 nm (data not shown). Thus it was identical in both shape and position to that previously described for adult albumin (22). However, the relative fluorescence intensity with adult albumin was 25% lower than that with cord albumin. The fact that the shape of the spectra and the positions of the maxima were the same for both complexes indicates that the conformations of the bound chromophores do not appreciably differ. Theoretically, the lower fluorescence quantum yield observed with the adult albumin complex could be due to any of the following three mechanisms (31–33): (*i*) absorption of irradiating light by extraneous yellow pigments, thereby preventing the excitation of bilirubin; (*ii*) absorption of radiation emitted from excited bilirubin by a red pigment; or (*iii*) conversion of the excitation energy into heat by dissipation through vibrational pathways (34). In our samples, modes *i* and *ii* can be excluded because the absorption spectra of the albumin preparations gave no indication of the presence of colored contaminants. On the other hand, radiationless energy transfer (mode *iii*) may account for the difference in fluorescence intensity observed between complexes with cord and adult albumin. This may result from minor conformational differences elicited by the possible presence of covalently attached small substitutents such as methyl, acetyl, phosphoryl, and glycosyl residues (35–37). The presence of noncovalently bound ligands, such as fatty acids, cannot be invoked because these were removed by charcoal treatment (14). Thus, the observed differences in fluorescence intensity does not necessarily imply different binding sites. Rather, the qualitative conformity of the two fluorescence spectra support the probable identity of the bilirubin-binding sites.

The primary association constants (±SD) of the bilirubin-albumin complexes were studied by using the peroxidase method (23, 24) and were found to be $3.7 \pm 0.7 \times 10^7\ M^{-1}$ for cord and $2.9 \pm 0.3 \times 10^7\ M^{-1}$ for adult albumin. Thus the association constants not only were in good agreement with the published value of $7 \times 10^7\ M^{-1}$ (23) but also were not significantly different from each other ($P < 0.001$, $n = 20$).

CONCLUDING REMARKS

The results presented above demonstrate that cord and adult albumin were indistinguishable in size, charge, antigenicity, amino acid composition, and terminal sequences. Moreover, peptide maps were identical and circular dichroic spectra in the ultraviolet suggested the same secondary structure content for both albumins. Furthermore, bilirubin binding properties, as determined by circular dichroism and association constants, were also the same for the two proteins. Closely similar bilirubin binding was further supported by the identical shape of the two fluorescence spectra and the presence of both emission maxima at 520 nm. The only difference found between the two albumins was in the fluorescence intensity of the bilirubin complexes. However this is insufficient evidence to conclude nonidentity at the binding sites because it might reflect the presence of minor covalent substituents eliciting small conformational modulations. Rather, combined evidence strongly favors identity of the two albumins, not only in structure but also in bilirubin binding.

The existence of an immature fetal type of albumin was postulated to account for the lower bilirubin-binding capacity of cord sera. This postulate was based on the findings that cord albumin had electrophoretic and chromatographic properties different from those of adult albumin and that it was more resistant to alkaline denaturation and proteolytic cleavage (5, 6). However, these observations were made on preparations obtained by a simple salting-out technique and their purity was never adequately demonstrated. In fact, Tuilié and Lardinois (9) showed such preparations to contain contaminating plasma proteins. Furthermore, we have found that this procedure does not remove small molecular weight ligands from albumin. It is therefore questionable whether the observed differences can be regarded as intrinsic properties of the two albumins. We have not found it pertinent to reemploy the methods used in the early sixties by Miyoshi *et al.* (5, 6) to compare the two albumins because more refined and sensitive techniques are now available for characterizing proteins.

On the basis of the above considerations we conclude that a fetal type of albumin does not exist, although final proof would require complete structure elucidation of both proteins.

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