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Albumin difference spectroscopy
bilirubin erythrocytes

Influence of Intravenous Nutrients on Bilirubin Transport. I. Amino Acid Solutions

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Summary

The effects of synthetic amino acids on bilirubin transport were investigated with competitive binding assays, peroxidase assays, isotopic studies of bilirubin uptake by red cells, and difference spectroscopy. Results indicated that amino acids had no significant effect on the distribution of bilirubin at pigment to albumin molar ratios likely to be encountered in clinical situations.

Speculation

Amino acids in solution do not appear to interfere with bilirubin transport. Parenteral alimentation with amino acids should be a safe procedure in jaundiced patients.

Parenteral feeding is frequently required in premature and sick newborn infants who are unable to take adequate nutrition by mouth. The past decade has brought technical advances which make it possible to supply not only carbohydrate, but also fat and amino acids by an intravenous route. By these means, sustained growth has been achieved in both premature and term infants (2, 3).

Unconjugated hyperbilirubinemia is frequently present in sick newborns receiving high concentrations of amino acids and/or lipids. Since the possibility of kernicterus is known to be increased by therapeutic agents which interfere with binding of bilirubin to plasma albumin (7, 11), it is important to evaluate the effect of parenteral nutrient solutions on bilirubin transport. This study evaluates the influence of a commercially available synthetic amino acid solution on bilirubin binding to human serum albumin and red blood cells.

MATERIALS AND METHODS

A stock 8% solution of amino acids in electrolyte medium (Veinamine, Cutter Laboratories, Inc., Berkeley, California) was used in all experiments. This preparation contains all essential amino acids and a balanced mixture of nonessential amino acids (Table 1). Glycine is a principal constituent (3.4 g/100 ml). The osmolality of the stock solution is approximately 932 mOsm/liter.

Bilirubin-human albumin solutions were prepared by dissolving bilirubin (Sigma) in 0.5 N NaOH and adding the bilirubin to albumin (crystalline, Cutter) dissolved in 0.055 M phosphate buffer, pH 7.4, ionic strength 0.15. All bilirubin solutions were prepared and maintained in the dark or subdued light. Final pH of bilirubin-albumin solutions was 7.4.

The interaction of amino acids with bilirubin-albumin complexes and transport were studied by competitive binding assays using cholestyramine and Sephadex gel filtration, the peroxidase assay, red cell uptake of radioactive bilirubin, and by an analysis of absorption spectra.

CHOLESTYRAMINE BINDING

Competitive binding between albumin and cholestyramine (12) was studied using a procedure modified from Schmid *et al.* (10). Bilirubin-albumin solutions were prepared to contain 5 g/100 ml human serum albumin; bilirubin/albumin molar ratios of 0.5, 1.0, 2.0; and amino acid concentrations of 0, 0.80, and 2.67 g/100ml. An equal volume of prewashed cholestyramine (12) suspension was added and the mixture was stirred constantly. Aliquots were removed at 0, 15, 30, 60, and 90 min of exposure and filtered free of resin through glass wool. The concentration of bilirubin remaining in individual filtrates was determined by the method of Malloy and Evelyn (6). Control experiments in

Table 1.

	Concentration
Amino acid (g/100 ml)	
Amino acetic acid	3.387
Arginine	0.749
Aspartic acid	0.4
Glutamic acid	0.426
Histidine	0.237
Isoleucine	0.493
Leucine	0.347
Lysine-HCl	0.667
Methionine	0.427
Phenylalanine	0.400
Proline	0.107
Threonine	0.160
Tryptophan	0.080
Valine	0.253
Electrolyte (mEq/liter)	
Na	40
K	30
Mg	6
Cl	50
Acetate	50

the absence of cholestyramine indicated no detectable loss of bilirubin for at least 5 hr under the conditions employed. Bilirubin adsorbed to cholestyramine could be recovered (90–95%) by repeated extraction of the resin with concentrated albumin solution.

SEPHADEX FILTRATION

Five-centimeters columns of Sephadex G-25 gel were prepared in Pasteur pipettes and equilibrated with phosphate buffer. Fifty-microliter aliquots of a solution containing 1.5 g/100 ml albumin and 15.3 mg/100 ml bilirubin (molar ratio 1.15) were filtered with complete recovery of albumin in a 2.0 ml eluate. Bilirubin which was adsorbed to the Sephadex column was eluted with albumin (3.0 g/100 ml). The concentration of bilirubin in each eluate was determined by direct spectrophotometric measurement assuming a molar extinction of albumin-bound bilirubin of 48.0 mM cm^{-1} at 460 nm.

PEROXIDASE ASSAY

The peroxidase enzymatic assay for unbound bilirubin can detect very low concentrations of unbound bilirubin and, in contrast to Sephadex filtration, may be used to analyze binding below a 1:1 molar ratio (5). In this method, unbound bilirubin is oxidized to colorless compounds by ethyl hydroperoxide in the presence of horseradish peroxidase. The initial rate of oxidation, measured by the decrease in absorbance at 460 nm, is proportional to the unbound bilirubin concentration. By adding bilirubin to albumin and repeating the assay, the relationship of total bilirubin concentration and bilirubin to albumin molar ratio to the unbound bilirubin concentration can be evaluated. This "titration" of albumin with bilirubin was used to determine the influence of amino acids on the binding capacity (molar ratio at which the high affinity sites are saturated) and binding affinity (apparent association constant) of albumin for bilirubin.

UPTAKE OF BILIRUBIN BY RED CELLS

Plasma and red cells from cord blood were separated and the plasma albumin concentration determined. The plasma was mixed (2:1) with stock amino acid solution or isotonic phosphate buffer, pH 7.4, and the plasma albumin concentration reduced to 1 mg/ml by the addition of phosphate buffer containing 10 mM glucose. Aliquots of the plasma-amino acid solution were introduced into a series of duplicate incubation flasks containing [^{14}C]bilirubin (prepared biosynthetically from labeled precursors (9)). The amount of labeled bilirubin was kept constant while plasma and buffer were varied to produce a range of bilirubin to albumin molar ratios from 0.4 to 1.2 in a total volume of 10 ml.

Red cells from cord blood were washed and resuspended in the glucose-phosphate buffer. Aliquots (0.5 ml) of cell suspension (20%) were added to each incubation flask and incubated for 15 min at 37°. At the end of incubation, red cells were separated by centrifugation and washed free of medium. The radioactivity associated with the cells, which represents absorbed bilirubin, was measured in a liquid scintillation counting system. A portion of the red cell suspension was lysed with distilled water to dissociate membranes from cell contents, the membranes recovered by centrifugation and membrane radioactivity determined.

DIFFERENCE SPECTRA

Difference spectra were performed using a Cary model 16 recording spectrophotometer with tandem cells in the sample and reference compartments. Bilirubin-albumin solutions (3.0 ml) containing 10 μm bilirubin were added to the anterior reference and sample cuvettes, and 3.0 ml buffer were added to the remaining cuvettes. Amino acids were added to the sample (bilirubin) and reference (buffer) using a micrometer syringe. An equal volume of buffer was added to the reference bilirubin. Thus, both sample and reference light paths were exposed to identical concentrations of bilirubin, albumin and amino acids.

RESULTS

CHOLESTYRAMINE BINDING

The stability of bilirubin-albumin complexes in the presence of resin with a high affinity for bilirubin was unaltered by amino acids in high concentrations. The disappearance of bilirubin from solution during 90 min of exposure to resin was similar in solutions containing 0% and 10% (0.8 g/100 ml) amino acid solution. At a bilirubin to albumin molar ratio of 2:1, however, high concentrations of amino acids (2.7 g/100 ml) resulted in a slower extraction of bilirubin (Fig. 1). Pretreatment of the resin with amino acids had no effect on these results.

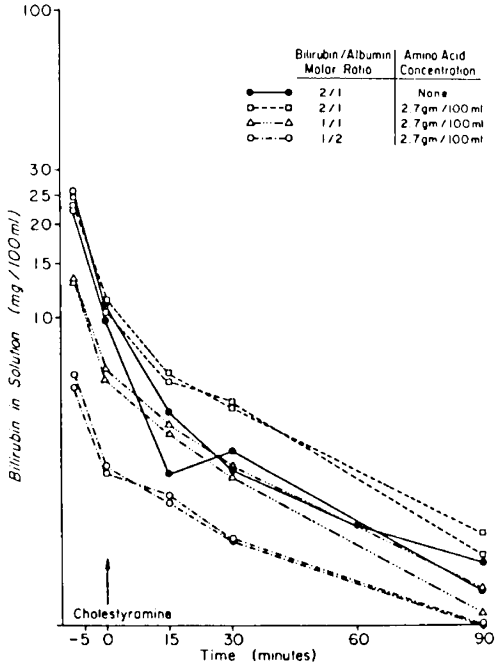

Fig. 1. Cholestyramine resin extraction of bilirubin. At a 2:1 bilirubin to albumin molar ratio, 2.7 g/100 ml amino acids inhibited adsorption of billirubin by cholestyramine. Extraction of bilirubin at 1:1 and 1:2 ratios was not affected by amino acids (shown paired with corresponding control curves).

SEPHADEX FILTRATION

Sephadex filtration was performed with a bilirubin albumin molar ratio which exceeds that normally found under clinical conditions. High concentrations of amino acid solution appeared to displace bilirubin slightly, but the amount of displaced bilirubin was very small and did not reach statistical significance until the amino acid concentration reached 3,721 mg/100 ml (Fig. 2). At these extremely high concentrations of amino acids, the ionic strength was also increased; the effect of ionic strength on binding was not examined. Within the amino acid concentration range anticipated with clinical use, no displacement could be demonstrated.

PEROXIDASE ASSAY

Under most clinical conditions the concentration of bilirubin is below the molar concentration of albumin. In the presence of excess albumin, amino acids displaced little bilirubin from albumin (Figs. 3 and 4). Binding to the secondary binding sites on albumin (beyond a 1:1 molar ratio) is considerably weaker than binding at the high affinity primary site (4). The unbound bilirubin increases rapidly with increasing molar ratio beyond 1:1 and under these nonphysiologic conditions displacement by amino acids was observed. At a bilirubin to albumin ratio of 1.5, the unbound bilirubin concentration was 1.45 μmol/liter (0.085 mg/100 ml) in the control sample and increased to 2.2 μmol/liter when the amino acid concentration reached 380 mg/100 ml.

In interpreting these results, it should be noted that the apparent high affinity site binding constant of the albumin preparation used was $4 \times 10^7\ M^{-1}$ (Fig. 4), which is considerably lower than that found in native serum ($1\text{–}3 \times 10^8\ M^{-1}$) (5). We were unable to demonstrate any alteration in the apparent association constant of the primary binding site until the amino acid concentration reached 400 mg/100 ml and were never able to demonstrate an influence of amino acids on binding capacity (*i.e.*, molar ratio at saturation) (see legend to Fig. 4).

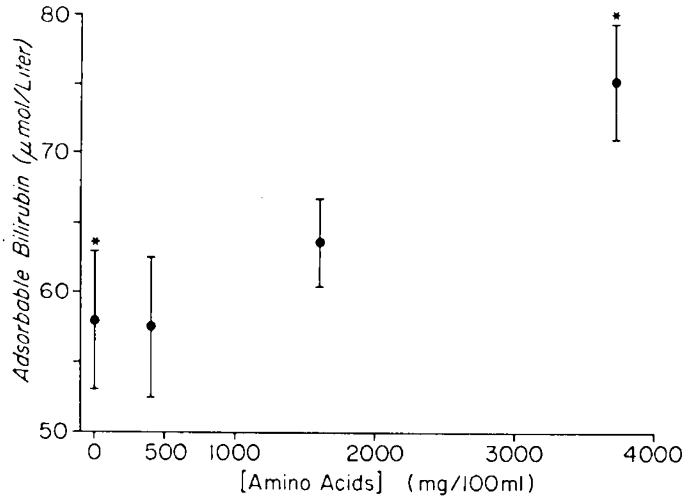

Fig. 2. Sephadex G-25 gel filtration. Model serum contained 15.3 mg/100 ml (262 μmol/liter) bilirubin. Bilirubin adsorbed to the column (mean ± SD) increased at high amino acid concentrations; (*) $P < 0.025$.

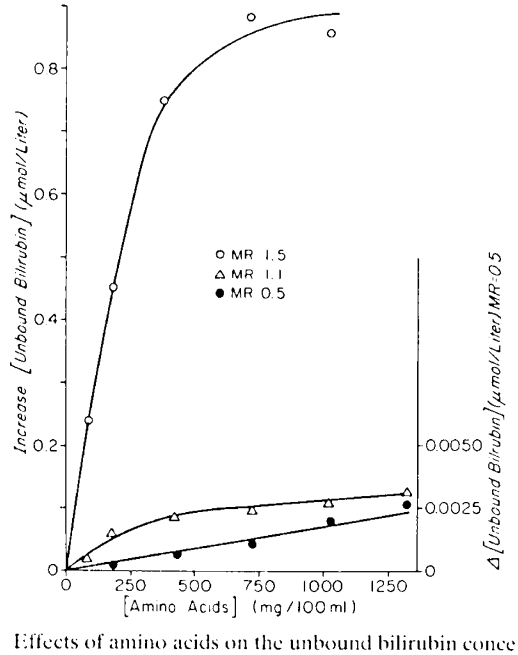

Fig. 3. Effects of amino acids on the unbound bilirubin concentration determined by the peroxidase assay.

RED CELL BINDING

The uptake of radioactive bilirubin by red cells is shown in Figure 5. The amount of bilirubin absorbed by red cells is a function of the bilirubin to albumin molar ratio in plasma. Thus, under these experimental conditions, the bilirubin removed by red cells from medium increased from less than 10% to more than 20% of the total bilirubin in solution as the bilirubin to albumin molar ratio increased from 0.6 to 1.2. In this range, amino acids had no effect on uptake of pigment by cells. Red cells approached saturation with bilirubin at molar ratios greater than 1.2. The percentage of total bilirubin in solution adsorbed by red cells or by their isolated membranes at the saturation point (molar ratio 1.4) was approximately 2% greater in the presence of amino acids (Figures 5 and 6).

Binding of bilirubin to red cell membranes followed a similar pattern (Fig. 6). High concentrations of amino acids produced a definite increase in membrane bound pigment after the high affinity albumin binding sites became saturated.

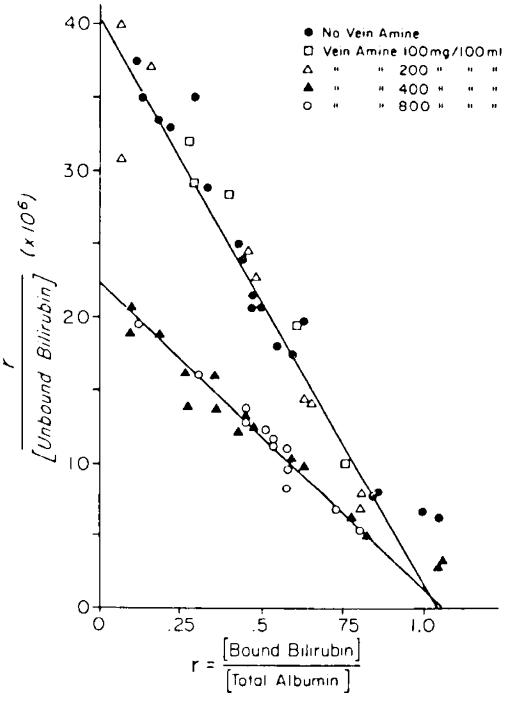

Fig. 4. Scatchard graph, illustrating the effects of amino acids on high affinity binding of bilirubin. The intercept with the abscissa represents the average number of high affinity binding sites on the albumin molecule (the binding capacity, n). The intercept with the ordinate is the binding constant (nk). High concentrations of amino acids depress the apparent binding affinity of albumin without affecting the binding capacity.

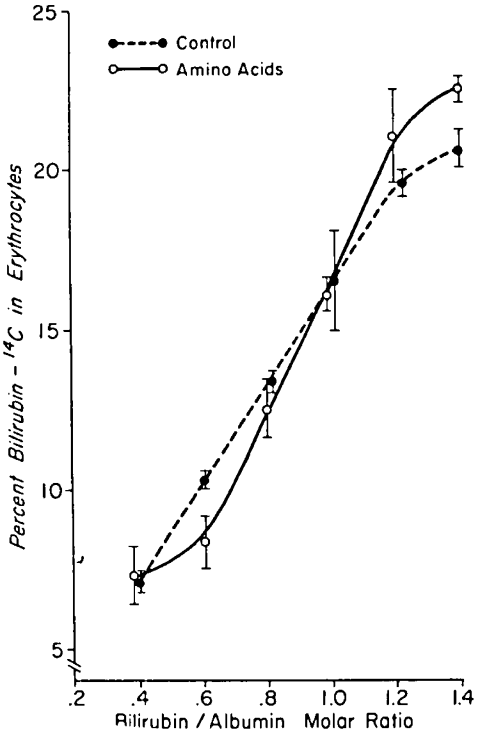

Fig. 5. Bilirubin uptake by red cells at various bilirubin to albumin molar ratios (mean ± SD). The total bilirubin remained constant; albumin concentration varied.

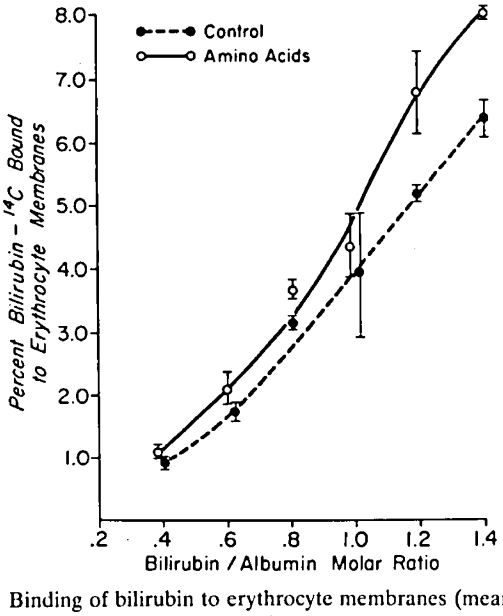

Fig. 6. Binding of bilirubin to erythrocyte membranes (mean SD) at various bilirubin to albumin molar ratios.

DIFFERENCE SPECTRA

Slight alterations in the absorption spectrum can be more easily analyzed using difference spectra. The concentration of the chromophore (bilirubin) is the same in both reference and sample cuvettes; the perturbing reagent (amino acids) is added to the sample and an equal volume of buffer is added to the reference. Difference spectra were performed using molar ratios (bilirubin to albumin) of 0.1, 1.1, and 1.5 (Figs. 7 and 8).

Amino acids produced alterations in the absorption spectrum of bilirubin bound to albumin even at a molar ratio of 0.1. These

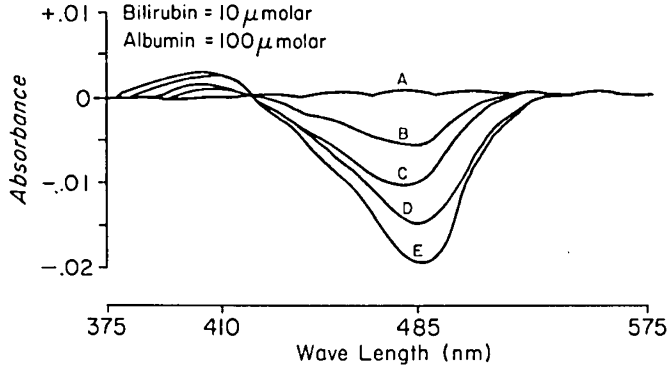

Fig. 7. Perturbation of the bilirubin to albumin absorption spectrum by amino acids. Bilirubin to albumin molar ratio 0.1. Amino acids were added to the sample cuvette, buffer to the reference, the bilirubin concentration remained equal in both cuvettes. A, baseline with no amino acids added; B, amino acid concentration increased to 66 mg/100 ml; C, 131 mg/100 ml; D, 258 mg/100 ml; E, 381 mg/100 ml.

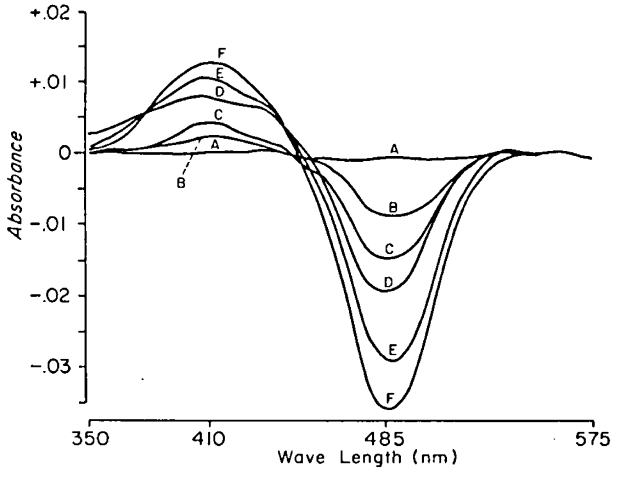

Fig. 8. Difference spectra, molar ratio 1.5. Amino acid concentrations in sample cuvette were (A) none, (B) 98 mg/100 ml, (C) 195 mg/100 ml, (D) 381 mg/100 ml, (E) 1,043 mg/100 ml, (F) 2,074 mg/100 ml.

spectral alterations have been previously interpreted to represent displacement of bilirubin from albumin (1, 8). However, the decrease in absorbance at 460–485 nm was essentially identical at both a 0.1 and 1.1 molar ratio (latter not shown). One would anticipate a greater displacing effect as bilirubin approached saturation. Furthermore, the difference spectrum of albumin-bound bilirubin (reference) vs. unbound bilirubin (sample) shows a sigmoidal curve with a maximum of 410 nm and a minimum at 485 nm (Fig. 9, B). Difference spectra using sulfisoxazole as a competitor produced a pattern compatible with displacement (Fig. 9, C), but amino acids produced primarily an alteration at 485 nm with little increase at 410 nm at a molar ratio of 1.0 (Fig. 9, D). We have observed that similar spectral changes at 485 nm will occur simply by adding additional albumin to a bilirubin to albumin solution, even when the albumin is not saturated (Fig. 9, E). Although the chemical nature of this decrease in extinction at 485 nm is not known, we conclude that the perturbation in the absorption spectrum by amino acids is most likely due to alterations in the excitability of the bilirubin:albumin rather than to displacement.

These observations suggest that alterations in the absorption spectrum of bilirubin to albumin complexes can be induced by drugs which bind both competitively and noncompetitively to albumin. Thus, the interpretation of shifts in the absorption

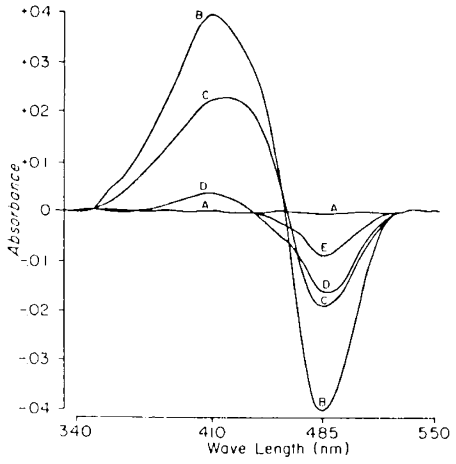

Fig. 9. Perturbations of the bilirubin-albumin absorption spectrum as measured by difference spectra. A, base line (conditions are identical in sample and reference). B, bilirubin, 4.0 μmol/liter in sample and reference (sample has no albumin, reference has albumin, 8 μmol/liter; *i.e.*, the spectrum of unbound vs. bound bilirubin). C, bilirubin and albumin = 10 μmol/liter (molar ratio 1.0), sulfisoxazole (10.6 mg/100 ml in sample). D, bilirubin and albumin, 10 μmol/liter, amino acids (500 mg/100 ml) in sample. E, bilirubin, 10 μmol/liter; albumin, 100 μmol/liter in sample (molar ratio 0.1) and 20 μmol/liter in reference (molar ratio 0.5); the decrease at 485 nm is not accompanied by a change in absorbance at 410 nm.

spectrum of bilirubin (1, 7, 8) must be made with great caution and only under well controlled conditions.

CONCLUSION

The results of these studies indicate that certain amino acids interact with human albumin inducing slight spectral alterations in the bilirubin: albumin complex. At high molar ratios of bilirubin:albumin and in the presence of high amino acid concentrations, displacement of bilirubin occurs, but the magnitude of displacement is small. Thus, the administration of parenteral solutions containing amino acids to sick infants with unconjugated hyperbilirubinemia would be unlikely to increase the risk of bilirubin encephalopathy.

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Albumin erythrocytes
bilirubin lipids

Influence of Intravenous Nutrients on Bilirubin Transport. II. Emulsified Lipid Solutions

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Summary

The effects of an emulsified intravenous fat preparation (Intralipid) on bilirubin transport were analyzed by cholestyramine extraction, spectrophotometric analysis, Sephadex gel filtration, peroxidase assay, bilirubin uptake by red blood cells, and by toxicity in tissue culture (L-929) cells. Intralipid is capable of binding bilirubin, but does not compete effectively with bilirubin bound to high affinity sites on albumin. The emulsified fat appears to have a higher affinity for bilirubin than for cell membranes. Red blood cells become coated with Intralipid, resulting in an increased association of bilirubin with the cell