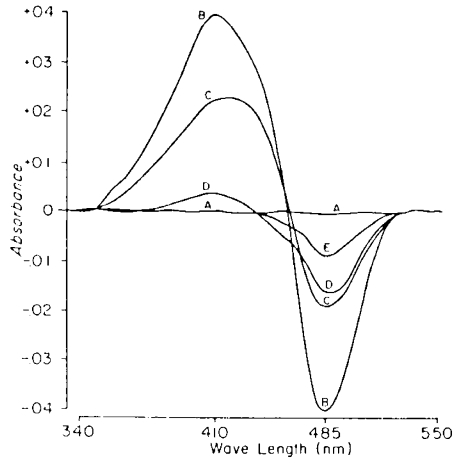

Fig. 9. Perturbations of the bilirubin-albumin absorption spectrum as measured by difference spectra. A, base line (conditions are identical in sample and reference). B, bilirubin, 4.0 μmol/liter in sample and reference (sample has no albumin, reference has albumin, 8 μmol/liter; *i.e.*, the spectrum 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.

REFERENCES AND NOTES

1. Coutinho, C. B., Lucek, R. W., Cheripko, J. A., and Kuntzman, R.: A new approach to the determination of protein-bound bilirubin-displacement and its applications. Ann. N. Y. Acad. Sci., *226:* 238 (1973).
2. Driscoll, J. M., Jr., Heird, W. C., Schullinger, J. N., Gongaware, R. D., and Winters, R. W.: Total intravenous alimentation in low-birth-weight infants: A preliminary report. J. Pediat., *81:* 145 (1972).
3. Gustafsson, A., Kjellmer, I., Olegard, R., and Victorin, L: Nutrition in low-birth weight infant. Acta Pediat. Scand., *61:* 149 (1972).
4. Jacobsen, J.: Binding of bilirubin to human serum albumin. Fed. Eur. Biochem. Soc. Lett., *5:* 112 (1969).
5. Jacobsen, J., and Wennberg, R. P.: Determination of unbound bilirubin in the serum of newborns. Clin. Chem., *20:* 783 (1974).
6. Malloy, H. T., and Evelyn, K. A.: The determination of bilirubin with the photoelectric colorimeter. J. Biol. Chem., *119:* 481 (1937).
7. Odell, G. B.: Dissociation of bilirubin from albumin and its clinical implications. J. Pediat., *55:* 268 (1959).
8. Odell, G. B.: Studies in kernicterus. I. The protein binding of bilirubin. J. Clin. Invest., *38:* 823 (1959).
9. Ostrow, J. D., Hammaker, L., and Schmid, R.: The preparation of crystalline bilirubin-C^{14}. J. Clin. Invest., *40:* 1442 (1961).
10. Schmid, R., Diamond, I., Hammaker, L., Gunderson, C. B.: Interaction of bilirubin with albumin. Nature, *206:* 1041 (1965).
11. Silverman, W. A., Anderson, D. H., Blanc, W. A., and Crozier, D. N.: A difference in mortality rate and incidence of kernicterus among premature infants allotted to two prophylactic antibacterial regimens. Pediatrics, *18:* 614 (1956).
12. Questran, Mead-Johnson.
13. This research was supported in part by United States Public Health Service Grant HD-03148 and HD-04665 and by United Cerebral Palsy grants R-263 and R-265.
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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

surface, but a decrease in pigment actually incorporated into the red cell membrane. In tissue culture experiments, Intralipid protected the cells from bilirubin toxicity. It is concluded that Intralipid may enhance the carrying capacity of serum for bilirubin, and thus exert a protective effect on tissues.

Speculation

Emulsified fat appears to interact with serum bilirubin not bound (or loosely bound) to albumin. Higher affinity for bilirubin of Intralipid compared with cellular surface membranes suggests that emulsified fat infusions may act as a temporary barrier to entry of bilirubin into tissues.

Intravenous administration of fat is a potentially useful means of supplying calories to malnourished newborn infants and patients with severe gastrointestinal deficiencies (1, 6, 7, 20). Premature infants treated with an emulsified fat infusion have demonstrated weight gain which closely approximated the intrauterine growth rate (3, 8, 13). Most newborn infants and premature infants clear emulsified fat rapidly, although a decreased clearance has been noticed in some premature and small for date newborns (8, 18). Since bilirubin may form complexes with lipids (10, 11), infused fat emulsion could interfere with bilirubin transport and binding to plasma albumin. For these reasons we have investigated the effects of fat emulsion on albumin binding and on bilirubin distribution between albumin and tissue.

MATERIALS AND METHODS

Intralipid (obtained from Cutter Laboratories, Berkeley, Calif.) is an emulsified fat preparation containing 10% soy bean oil, 1.2% egg phospholipids, 2.25% glycerin, and pyrogen-free water with an osmolality of 280 mOsm/liter. Various concentrations of Intralipid were diluted in isotonic phosphate buffer, pH 7.4. Bilirubin-albumin solutions were prepared by dissolving bilirubin in 0.05 N NaOH, and then mixing with a solution of purified human serum albumin (fraction V, Sigma) or crystalline albumin (Cutter). All experiments were conducted in 0.055 M phosphate buffer, pH 7.4, except for tissue culture experiments, which were performed in HEPES-buffered Hanks' solution, pH 7.4, at 37°. Crystalline [^{14}C]bilirubin was prepared by the method of Ostrow *et al.* (12). All experiments were performed in subdued light.

Spectrophotometric analysis of bilirubin-albumin and bilirubin-Intralipid complexes were performed using a Cary model 16 recording spectrophotometer with a high intensity light source. Studies were conducted at 27°.

Cholestyramine resin binding was performed using a procedure modified from Schmid *et al.* (14). Each incubation flask contained a solution of bilirubin-albumin at a molar ratio of 2:1, including 3 μg [^{14}C]bilirubin (specific activity 26,000 dpm/μg), and Intralipid at a final concentration of 1,000 mg/100 ml. A prewashed suspension of buffered cholestyramine or cholestyramine pretreated with Intralipid (10 mg/100 mg resin) was added with constant stirring. Aliquots removed at specified intervals were immediately filtered free of resin and the pigment remaining in solution was measured by counting the radioactivity in the clear filtrate. Control incubations contained all components with the exception of Intralipid, which was replaced by an equivalent volume of buffer. Studies of the interaction between bilirubin and Intralipid in the absence of albumin were conducted using a [^{14}C]bilirubin concentration of 9 mg/100 ml.

Sephadex G-25 gel filtration, the peroxidase assay for unbound bilirubin, and red cell uptake of bilirubin were performed as previously described (9, 19).

Cell toxicity was evaluated in cultured L cells. L-929 cells were grown with 5% CO_2 and air in Eagle's minimal essential medium containing Hanks' balanced salt solution and 5% fetal calf serum. Cells were harvested by scraping and diluted to 100 cells/ml with medium. One-milliliter aliquots of cell suspension were pipetted into 35-mm plastic Falcon dishes and allowed to attach overnight. On the following day, the plates were washed three times with 1 ml HEPES-buffered Hanks' solution, pH 7.4, containing 1.5 g/100 ml albumin and various concentrations of bilirubin, and incubated in the dark at 37°. After a 60-min incubation, the cells were washed twice with tissue culture medium and allowed to grow for 3 days. The cells were fixed, stained with Giemsa, and colonies were counted. The plates were exposed either to albumin or albumin and bilirubin, with or without the presence of Intralipid. Three test plates were incubated and counted for each set of experimental conditions. Viabilities were calculated by dividing the mean colony count of the bilirubin-exposed cultures by the mean colony count of appropriate control plates. The mean coefficient of variation in counting was 21%.

RESULTS

INTERACTION OF BILIRUBIN WITH INTRALIPID

The influence of Intralipid on the absorption spectrum of aqueous bilirubin is illustrated in Figure 1. Bilirubin forms an unstable solution at pH 7.4 and is thought to have a solubility of approximately 1 μmol/liter (2). The initial bilirubin concentration was 12.5 μmol/liter; in the absence of Intralipid, the absorptivity declined slowly over a period of hours as colloidal bilirubin formed. This process was greatly accelerated in the presence of very low concentrations of Intralipid (0.12 mg/100 ml) (Fig. 1, B). The mixture became turbid over a period of 3–4 min and, on microscopic examination, aggregates of heavily stained lipid particles were observed. As the concentration of Intralipid increased beyond 1 mg/100 ml, however, increased turbidity was not visually observed, bilirubin did not precipitate even with shaking (in contrast to aqueous bilirubin), and the absorption spectrum assumed the characteristics of bilirubin bound to mitochondria as reported by Mustafa and King (11). Greater increase in the Intralipid concentration produced a gradual shift in the absorption spectrum approaching that of bilirubin dissolved in chloroform or olive oil.

Aqueous solutions of bilirubin at pH 7.4 (no albumin) were rapidly cleared of pigment by both pretreated and untreated cholestyramine resin (Fig. 2). Intralipid, however, inhibited the removal of bilirubin from solution.

INFLUENCE OF INTRALIPID ON ALBUMIN BINDING OF BILIRUBIN

Intralipid had no detectable effect on the absorption spectrum of bilirubin bound to human serum albumin (fraction V) until

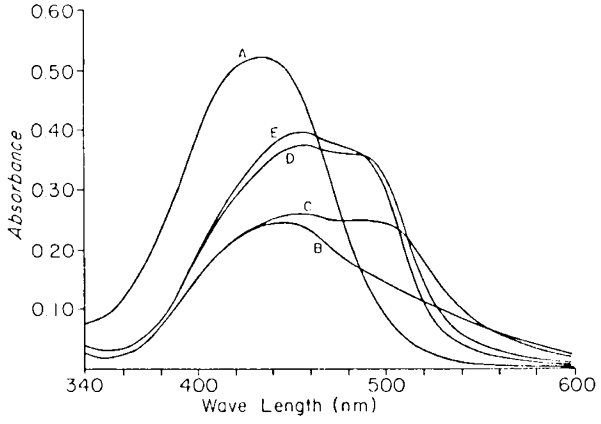

Fig. 1. Effects of Intralipid on the absorption spectrum of bilirubin. Solutions contained phosphate buffer, pH 7.4, and bilirubin, 12.5 μmol/liter. Intralipid concentration was (A) 0 mg/100 ml, (B) 0.125 mg/100 ml, (C) 1.0 mg/100 ml, (D) 4.0 mg/100 ml, (E) 128 mg/100 ml.

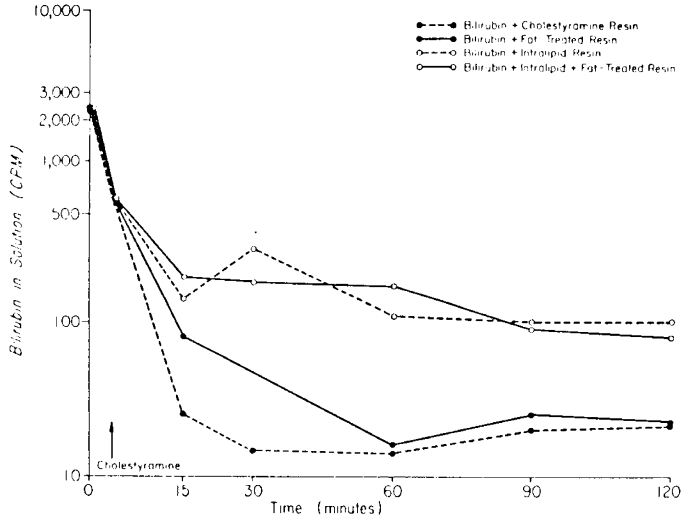

Fig. 2. Effects of Intralipid on cholestyramine extraction of bilirubin. Representative of six individual experiments. Solutions contained bilirubin and phosphate buffer, pH 7.4, but no albumin.

Table 1. *Effects of Intralipid on albumin binding of bilirubin: Spectrophotometric analysis*

Molar ratio, bilirubin-albumin	Ratio of absorbance, 490 nm/460 nm		
	No lipid	Intralipid, 34 mg/100 ml	Change in ratio
0.5	0.54	0.53	−0.01
0.8	0.54	0.57	+0.03
1.2	0.54	0.59	+0.05
2.0	0.53	0.59	+0.05
No albumin	0.39	0.98	+0.59

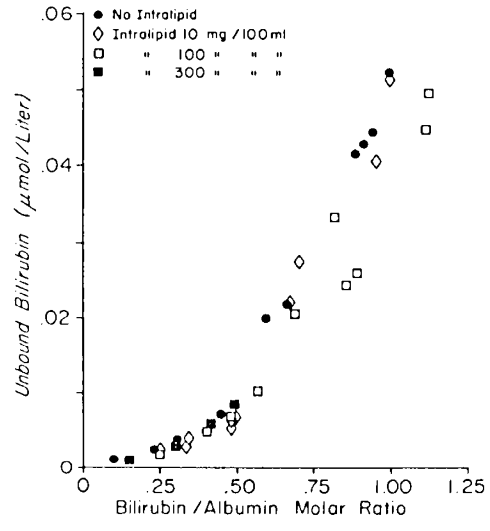

Fig. 3. Effect of Intralipid on the unbound bilirubin concentration. Solutions contained phosphate buffer, pH 7.4, albumin 20 μmol/liter, and various bilirubin concentrations. Peroxidase assays were performed at 27°.

the molar ratio of bilirubin to albumin approached 1:1. Even with a molar ratio 2:1, alterations in the absorption spectrum were minimal (Table 1).

The peroxidase assay was used to detect small changes in unbound bilirubin before the molar ratio (bilirubin-albumin) reached 1:1 (*i.e.*, in the bilirubin concentration range usually observed in clinical situations). The effect of Intralipid on the unbound bilirubin concentration at various molar ratios of bilirubin to albumin is summarized in Figure 3. Intralipid had little influence on the concentration of unbound bilirubin until the molar ratio of bilirubin to albumin exceeded 1:1, after which lower unbound bilirubin concentrations were observed in the presence of Intralipid.

Less bilirubin became associated with cholestyramine resin in the reaction mixture in the presence of Intralipid than when no Intralipid was added (Fig. 4), indicating that fat interfered with transfer of pigment from albumin to resin. Pretreatment of cholestyramine with Intralipid did not change the affinity of the resin for bilirubin, suggesting that the slower uptake of bilirubin in the presence of Intralipid was not due to competition between lipid and pigment for binding sites on the resin.

Binding of bilirubin to albumin at a molar ratio of 2:1 was stronger than binding of bilirubin to emulsified fat. Immediately after addition of resin to the bilirubin-albumin solution, 10% of the pigment was removed from solution, compared with 25% removed from the buffered bilirubin-Intralipid suspension. As seen by comparing Figures 2 and 4, twice as much bilirubin was maintained in solution by albumin compared with Intralipid after 30 min of incubation. Albumin and Intralipid in combination appeared to have an additive effect on the ability of bilirubin to stay in solution (Fig. 4).

Analogous results were obtained with Sephadex filtration. Rather than causing increased displacement of bilirubin from albumin, Intralipid actually decreased the amount of bilirubin staining the gel (Fig. 5). A portion of the filtered bilirubin was presumably bound to the Intralipid.

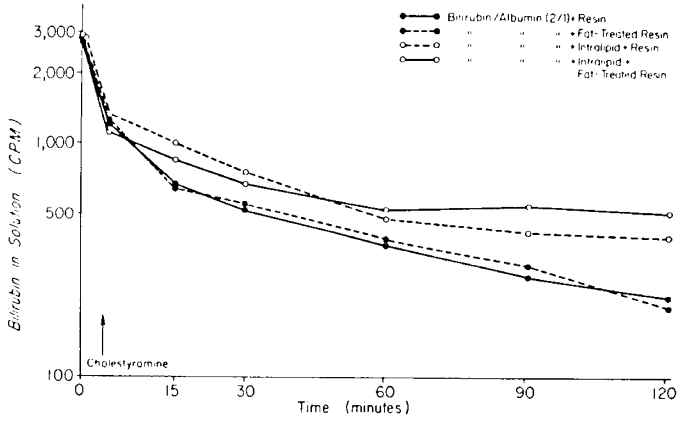

Fig. 4. Cholestyramine extraction of bilirubin in the presence of albumin. Representative of eight individual experiments.

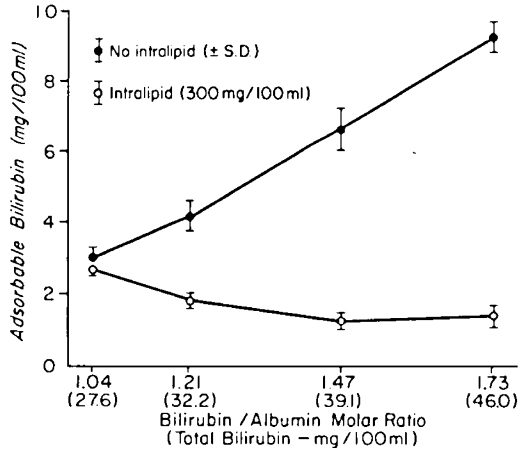

Fig. 5. Sephadex G-25 gel filtration. Bilirubin adsorbed by the gel was eluted with albumin.

EFFECTS OF INTRALIPID ON BILIRUBIN TRANSPORT

In the absence of Intralipid, the amount of bilirubin adsorbed by red cells depended on the bilirubin-albumin molar ratio in plasma (Fig. 6). Approximately 7% of the pigment in the plasma was removed by red cells at a bilirubin to albumin ratio of 0.4, compared with 22% of a molar ratio of 1.4. Intralipid produced

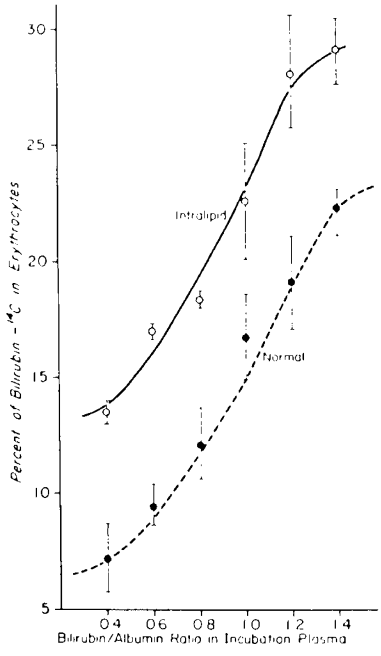

Fig. 6. Effects of Intralipid on red blood cell uptake of bilirubin (mean ± SD).

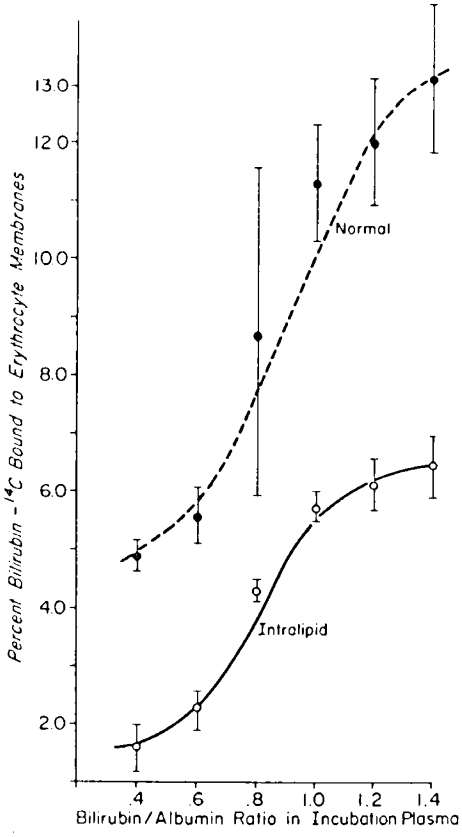

Fig. 7. Effect of Intralipid on red cell membrane binding of bilirubin (mean ± SD).

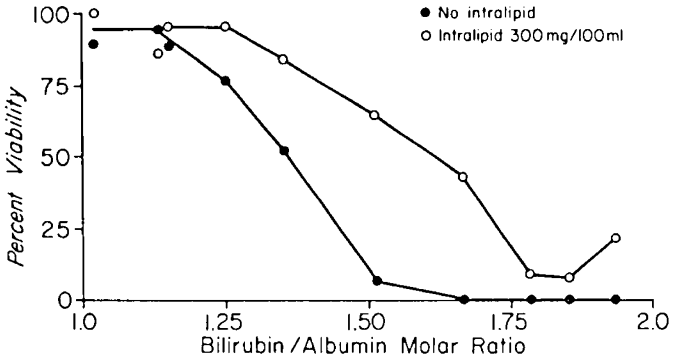

Fig. 8. Intralipid protection of tissue culture cells from bilirubin toxicity. Survival of plated L-929 cells was determined by counting cell colonies in treated and control plates 2 days after exposure to bilirubin.

a striking increase in association of pigment with red cells. Thus, at the lowest bilirubin to albumin molar ratio tested (0.4), nearly twice as much bilirubin was carried by red cells in the presence of Intralipid. This increase in capacity of red cells for bilirubin in the presence of emulsified fat persisted at all molar ratios tested.

In contrast to results obtained with intact red cells, significantly less bilirubin remained bound to membranes isolated from erythrocytes exposed to Intralipid in plasma compared with controls (Fig. 7). Decreased membrane binding was observed throughout the entire range of bilirubin to albumin molar ratios from 0.4 to 1.4. The differences can be explained by adsorption of Intralipid to red cell membranes; fat adsorption increased the bilirubin carrying capacity of red cells, but at the same time decreased the amount of pigment actually bound to cellular elements. The halo of pigmented fat was removed by washing during the process of membrane isolation.

Intralipid inhibition of bilirubin uptake by cells was reflected in decreased cellular toxicity in tissue culture. When plated L cells were incubated with various molar ratios of bilirubin-albumin at concentrations compared with clinically observed values (3 g/100 ml albumin, 25–50 mg/100 ml bilirubin), Intralipid provided considerable protection from bilirubin toxicity (Fig. 8). Fifty percent cell death occurred at a bilirubin albumin molar ratio of about 1.36 (initial concentration) in the absence of Intralipid in comparison with 1.62 in the presence of 300 mg/100 ml Intralipid. With an albumin concentration of 3 g/100 ml, these bilirubin values are equivalent to total initial bilirubin concentrations of about 35 mg/100 ml and 42 mg/100 ml, respectively. The toxic threshold occurred at a bilirubin concentration of 28 mg/100 in control cells and at 33 mg/100 ml in the presence of Intralipid. At the same initial bilirubin concentration, the unbound pigment was consistently lower in media containing Intralipid.

DISCUSSION

Intralipid binds bilirubin avidly, but does not interact with albumin binding sites. As such, it serves as a potential vehicle for serum transport of bilirubin, and, with the tissues tested, Intralipid competed effectively with cells for the bilirubin pool. Our results in this respect are consistent with the observations of Chan and Schiff (4), who reported minimal interaction of Intralipid with albumin binding of bilirubin as measured by Sephadex filtration and red cell uptake.

Whether Intralipid provides substantial protection from kernicterus in jaundiced human neonates will depend on the clearance of the fat particles, the clearance of the free fatty acids produced by the particle breakdown, and the fate of lipid-bound bilirubin during this process.

Experiments in jaundiced Gunn rats may come closer to elucidating these potentially complex effects of emulsified fat infusions on bilirubin metabolism. Rat albumin has a considerably lower affinity for bilirubin compared with human albumin

(14). Hence, potential effects of Intralipid or of fatty acids derived from its constituents *in vivo* on bilirubin-albumin associations should be exaggerated in rats. Injections or infusions of Intralipid produced no changes in bilirubin kinetics and distribution in blood and tissues of Gunn rats whose total pigment pool had been uniformly labeled with [^{14}C]bilirubin (16).

Although free fatty acids are elevated in human infants receiving Intralipid, the levels attained would be unlikely to affect the binding of bilirubin to human albumin (5, 15, 17), especially when the fat is administered as a constant rather than periodic infusion (3).

Short of recommending Intralipid as specific therapy for hyperbilirubinemia, our data indicate that Intralipid does not interact with albumin binding of bilirubin and should, therefore, exert little influence on bilirubin transport when the bilirubin to albumin ratio is less than unity.

REFERENCES AND NOTES

1. Borrensen, H. C., Coran, A. G., and Knutsud, O.: Metabolic results of parenteral feeding in neonatal surgery. Ann. Surg. *172:* 291 (1970).
2. Brodersen, R., and Theilgaard, J.: Bilirubin colloid formation in neutral aqueous solution. Scand. J. Clin. Lab. Invest., *24:* 395 (1969).
3. Cashore, W. J., and Usher, R. H.: Intravenous nutrition of small premature infants. The Role of Fat in Intravenous Feeding of the Newborn, pp. 122–128, In: P. Hahn, S. Segal, and S. Israels: (Pharmacia, Dorral, Quebec, 1974).
4. Chan, G., and Schiff, D.: In vitro studies of the relationship between Intralipid and bilirubin albumin binding. In: P. Hahn, S. Segal, and S. Israels: The Role of Fat in Intravenous Feeding of the Newborn, pp. 27–38. (Pharmacia, Dorral, Quebec, 1974).
5. Chan, G., Schiff, D., and Stern, L.: Competitive binding of free fatty acids and bilirubin to albumin: differences in HBABA dye versus Sephadex G-25 interpretation of results. Clin. Biochem., *4:* 208 (1971).
6. Coran, A. G., and Nesbakken, R.: The metabolism of intravenously administered fat in adult and newborn dogs. Surgery, *66:* 922 (1969).
7. Coran, A. G.: The intravenous use of fat for the total parenteral nutrition of the infant. Lipids, *7:* 455 (1972).
8. Gustafson, A., Kjellmer, I., Olegard, R., and Victorin, L. H.: Nutrition in low birth-weight infants. II. Repeated intravenous injections of fat emulsion, Acta Pediat. Scan. *63:* 177 (1974).
9. Jacobsen, J., and Wennberg, R. P.: Determination of unbound bilirubin in the serum of newborns. Clin. Chem., *20:* 783 (1974).
10. Kapoor, C. L., Krishna, Murti, C. R., and Bajpai, P. C.: Uptake and release of bilirubin by skin. Biochem. J., *136:* 35 (1975).
11. Mustafa, M. G., and King, T. E.: Binding of bilirubin with lipid; a possible mechanism of its toxic reactions in mitochondria. J. Biol. Chem., *245:* 1084 (1970).
12. Ostrow, J. D., Hammaker, L., and Schmid, R.: The preparation of crystalline bilirubin-^{14}C J. Clin. Invest., *40:* 1442 (1961).
13. Pendray, M. R.: Peripheral vein feeding in infants. In: Intravenous Feeding of the Newborn, pp. 158–175. The Role of Fat in P. Hahn, S. Segal, and S. Israels: (Pharmacia, Dorral Quebec, 1974).
14. Schmid, R., Diamond, I., Hammaker, L., and Gunderson, C. B.: Interaction of bilirubin with albumin. Nature, *206:* 1041 (1965).
15. Starinsky, R., and Shafrir, E.: Displacement of albumin-bound bilirubin by free fatty acids: Implications for neonatal hyperbilirubinemia. Clin. Chem. Acta, *24:* 311 (1970).
16. Thaler, M. M., and Pelger, A.: III. Influence of Emulsified fat infusions on bilirubin metabolism. Pediat. Res., *11:* 000 (1977).
17. Thiessen, J., Jacobsen, J., and Brodersen, R.: Displacement of albumin bound bilirubin by fatty acids. Acta Paediat. Scand., *61:* 285 (1972).
18. Wolf, H., and Lohr, H.: Fettinfusionen bei fruhgeborenen am ersten lebenstag. M. Schr. Kinderheilk. *116:* 262 (1968).
19. Wennberg, R. P., and Thaler, M. M.: Influence of intravenous nutrients on bilirubin transport. I. Amino acid solutions. Pediat. Res., *11:* 000 (1977).
20. Wretlind, O. A.: Complete intravenous nutrition: Theoretical and experimental background. Nutr. Metabol., Suppl. *14:* (1972).
21. This work was supported in part by United States Public Health Service Grants HD-03148 and HD-04665 and by United Cerebral Palsy Grants R-263 and R-265.
22. Requests for reprints should be addressed to: M. M. Thaler, M.D., Department of Pediatrics, University of California, San Francisco, Calif. 94143 (USA).
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Bilirubin malnutrition
Intralipid newborns
jaundice

Influence of Intravenous Nutrients on Bilirubin Transport. III. Emulsified Fat Infusion

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Summary

Infusions and injections of Intralipid were administered to congenitally jaundiced rats (Gunn) in whom the total body bilirubin pool was uniformly labeled with radioactive bilirubin. These isotopic studies indicated that Intralipid treatment had no effect on the kinetics of bilirubin formation, transport, tissue distribution, and clearance. There were no significant differences between control and fat emulsion-treated animals in the total bilirubin pool, plasma bilirubin pool, biologic half-life of bilirubin, fractional turnover, and exponential decline in plasma bilirubin specific activity at steady state. Bilirubin concentrations in blood, plasma, liver, kidneys, brain, adipose tissue, and muscle were unaltered during the course of continuous infusions (up to 24 hr) of Intralipid. These results indicate that Intralipid administered in clinically applicable doses and regimens has no discernible effects on bilirubin metabolism in jaundiced rats.

Speculation

These experimental procedures and results obtained in unperturbed animals provide a basis for studies of the effects of Intralipid infusion on bilirubin metabolism in presence of experimentally induced hypoxia, hypoglycemia, metabolic acidosis, and starvation.

The caloric needs of patients requiring parenteral alimentation can be conveniently supplied or supplemented with infusions of emulsified fat (Intralipid (20)) infused directly into peripheral