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Ceftriaxone Binding to Human Serum Albumin: Competition with Bilirubin

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SUMMARY

Ceftriaxone, a cephalosporin, is bound reversibly to defatted human serum albumin from adults, with a first stoichiometric binding constant of 60,000 M^{-1}, as found by equilibrium dialysis at pH 7.4, 37°. A second molecule is weakly bound, with a binding constant of 500 M^{-1}. Possible cobinding with bilirubin was studied by the peroxidase method and by equilibrium dialysis with and without added bilirubin. Results indicated competitive binding; formation of an albumin complex containing both bilirubin and ceftriaxone could not be demonstrated. Light absorption spectra of bilirubin-albumin showed little change on addition of ceftriaxone, in agreement with the competitive biding mechanism. Binding to serum albumin from newborn infants is weaker than to the protein from adults, with the first binding constant being about 36,000 M^{-1}. Cobinding of ceftriaxone and bilirubin to albumin from newborn infants is likewise competitive. It is concluded that ceftriaxone is a strong bilirubin displacer with a potential of inducing bilirubin encephalopathy in predisposed newborns.

Ceftriaxone is a "third-generation" cephalosporin that is active against many gram-positive and gram-negative organisms. The drug is widely used because of its broad spectrum of antibacterial activity, infrequent side-effects, and long serum half-life and it has recently been recommended as the drug of choice for use in newborn infants exposed to *Neisseria gonorrhea* during delivery (1). The long half-life results partially from the binding to serum albumin (2). Ceftriaxone competes with bilirubin for albumin binding (3–5) and should not be used in neonates in danger of kernicterus (5).

Previous ceftriaxone binding studies have mainly been concerned with serum albumin from adults (Refs. 6 and 7 and references therein). It has, however, previously been found that a few drugs are bound differently to albumin from adults and to albumin from newborn infants. Also, displacement of bilirubin may be different for the two types of albumin (8–10). There is thus a need for further data on ceftriaxone binding to albumin from neonates. The present work was carried out to describe more completely the binding equilibria of ceftriaxone to human serum albumin from adults and newborns and the interaction with binding of bilirubin.

Materials and Methods

Human serum albumin was from AB Kabi (Stockholm, Sweden) and was defatted with charcoal in acid solution (11), dialyzed and lyophilized. Horseradish peroxidase Type 1 and bilirubin were obtained from Sigma Chemical Co. (St. Louis, MO). Bilirubin was purified acording to the method of McDonagh and Assisi (12). Ceftriaxone (disodium salt; molecular weight, 661.59) was from Hoffmann-La Roche (Basel, Switzerland).

Albumin concentrations were measured by the method of Laurell (13).

Equilibrium dialysis for determination of ceftriaxone binding was performed in 1-ml dialysis chambers (Diachema AG, Langnau-Zurich, Switzerland) at a rotation speed of 24 rpm, using thin cellophane membranes (Diachema Type 10.17; thickness, 7.5 μm). Equilibrium was reached in less than 2 hr. Dialysis was performed for 3 hr at 37°. Ceftriaxone concentration was measured in the protein-free compartment by light absorption at 270 nm. The molar extinction coefficient was 0.2953×10^5 M^{-1} cm^{-1}. The results for binding of ceftriaxone to albumin in the absence of bilirubin were analyzed by computerized curve fitting, using the general binding equation (14). The variation of binding constants was investigated by the method of several acceptable fits (15).

Equilibrium dialysis experiments were also conducted with a mixture of albumin (30 μM), bilirubin (15 μM), and varying ceftriaxone concentrations in buffered solution on the left side of the membrane and buffer on the right side. Bilirubin is tightly bound and remains in the left chamber. Measurement of ceftriaxone equilibrium concentrations on the right side provides information on displacement of ceftriaxone by bilirubin and, thus, on displacement of bilirubin by ceftriaxone. Dialysis experiments with ceftriaxone with and without bilirubin were also performed with diluted umbilical cord serum.

The effect of ceftriaxone on bilirubin-albumin binding was further measured by the peroxidase method, with defatted human serum albumin (16). Experiments were also conducted in diluted umbilical cord

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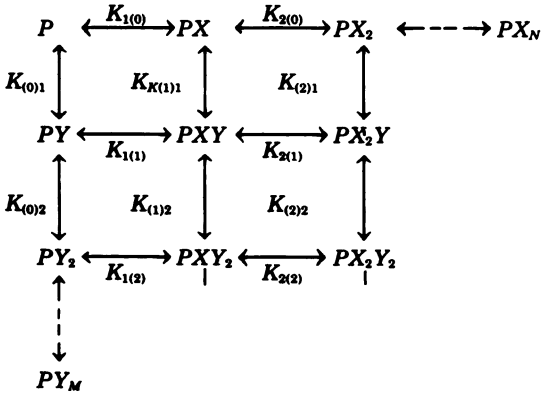
ABBREVIATION: MADDS, monoacetyl-4,4′-diaminodiphenyl sulfone.

serum with peroxidase, using ethyl hydrogen peroxide as the oxidizing substrate (17), with varying ceftriaxone concentrations. Light absorption spectra of the bilirubin-albumin and bilirubin-albumin-ceftriaxone mixtures were recorded on a Beckman Acta M VI spectrophotometer.

Cobinding Theory and Methodology

When two ligands are in multiple binding equilibria with one carrier, a number of complexes may be formed, as shown in model A.

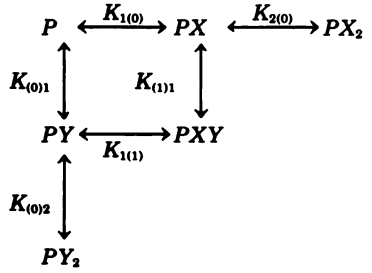


Model A

The carrier, P, in the present case is human serum albumin and the ligands, X and Y, are bilirubin and ceftriaxone, respectively. Stoichiometric binding constants are shown as well.

Note that all complexes and binding constants are understood in the stoichiometric sense, not considering binding to specific sites but only to the carrier molecule as a whole.

In blood plasma from icteric individuals, we have complexes of one albumin with one and two bilirubin molecules, PX and PX_2, while the concentration of PX_3 is negligible (10). At high concentrations of ceftriaxone, a maximum of two molecules of this drug may be bound, as shown in Results. We further find that binding of ceftriaxone strongly reduces binding affinity for bilirubin and vice versa. We will, accordingly, disregard formation of complexes with a total of more than two ligand molecules bound. Model A thus reduces to model B.

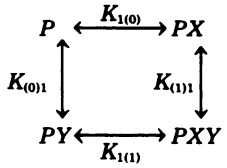


Model B

The binding constants for bilirubin in the absence of ceftriaxone are known from previous work (18). The stoichiometric constants are $K_{1(0)} = 5.9 \times 10^7\ \text{M}^{-1}$ and $K_{2(0)} = 4.1 \times 10^6\ \text{M}^{-1}$. The binding constants for ceftriaxone in the absence of bilirubin, $K_{(0)1}$ and $K_{(0)2}$, will be found from equilibrium dialysis measurements with ceftriaxone and albumin. Only $K_{(1)1}$, the first binding constant for ceftriaxone to a complex of one albumin with one bilirubin molecule, remains to be determined. Knowledge of this particular constant is decisive for knowing whether ceftriaxone displaces bilirubin. Should $K_{(1)1}$ be found to be equal to $K_{(0)1}$, then we may conclude that the first molecule of bilirubin and the first of ceftriaxone are bound independently to albumin and ceftriaxone would then be nondisplacing. On the other hand, if $K_{(1)1}$ is 0, then the complex PXY does not exist, ceftriaxone and bilirubin cannot be bound together, and ceftriaxone is a strong displacer, occupying albumin that would otherwise be available for binding of bilirubin.

We have two main methods for determination of $K_{(1)1}$, the peroxidase method and equilibrium dialysis for determination of ceftriaxone binding to albumin with and without bound bilirubin. Additional information on displacement can be obtained by studies of light absorption spectra and by measuring reserve albumin concentration for binding of MADDS with varying concentrations of ceftriaxone (5).

Theory of the peroxidase method. To determine $K_{(1)1}$ by the peroxidase method, we use a solution of defatted human serum albumin, (30 μM) with 15 μM bilirubin in a 66 mM sodium phosphate buffer, pH 7.4, at 37°. The rate of oxidation of bilirubin is proportional to the equilibrium concentration of unbound bilirubin and is measured in the presence of varying concentrations of the drug. The oxidation rate in the presence of drug divided by the rate in its absence is plotted against the free drug concentration and the slope of the curve, K_D, at zero drug concentration, is measured. The binding constants for bilirubin described above are applied under these circumstances and we calculate that the solution contains about 14 μM complex PX and 1 μM PX_2. The free bilirubin concentration is 17 nM. In a reasonably good approximation, we may disregard the presence of PX_2 and take the concentration of PX as 15 μM. We then have the binding model C,



Model C

and the binding equilibrium equation,

$$r_x = \frac{xK_{1(0)}(1 + yK_{(1)1})}{1 + yK_{(0)1} + xK_{1(0)}(1 + yK_{(1)1})} \qquad (1)$$

as derived from the previously published equation for binding of two ligands to one carrier (19). The free concentrations of bilirubin and ceftriaxone are x and y, respectively and r_X is the concentration of bound bilirubin divided by the concentration of albumin.

In the absence of ceftriaxone, the free bilirubin concentration in this solution is $1/K_{1(0)}$. We measure the oxidation rate for bilirubin. This rate is proportional to the free bilirubin concentration. If the rate is v_0 in the absence of ceftriaxone and v when ceftriaxone is present at the free concentration y, we have

$$v/v_0 = xK_{1(0)} \quad \text{and} \quad r_X = ½$$

Entering this in the binding Eq. 1 and rearranging gives

$$\frac{v}{v_0} = \frac{1 + yK_{(0)1}}{1 + yK_{(1)1}} \qquad (2)$$

which describes the relative oxidation rate with varying free ceftriaxone concentrations.

Differentiation of v/v_0 with respect to y in Eq. 2 and putting $y = 0$ gives the slope, at zero ceftriaxone concentration, of the curve, v/v_0 plotted against y.

$$K_D = \left[\frac{d\frac{v}{v_0}}{dy}\right]_{y=0} = K_{(0)1} - K_{(1)1} \qquad (3)$$

Determination of the slope thus serves for approximate calculation of $K_{(1)1}$ when $K_{(0)1}$, the first stoichiometric binding constant for ceftriaxone, is known.

Theory of equilibrium dialysis with and without bilirubin. Determination of the first stoichiometric binding constant for ceftriaxone to a complex of one albumin with one bilirubin might, in principle, be accomplished by equilibrium dialysis using a solution of the 1:1 complex and varying ceftriaxone concentrations. Mixing equal molar amounts of albumin and bilirubin, however, results in a solution that, besides PX, contains substantial amounts of P and PX_2. In a solution of 30 μM albumin and 30 μM bilirubin, the concentration of PX is 20 μM and of PX_2 is 5 μM, and 5 μM albumin is free. These amounts will, furthermore, vary on addition of ceftriaxone.

We use, accordingly, a solution of 30 μM albumin with 15 μM bilirubin, the same as for the peroxidase method. Ceftriaxone is added in varying amounts and free ceftriaxone concentrations are measured

by equilibrium dialysis. Results are plotted as bound versus log free ceftriaxone, with and without added bilirubin. It can be seen from the graph whether addition of bilirubin causes reduced binding of ceftriaxone. If so, it is concluded that the drug displaces bilirubin. Exact calculation of the displacing effect from these experiments is complicated and nonrewarding unless an unusually high precision of measurements can be achieved.

Light absorption spectra. The light absorption spectrum of PXY is expected to be different from the spectrum of PX, even if Y is a colorless substance such as ceftriaxone. If the spectrum of a bilirubin-albumin solution is markedly changed by addition of ceftriaxone, we may conclude that the two ligands, bilirubin and ceftriaxone, can be bound to the same albumin molecule, which would indicate relatively weak displacing properties of the drug. On the other hand, lack of spectral change indicates a strong bilirubin displacement. However, even if the ternary complex, PXY, cannot be formed and Y is strongly displacing, a slight spectral change may be observed by formation of larger amounts of PX_2 (20).

Results

Ceftriaxone Binding

Fig. 1 presents the binding isotherm for ceftriaxone as fitted to 63 observed points. Experiments were conducted with three different albumin concentrations, 100, 300, and 600 μM. A continuous curve was obtained, indicating that binding equilibria were not influenced by reversible albumin-to-albumin interaction. From the curve, it is seen that saturation of binding was not obtained. More than one molecule of ceftriaxone can be bound to one of albumin. The best-fit stoichiometric binding constants are $K_1 = 60{,}200\ M^{-1}$ and $K_2 = 506\ M^{-1}$. In Fig. 1 is seen a slight indication of a step when saturation of the 1:1 complex is approached. This rare finding is due to the unusually wide spacing of the two K values, by a factor of more than 100.

In order to elucidate the variation of the binding constants, the curve-fitting procedure was repeated to generate thirty acceptable fits (15) at each of two probability levels, 0.75 and 0.99. With 75% probability, the first stoichiometric binding constant is within 57,000 to 63,800 M^{-1} and the second is between 440 and 610 M^{-1}. With 99% probability, the first binding constant is within 54,400 to 66,700 M^{-1} while the second is between 300 and 700 M^{-1}.

Klotz affinity profiles are shown in Fig. 2, where iK_i is plotted versus i for the 30 sets of binding constants at 75% probability. A strongly negative interaction of binding is seen (21).

Competition with Binding of Bilirubin

Peroxidase kinetic studies. Results obtained with the peroxidase method are shown in Fig. 3, where the rate of bilirubin oxidation with hydrogen peroxide and peroxidase is pictured as a function of the free ceftriaxone concentration. A marked increase in oxidation rate is observed with increasing ceftriaxone concentration, indicating displacement of bilirubin

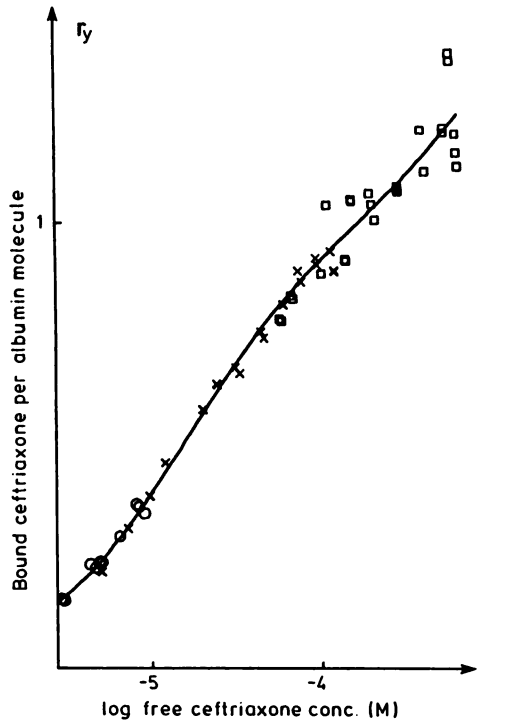


Fig. 1. Ceftriaxone-human serum albumin binding isotherm. The *ordinate* is the concentration of bound ceftriaxone divided by the albumin concentration. The *abscissa* is the logarithm of the free ceftriaxone concentration. Human serum albumin concentrations were 100 (○), 300 (×), and 600 μM (□) and ceftriaxone concentrations varied from 20 μM to 2 mM. The *curve* represents the best fit, obtained by a computerized procedure (15).

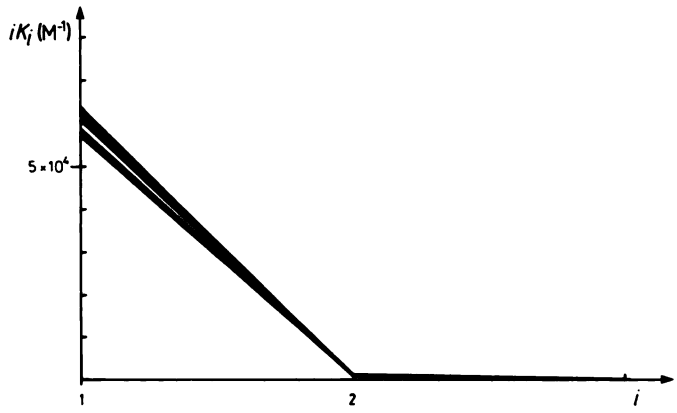


Fig. 2. Klotz affinity profiles for binding of ceftriaxone to human serum albumin. The meaning of this plot is explained in Ref. 21. Thirty acceptable values of the first stoichiometric binding constant are plotted on the *ordinate* indicating the variation within a probability limit of 0.75. Two times the second stoichiometric binding constant is likewise shown at the *abscissa* value of 2.

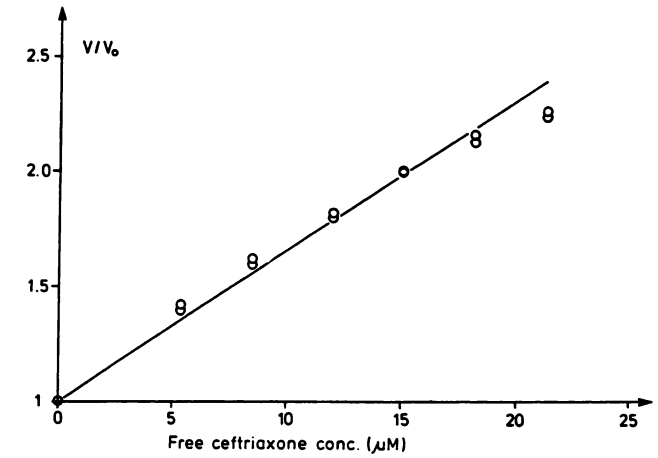


Fig. 3. Effect of ceftriaxone on bilirubin-albumin binding by human serum albumin. The *ordinate* is the peroxidase oxidation velocity, v, of unbound bilirubin at varying drug concentrations, relative to velocity in the absence of drug, v_o. Incubation consists of 15 μM bilirubin, 30 μM human serum albumin, 12 μM hydrogen peroxide, 4–12 nM horseradish peroxidase, and varying concentrations of ceftriaxone in 66 mM sodium phosphate buffer, pH 7.4. The reaction is run at 37° and the time for a 20% oxidation is determined.

on binding of the drug. Ceftriaxone at higher concentrations inhibits the enzyme.

The slope, K_D, of the line in Fig. 3 is 65,000 M^{-1}. This value is in the upper range of the first stoichiometric binding constant for ceftriaxone, $K_{(0)1}$, in the absence of bilirubin. According to Eq. 3, we obtain $K_{(1)1} = 0$, indicating that binding of ceftriaxone to the 1:1 bilirubin-albumin complex could not be demonstrated. A model without the complex PXY is consequently sufficient to describe our findings. This leads to model D, in which bilirubin and ceftriaxone compete for binding to albumin, being incapable of binding to the same albumin molecule.

$$\begin{array}{ccccc} & & K_{1(0)} & & K_{2(0)} \\ & P & \leftrightarrow & PX & \leftrightarrow & PX_2 \\ K_{(0)1} & \updownarrow & & & \\ & PY & & & \\ K_{(0)2} & \updownarrow & & & \\ & PY_2 & & & \end{array} \qquad \text{Model D}$$

Equilibrium dialysis studies. Equilibrium dialysis of ceftriaxone-albumin mixtures, with and without bilirubin, indicated weaker binding of ceftriaxone when bilirubin is present, as shown in Fig. 4. Bilirubin thus displaces ceftriaxone and it is concluded that ceftriaxone displaces bilirubin. The findings are in agreement with the competitive binding model D and are compatible with those of the peroxidase method.

Light absorption spectra. Spectra were recorded of solutions containing 30 μM albumin, 15 μM bilirubin, and varying concentrations of ceftriaxone. Changes were slight, consistent with the model of competitive binding, model D.

Studies with Albumin from Newborn Infants

Fig. 5 presents equilibrium dialysis results using diluted umbilical cord serum. The first stoichiometric binding constant is 36,000 M^{-1}. Thirty acceptable variations of the curve fitting gave the range 25,000–46,000 M^{-1} at the probability limit 0.75. Binding of ceftriaxone to albumin from newborns is weaker than to albumin from adults.

Fig. 6 shows the peroxidase oxidation rate of bilirubin in serum from newborns with varying concentrations of ceftriaxone. The K_D is 25,000 M^{-1}, not significantly different from the first stoichiometric biding constant for ceftriaxone to albumin from newborns. The findings are, thus, compatible with a model of competitive binding of bilirubin and ceftriaxone to albumin from newborn infants as well as to the protein from adults.

Equilibrium dialysis results of ceftriaxone mixtures with diluted serum from newborn infants in the presence of bilirubin are plotted in Fig. 5. It is seen that the addition of bilirubin

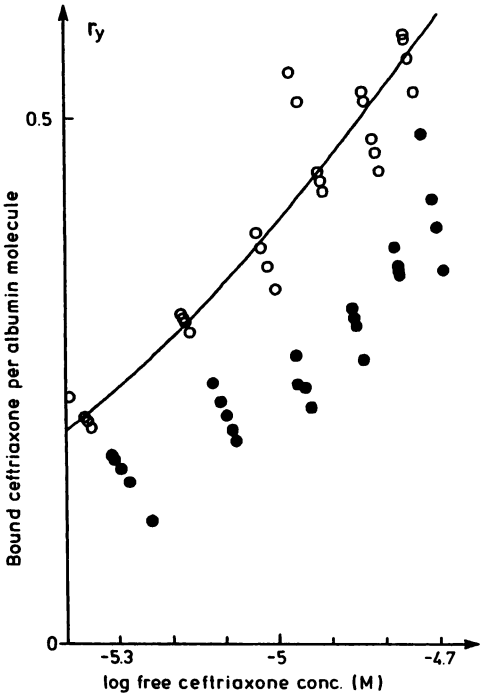


Fig. 4. Equilibrium dialysis of ceftriaxone and human serum albumin, with and without added bilirubin. Human serum albumin from adults (30 μM), without bilirubin (O) and with bilirubin (15 μM) (●), was dialyzed against buffer with varying concentration of ceftriaxone. It is seen that addition of bilirubin lessens the binding of ceftriaxone.

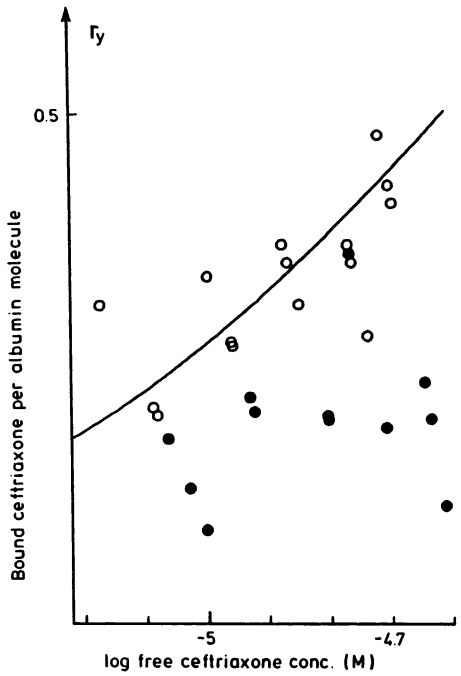


Fig. 5. Equilibrium dialysis experiment, as in Fig. 4, but with diluted serum from newborn infants. Albumin and bilirubin concentrations were as in Fig. 4.

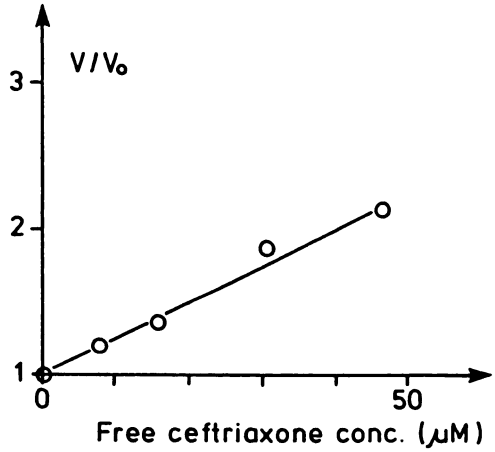


Fig. 6. Effect of ceftriaxone on bilirubin-albumin binding in diluted newborn serum. The *ordinate* is the peroxidase oxidation velocity, v, of unbound bilirubin at varying drug concentrations, relative to velocity in the absence of drug, v_0. Incubation consisted of 7 μM bilirubin, umbilical cord serum diluted to an albumin concentration of 15 μM, 0.9 mM ethyl hydrogen peroxide, 125–250 nM horseradish peroxidase, and varying concentrations of ceftriaxone, in 66 mM sodium phosphate buffer, pH 7.4. The oxidation was run at 37° for 2 min.

lessens binding of ceftriaxone and it is concluded that ceftriaxone in turn displaces bilirubin.

Light absorption spectra of bilirubin-newborn albumin with varying concentrations of added ceftriaxone are shown in Fig. 7. As found with albumin from adults, the spectral shift is small, compatible with competitive binding of bilirubin and ceftriaxone.

Discussion

Binding of ceftriaxone. The equilibrium dialysis binding studies indicate that one molecule of ceftriaxone is reversibly bound to human serum albumin from adults with rather high affinity. A second molecule is more loosely bound. Saturation of the albumin molecule could not be reached, in agreement with our previous finding that serum albumin is a nonsaturable carrier (22).

Binding site model. All the above considerations concern stoichiometric binding models, dealing with binding equilibria to the albumin molecule as a whole but not considering localization of bound ligand molecules to specific binding sites. It is generally not possible to draw conclusions about the presence of sites from measurements of binding equilibria alone (23). In the present case, however, an unusually large gap exists between the first and second binding constants. Under these particular circumstances, it is reasonable to conclude that ceftriaxone in the complex with albumin is located in one specific site. It must be stressed, on the other hand, that this does not mean that a preformed site for binding of ceftriaxone is present in the albumin molecule when ceftriaxone is absent. It is probable that the albumin molecule undergoes considerable conformational changes during the process of binding, swadling itself around the ligand molecule (24). Distant areas of the albumin molecule may be brought together during the binding process and form a suitable site for binding of ceftriaxone.

It has previously been concluded from spectroscopic and kinetic investigations that one bilirubin is bound to a specific site in the bilirubin-albumin complex (25). A second bilirubin or a sulfonamide molecule can be bound in immediate contact with the first bilirubin. Although it might appear tempting to guess that the bilirubin and ceftriaxone sites are identical, such a conclusion is not justified. Albumin is a flexible molecule and bilirubin and ceftriaxone are not chemically related. It is more likely, therefore, that albumin folds into different conformations when binding these two ligands. Formation of the site for bilirubin prevents formation of the site for ceftriaxone, excluding cobinding of both substances.

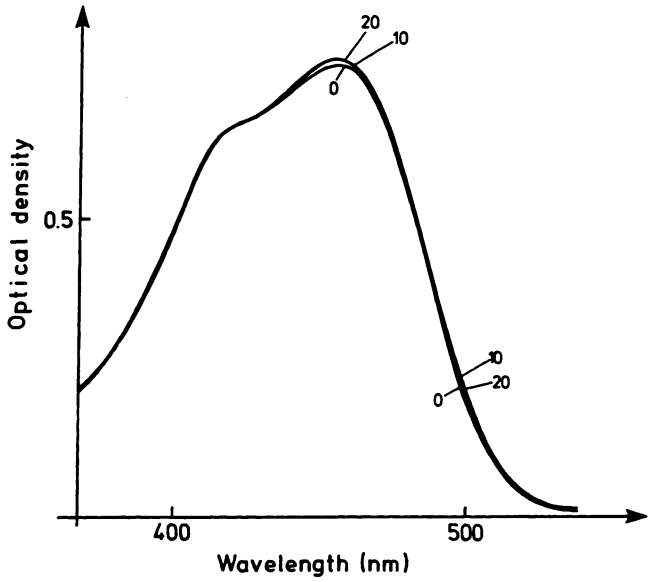


Fig. 7. Light absorption spectra of bilirubin-newborn serum, with and without ceftriaxone added. Spectra were recorded from newborn infants, diluted serum containing 15 μM albumin, with 7 μM bilirubin added, in a phosphate buffer, pH 7.4 Ceftriaxone concentrations were 0, 10, and 20 μM, as indicated in the figure.

Displacement of bilirubin. Silverman *et al.* (26) observed an increased mortality rate when sulfisoxazole was given to a group of jaundiced newborns and Odell (27) has explained this as a result of displacment of bilirubin by binding of the sulfonamide to serum albumin. Since then, sulfonamides have been taken to be contraindicated for the treatment of neonates and Stern (28) has suggested that all drugs used for newborns should be tested for bilirubin-displacing properties.

The decision whether an albumin-bound drug should be regarded as bilirubin displacing involves a two-step consideration (16). In the first step, we investigate the mechanism of interaction of the drug with bilirubin-albumin binding, irrespective of its plasma concentration. If the drug binds independently of bilirubin, no displacement will occur. If the drug binds competitively with bilirubin, strong displacement takes place. Intermediate mechanisms may be found. The displacing effect of the drug is expressed by its K_D, which is 0 if drug and bilirubin are bound independently and is equal to the first stoichiometric binding constant of the drug if the two ligands bind competitively.

In the second step, a maximal displacement factor, δ, is calculated,

$$\delta = K_D d + 1 \qquad (4)$$

This calculation takes into account the value of K_D and the free concentration, d, of the drug in blood plasma. The maximal displacement factor indicates the relative increase of free bilirubin concentration expected as a result of giving the drug. When alternative drugs are at hand, the physician should generally choose one with a low value of δ. An upper safe limit for δ cannot be given but the authors suggest that values above 1.2 should be avoided unless a compelling indication is present.

The mean peak plasma concentration of ceftriaxone in adults is about 450 μM after a 1500 mg intravenous injection (29). The drug is 83% protein bound (29). Using the K_D reported above for albumin from adults, 65,000 M^{-1}, gives $\delta = 6.0$. For comparison, the maximal displacement factor for sulfisoxazole, obtained with data from adult albumin, is 2.4 (16). Such extremely high δ values, as calculated here for ceftriaxone, will hardly be encountered in the clinic, partly because it is recommended that ceftriaxone be given as a continuous infusion over 15–30 min, rather than as a bolus injection, and partly because conditions in the newborn are different (29).

In the newborn, maximal plasma concentrations of ceftriaxone have been reported as 136 to 173 μg/ml after a 15-min infusion of 50 mg/kg (30). The average free fraction of the drug in plasma from four newborn infants ranged from 0.25 to 0.44 (31). Using a guessed mean concentration of 155 μg/ml (234 μM), 0.35 for the free fraction, and, further, K_D = 25,000 M^{-1} for albumin from newborn infants, we calculate $\delta = 3.0$, however, with a considerable variation. It is hardly possible to obtain precise δ values for the newborn and actual degrees of displacement of bilirubin will probably vary considerably from

one infant to another. The possibility of strong displacement is obvious.

Gulian *et al.* (4) in 1986 found that ceftriaxone displaces bilirubin from albumin to human erythrocytes, and Fink *et al.* (5) have determined the displacing effect in quantitative terms by means of the peroxidase method and have further studied competition with binding of MADDS in neonatal serum. The reserve albumin concentration for binding of MADDS was reduced by 39% in the presence of 200 μM ceftriaxone. These results are in agreement with the present observations. Owing to the potential clinical importance of this issue, the present authors have undertaken the above, more complete study of ceftriaxone binding and bilirubin displacement, using albumin from adults as well as from newborn infants. The strongly displacing character of this drug has been confirmed.

In conclusion, ceftriaxone should not be given to infants at risk of kernicterus. The drug can probably be used safely in a healthy child if born to term. On the other hand, it should not be used in premature and in severely ill infants with respiratory disease, acidosis, or severe infection and in newborns with marked jaundice.

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