Competitive Binding of Bilirubin and Drugs to Human Serum Albumin Studied by Enzymatic Oxidation

R. BRODERSEN

From the Institute of Medical Biochemistry, University of Aarhus, 8000 Aarhus C, Denmark

ABSTRACT The mechanism of drug-induced displacement of bilirubin from the blood into tissues was studied. A model of simple, competitive binding of bilirubin and drug to one site on serum albumin was established. Variations of the free bilirubin concentration after addition of drugs were studied in vitro by measuring velocities of oxidation with hydrogen peroxide and horseradish peroxidase. In all cases, the results were in agreement with the model. The competitive effects of 20 drugs were measured and expressed quantitatively as binding constants to the bilirubin site on human serum albumin. Several drugs caused changes of the bilirubin-albumin light absorption spectrum, indicating simultaneous binding of both ligands, without an effect on the free bilirubin concentration. Noncompetitive site-to-site effects on bilirubin binding could not be demonstrated.

An equation is proposed for calculation of the maximal displacing effect of a drug from knowledge of its plasma concentration, the above-determined binding constant, and the degree of protein binding of the drug.

Comparison of these results with previous observations of bilirubin displacement in newborn humans and in experimental animals indicates a general agreement with a simple competitive mechanism of binding of bilirubin and drug to one site on the albumin molecule. Binding of drugs to other, noncompetitive sites is common.

INTRODUCTION

A number of drugs, if given to newborn children with unconjugated hyperbilirubinemia, may displace bilirubin from its binding to plasma albumin, with the possible effect that the pigment enters the central nervous system, causing kernicterus or various late manifestations of brain damage. Sulfisoxazole is known to exert such an effect clinically (1, 2). This and other sulfonamides may precipitate kernicterus in hyperbilirubinemic animals (3, 4) and show a bilirubin-displacing effect in vivo (3, 5) and in vitro (6, 7). Salicylate, given to humans (8) and to Gunn rats (9, 10), causes a drop of serum bilirubin concentration, presumably due to displacement of the pigment to extravascular receptors. Large doses of fatty acids, infused to a child, resulted in a marked increase of the concentration of unbound bilirubin (11).

Some investigators have doubted the danger of giving sulfonamides to icteric newborns (12, 13), and a competitive effect of salicylate has been questioned by Thaler and Schmid (14). The latter authors, invoking a different principle, warn against the use of acetylsalicylic acid in hyperbilirubinemic patients because this drug has been shown to acetylate serum albumin (15), impairing its affinity for nonpolar anions. This uncertainty calls for investigation of the mechanism involved in displacement of bilirubin with drugs. In the present paper, the problem is elucidated by kinetic studies of oxidation of free bilirubin with hydrogen peroxide and peroxidase. A method for quantitative assessment of displacing effects is proposed.

As a basis for calculation of the effect of a competing drug, a model is defined by the following five assumptions: (*a*) Free, monomer bilirubin anion is present in solutions containing bilirubin and a molar excess of human serum albumin at neutral pH. The free bilirubin concentration is very low and cannot be determined by conventional means. Relative values of the free bilirubin concentration can be measured by the rate of oxidation with hydrogen peroxide and peroxidase. (*b*) One molecule of bilirubin is bound reversibly to one high-affinity site on serum albumin. (*c*) The free bilirubin concentration remains small, compared to the total. (*d*) One molecule of the drug is bound competitively to the bilirubin-binding site. (*e*) Additional molecules of the competitor may be bound to other sites on albumin, without interfering with binding of bilirubin.

It will be shown that this model is sufficient to describe the experimental findings, and the validity of the assumptions will be discussed.

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METHODS

Oxidation of bilirubin with hydrogen peroxide and peroxidase follows Michaelis-Menten kinetics (16). K_m for bilirubin at pH 8.2 is 8×10^{-4} M and less than 2×10^{-6} M for hydrogen peroxide. It is not possible to determine these constants at pH 7.4, due to the low solubility of bilirubin in neutral solutions, but they are unlikely to be several orders of magnitude different from the values at pH 8.2. This means that the rate of oxidation is proportional to the bilirubin concentration in the nanomolar range and is independent of the hydrogen peroxide concentration when this is of the order of 100 μM. A convenient principle is hereby available for relative determinations of free bilirubin concentrations in equilibrium mixtures. The processes involved are

$$\begin{array}{c} BP \rightleftharpoons B + P, \\ \downarrow \\ X \end{array}$$

where B, P, and X, are bilirubin, albumin, and oxidation product, respectively. The rate of the total process, conversion of the bilirubin-albumin complex to free albumin and the oxidation product, is proportional to the equilibrium concentration of free bilirubin if the following two conditions are fulfilled: (*a*) the free bilirubin only is oxidized, while bound bilirubin is protected, and (*b*) the velocity of the dissociation of bilirubin from albumin is high compared to the oxidation, so that the latter process is rate determining. It has previously been found by Jacobsen (17) that the rate of oxidation varies with the ratio of bilirubin to albumin in accordance with these assumptions, and further evidence for their validity is reported in the present paper (Figs. 2 and 3).

Addition of a displacing drug to the equilibrium mixture causes an increase of the free bilirubin concentration and an increased oxidation rate. Determination of the initial oxidation rate as a function of drug concentration may serve as a basis for assessment of the displacing effect of the drug. The method is based on the following quantitative principles.

P, B, and D denote the total concentrations of albumin, bilirubin, and drug, and [BP] and [DP] the concentrations of albumin with bilirubin and drug, respectively, bound to the high affinity site for bilirubin. The concentration of albumin without a ligand on this site is p, and the free concentrations of bilirubin and drug are b and d. An index o is used for concentrations in the absence of drug. K_B and K_D are the binding constants for bilirubin and drug to the high-affinity bilirubin-site on albumin. We then have

$$\frac{[BP]}{pb} = \frac{[BP]_o}{p_o b_o} = K_B, \quad \frac{[DP]}{pd} = K_D,$$

$$P = p + [BP] + [DP] = p_o + [BP]_o,$$

and

$$B = b + [BP] = b_o + [BP]_o.$$

For $b << B$ (see Discussion) we obtain the approximations $B \simeq [BP] \simeq [BP]_o$, $p_o \simeq P-B$, $[DP] \simeq P-B-p$, and hence

$$\frac{b}{b_o} = \frac{p_o}{p} = K_D d + 1. \qquad (1)$$

Experimental determinations of K_D were based on this equation. A reaction mixture was prepared, containing bilirubin and human serum albumin in a molar ratio of 1:2. The velocity of oxidation with hydrogen peroxide and peroxidase was determined with and without added drug. The ratio of these velocities was equated with the ratio of free bilirubin concentrations, b/b_o.

The free drug concentration in Eq. 1 is generally not known. If enough of the drug is added to provide a considerable excess over the amount bound to albumin, we can use the total concentration as an approximation of the free and obtain

$$\frac{b}{b_0} = K_D D + 1, \qquad (2)$$

which is valid when the drug concentration is large, compared to that of albumin (see Discussion).

Determination of the initial rate of the oxidation process from the slope of the progress curve as soon as possible after addition of the enzyme was found to be less reproducible than determination of the time taken for a certain fraction of the total change of optical density. In experiments with varying enzyme concentration and with several drugs, it was found that the ratio of such time periods with and without added drug was independent of whether the fraction 0.1, 0.15, or 0.2 of the total change was used. These ratios are consequently equal to the inverse ratio of initial velocities. Thus

$$\frac{t_{0.2(0)}}{t_{0.2}} = \frac{t_{0.15(0)}}{t_{0.15}} = \frac{t_{0.1(0)}}{t_{0.1}} = \frac{v}{v_o} = \frac{b}{b_o} \qquad (3)$$

when v and v_o are the initial velocities with and without drug added. The best reproducibility was obtained by use of $t_{0.2}$.

With increasing concentrations of a competing drug, the values of $t_{0.2}$ decrease and the horseradish peroxidase concentration, [HRP], was lowered to obtain suitable rates. Results from determinations with and without drug added (the latter with index o) were entered in the expression

$$\frac{t_{0.2(0)}}{t_{0.2}} \times \frac{[HRP]_o}{[HRP]} = K_D D + 1, \qquad (4)$$

which is derived from Eqs. 2 and 3, if the velocity is proportional to the enzyme concentration (Fig. 3).

The left side of Eq. 4 was plotted as the ordinates, against the drug concentration D as the abscissa. A line was fitted with the intercept 1 on the ordinate axis. The slope of the line is K_D, the binding constant of the drug to the bilirubin site.

Materials. Two batches of human serum albumin were obtained from AB Kabi, Stockholm, and one (salt: precipitated) from Statens Seruminstitut, Copenhagen. The content of fatty acids was determined according to Dole (18) and was 1.5, 0.9, and 0.5 mol/mol protein, respectively. Oxidation velocities obtained with these albumins were identical, in agreement with previous observations (19–21) that less than 3 mol fatty acid/mol albumin does not influence the binding of bilirubin. Defatting according to Chen (22) or desalting on a mixed ion exchange bed likewise did not change the oxidation rates. The albumin preparations contained a certain amount of dimer and

oligomers, of the order of 7%. A few experiments were conducted with monomer albumin, obtained by Sephadex gel chromatography, with identical results. It has been found previously (23) that binding of bilirubin is not influenced by dimerization of the albumin. The derivation of Eq. 1 is also not changed by dimerization of the protein.

Bilirubin was from Sigma Chemical Co., Inc. (St. Louis, Mo.), $E_{454}^{1\%} = 1.02 \times 10^3$ in chloroform, and was used as such. Horseradish peroxidase was also a Sigma product, Type I, 20%. The mol wt of the enzyme was taken as 40,000. Samples of kanamycin sulfate were obtained from H. Lundbeck & Co., Copenhagen, gentamicin sulfate from Schering Diagnostics, Port Reading, N. J., and carbamazepine from Ciba-Geigy AG, Basel. Polymyxin B sulfate was from Novo Industry A/S, Copenhagen.

Bilirubin-albumin solution. 87 mg human serum albumin was dissolved in 20 ml water and the pH adjusted to about 9 with NaOH. Bilirubin, 4.0 mg, was dissolved in 200 μl 0.5 M NaOH and 5 ml water. HCl, 75 μl, 1 M, was added, and 0.5 ml of this solution was immediately mixed with the albumin. The mixture was kept in the dark and was stable for 5 days in the refrigerator, giving no significant changes of light absorption spectrum or oxidation velocity. Reproducible light absorption spectra and oxidation velocities were obtained when albumin and bilirubin were mixed at a slightly alkaline pH, as above, avoiding colloid formation, which takes place if the bilirubin is added to a neutral albumin solution.

Exposure to bright daylight was avoided.

Procedure. 1 ml bilirubin-albumin solution and 1 ml sodium phosphate buffer, 133 mM, pH 7.40, containing the drug to be tested, was mixed. The final pH was 7.40±0.05. The solution was placed in a 1-cm cell in the Beckman Acta CV spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) with thermostatic cell holder at 37.0 ±0.2°C. A light absorption spectrum was recorded between 580 and 350 nm. Absorbance at maximum (460 nm) was close to 0.81 in the absence of a binding drug, corresponding to a final bilirubin concentration of 15 μM in the reaction mixture.

The concentration of free bilirubin was about 0.015 μM, (17) increasing on addition of a strongly displacing drug beyond the limit of solubility, which is about 0.1 μM (24). In most cases, the pigment remained in a supersaturated solution. Colloid precipitation could be discovered by a progressive decrease of maximal light absorption and broadening of the spectrum. In selected cases, the stability of the solution was checked by measuring light scattering (23).

Hydrogen peroxide, 5 μl, 35 mM, freshly diluted, was added, and the light absorption was recorded as a function of time at constant wavelength, 455 nm, during 3 min. The absorbance should remain constant.

Peroxidase solution, 100 μl in water, was added. A final peroxidase concentration of 12 nM was used in experiments without displacement, and lower concentrations as needed with added drug. The event marker was activated at the moment of peroxidase addition, and recording of the absorbance at 455 nm was continued until below 0.55.

Stability of the hydrogen peroxide was checked by addition of another 5 μl of hydrogen peroxide solution at this point. No change of reaction rate was observed. The final absorbance, about 0.012, was reached after several hours, or at 45–50°C in 1 h.

The shape of the spectral curves varied somewhat with several of the drugs. The wavelength used for recording the progress of the oxidation, 455 nm, in all cases was close to the maximum. The final optical density after completed oxidation was not influenced much by the drugs.

The time taken for oxidation of the initial 0.2 of the total amount of bilirubin was determined from the graph. The peroxidase concentration was chosen so that $t_{0.2}$ was longer than 2 min, to keep within the range where the velocity is proportional to the enzyme concentration, and shorter than 10 min, for stability.

Control test without albumin. To examine the possible influence of the drug on the activity of the enzyme, parallel experiments were carried out without albumin. A bilirubin concentration of 1.2 μM was chosen, since lower concentrations were unstable and precipitation was common at higher levels. Also, the amount of bilirubin dimer is negligible in the diluted solution (25). 1 ml water and 1 ml buffer, with or without drug, was mixed with 2 μl alkaline bilirubin solution, 1.29 mM. Peroxidase concentration was 24 pM. Light absorption spectrum and time course were recorded at the sensitivity range 0.1 optical absorbance. Any untoward reaction taking place between the reactants, such as oxidation of the drug with hydrogen peroxide and peroxidase or reactions between drug and bilirubin, would be discovered in the test, in addition to interaction of the drug with the enzyme.

This control test is more difficult and less accurate than the test proper with albumin present. A high-performance spectrophotometer is needed for recording the low optical densities. The small volume of bilirubin stock solution, limited stability of bilirubin in the absence of albumin, and deposition of the pigment on the walls of the cuvette tend to limit the reproducibility. Variations occurred in the range of 15% of the rate of oxidation. The lack of sensitivity of the control test could in several cases be counterbalanced by using higher concentration of the drug, thus assuring inactivity of the concentrations used in the test proper. In special cases, the inaccuracy of the control test may theoretically be limiting for the determination of K_D. This, however, is probably not of major practical importance, since none of the 20 drugs tested here showed any effect in the control test.

Notes on the procedure. The hydrogen peroxide concentration, calculated from the added amount, was initially 80 μM. Determinations with an *o*-dianisidine reagent showed that it remained above 25 μM. The velocity was constant from below 10 μM hydrogen peroxide and up to 200 μM. Higher concentrations were inhibitory.

Drugs of low solubility were added in alcoholic solution to the buffered bilirubin-albumin solution. Ethanol, 1% vol/vol, influenced the oxidation rates only slightly. In critical cases, tests were done with low concentrations of albumin, 6 μM, and bilirubin, 3 μM, in a 4-cm cell, and peroxidase concentration 2.4 nM or less (Fig. 4, phenyl salicylate). The low concentration of bilirubin did not give rise to instability when albumin was present.

The suitability of the enzymatic system and the albumin preparation was tested by examining the variation of the oxidation velocity with albumin concentration (Fig. 2). Some albumin preparations, stored dry for about 1 yr at 4°C, gave increased velocities at high albumin concentrations. Defatted albumin was more stable during storage than albumin with natural fatty acids.

The reproducibility of the oxidation velocity determinations without added drug corresponded to a standard deviation of about ±3%, increasing to the order of ±5% in the presence of a strongly competing drug.

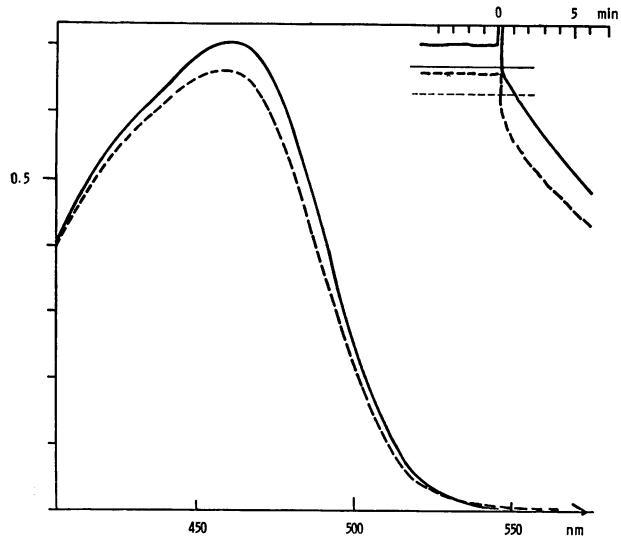

FIGURE 1 The peroxidase method. Full lines: light absorption spectrum (left) of a solution containing 30 μM human serum albumin, 15 μM bilirubin, and 50 μM hydrogen peroxide in phosphate buffer, pH 7.4. Time course (right) of the maximal extinction, before, and after addition of peroxidase at zero time. The thin horizontal line indicates the calculated extinction after dilution caused by the addition of enzyme solution. The stippled curves were obtained after previous irradiation of the bilirubin-albumin solution for 3 min in sunlight behind 1-cm window glass.

RESULTS

Investigation of the peroxidase method. The course of the oxidation of bilirubin, in a solution containing two mol albumin/mol bilirubin, with hydrogen peroxide and peroxidase, is seen in Fig. 1 (insert, full line). The optical density at 455 nm was recorded during 5 min before addition of the enzyme and remained constant, indicating stability of the system. Crude albumin preparations with a high content of hemoglobin gave a declining curve (16). On addition of the enzyme, the optical density initially decreased (to the level of the thin line, calculated) due to the dilution, and then proceeded to decline with somewhat decreasing velocity. A final level at optical density around 0.12 was reached in several hours. Oxidation is seen to result in product(s) with less light absorption at this wavelength. The chemical nature of the process is not known (16). The shape of the curve indicates that intermediate products with high optical density are not formed or are short-lived. Oxidation of bilirubin with hydrogen peroxide and peroxidase in this respect seems to be different from photooxidation, which proceeds through strongly colored intermediates (26–28). Monitoring of the optical density at 455 nm apparently is suitable for observing the progress of the enzymatic oxidation.

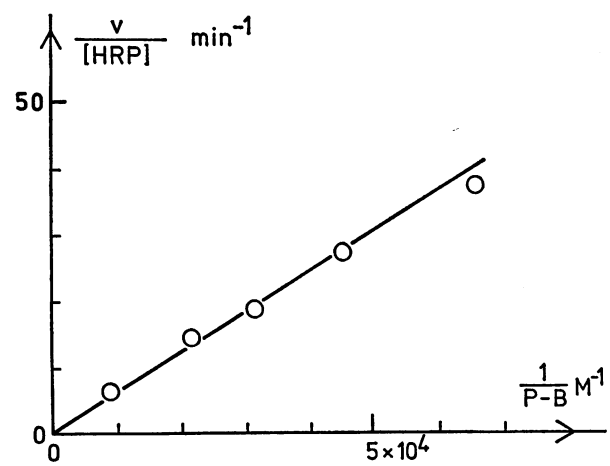

FIGURE 2 The initial velocity, relative to the enzyme concentration, of the oxidation of bilirubin with hydrogen peroxide and peroxidase (ordinates), in the presence of a molar excess of albumin, as a function of the reverse protein excess (abscissa). pH 7.4, 37°C.

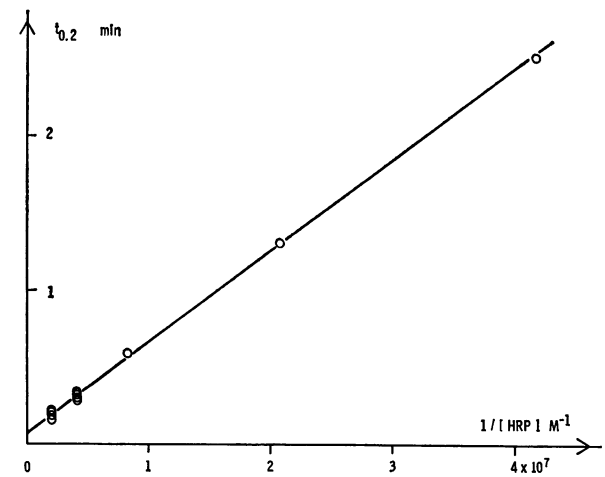

FIGURE 3 The time, $t_{0.2}$, taken for oxidation of 0.2 of the total amount of bilirubin (ordinates), as a function of the reverse enzyme concentration (abscissa). pH 7.4, 37°C.

Exposure of the bilirubin-albumin mixture to sunlight caused a slight decrease of the light absorption spectrum (Fig. 1, left, stippled curve). When the irradiated mixture was oxidized with hydrogen peroxide and peroxidase, the course of the optical density was initially steeper (insert, stippled curve). For practical purposes, this shows that exposure to strong light should be avoided. It seems possible that photodecomposition results in accumulation of a product that absorbs light at 455 nm and that is oxidized rapidly if hydrogen peroxide and peroxidase are added. This product, if it occurs as an intermediate in the enzymatic process, is short-lived and does not interfere with determination of the velocity of the rate-limiting step.

In the absence of a competing drug, the velocity of oxidation of bilirubin with hydrogen peroxide and peroxidase in mixtures with albumin decreases with increasing albumin concentration. This variation was examined and the results are pictured in Fig. 2. The added concentration of bilirubin was constant, 15 μM, and the concentration of albumin was varied from 30 to 120 μM. Free bilirubin concentrations in these solutions were of the order of 0.01 μM or less, since the binding constant is of the order 10^8 M^{-1}. The concentration of free bilirubin was

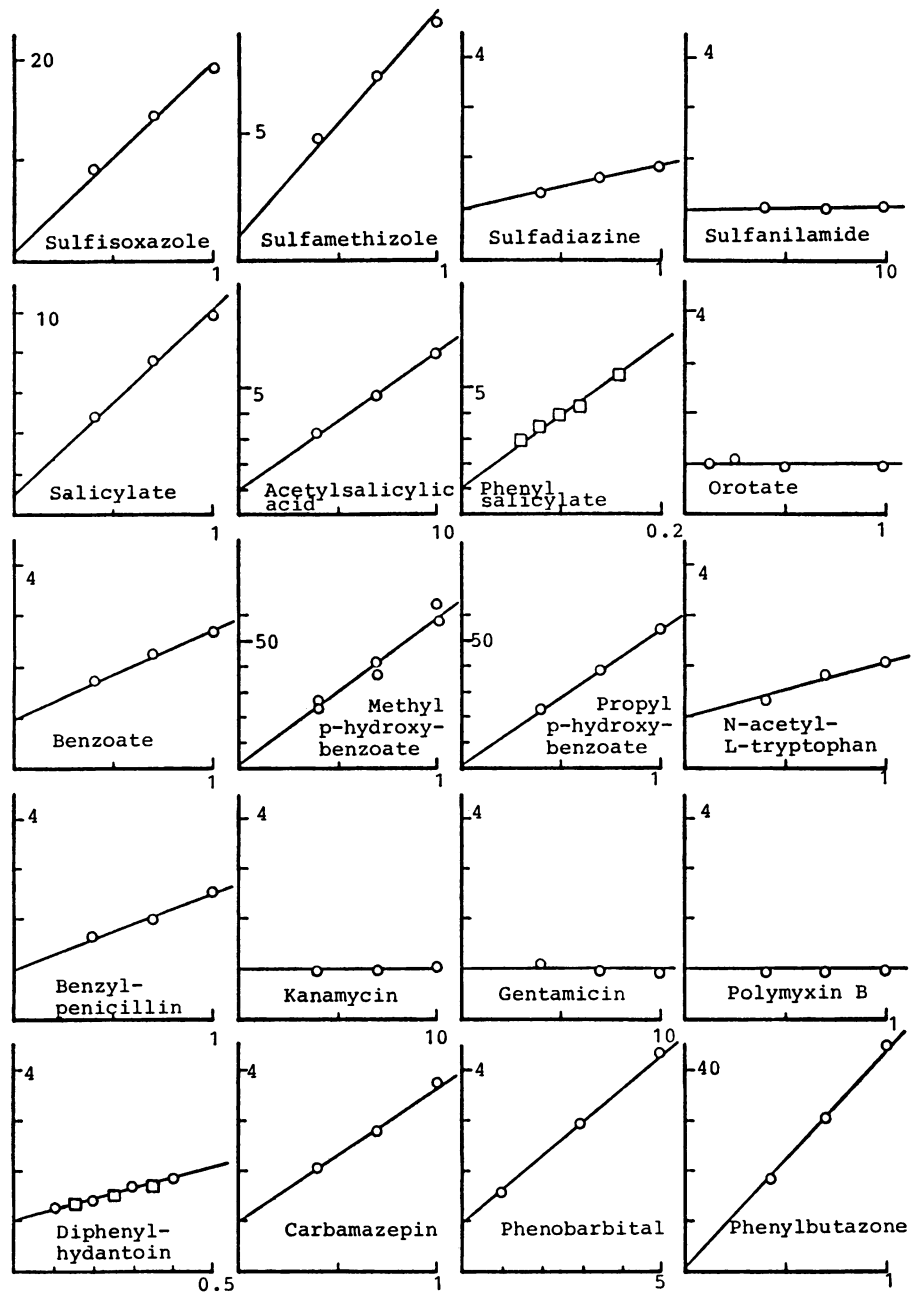

FIGURE 4 Free bilirubin concentrations, relative to the value without a displacer, measured as $t_{0.2(o)}[HRP]_o/(t_{0.2}[HRP])$, as a function of displacing drug concentration (in millimoles per liter). The linear course is consistent with a simple, competitive 1:1 displacement. The slope of the line is equal to the association constant of the drug at the high-affinity site for bilirubin. O, Albumin 30 μM, bilirubin 15 μM, 1-cm cell. □, Albumin 6 μM, bilirubin 3 μM, 4-cm cell. pH 7.4, 37°C.

TABLE I

Binding Constants of Drugs to the High-Affinity Bilirubin-Binding Site of Human Serum Albumin, at pH 7.4, 37°C, with 60 mM Sodium Phosphate Buffer

Drug	K_D
	M^{-1}
Sulfisoxazole	1.9×10^4
Sulfamethizole	8.6×10^3
Sulfadiazine	8.2×10^2
Sulfanilamide	$<2 \times 10^1$
Sodium salicylate	9.2×10^3
Acetylsalicylic acid	5.3×10^2
Phenyl salicylate	2.9×10^4
Sodium benzoate	1.7×10^3
Methyl *p*-hydroxybenzoate	5.7×10^4
Propyl *p*-hydroxybenzoate	5.3×10^4
Benzylpenicillin sodium	1.5×10^3
Kanamycin sulfate	$<1 \times 10^1$
Gentamicin sulfate	$<1 \times 10^1$
Polymyxin B sulfate	$<1 \times 10^2$
Diphenylhydantoin	2×10^3
Carbamazepin	2.7×10^3
Phenobarbital sodium	6.3×10^2
Phenylbutazone	4.3×10^4
N-acetyl-L-tryptophan	1.1×10^3
Sodium orotate	$<2 \times 10^2$

very small compared to that of bound. In the equilibrium equation, the total bilirubin concentration could thus be used in place of the bound. This gives

$$b = \frac{B}{K_B} \times \frac{1}{P - B}. \quad (5)$$

If the velocity of the oxidation process is proportional to the free bilirubin concentration b, and B and K_B are constant, we would expect to find a velocity proportional to $1/(P-B)$. As seen in Fig. 2, this was experimentally verified. This seems to confirm that a low concentration of free bilirubin is actually present in equilibrium with the bound. The oxidation apparently involved the free bilirubin only. Oxidation of the bound pigment would have resulted in a line with a positive intercept at the ordinate.

Variation of the oxidation velocity with the enzyme concentration was examined, with constant concentrations of bilirubin and albumin (Fig. 3). If the dissociation of bilirubin from the complex with albumin is very fast, compared to the oxidation, so that the latter process is rate limiting, the velocity should be proportional to the enzyme concentration, which means that $t_{0.2}$ should be proportional to the inverse enzyme concentration, 1/[HRP]. This was fulfilled with a good approximation at moderate velocities, as used in the present method. At very high rates, a delay of about 4 s was found, although this was of borderline significance. The line in Fig. 3 therefore intersects the ordinate axis slightly above the origin. It can easily be shown that this line is the expected result if dissociation occurs as a first-order process, with a velocity constant of 3 min^{-1}. This shows that the dissociation is so fast that the oxidation can be regarded as rate determining with good approximation, if the time taken for 0.2 of the total oxidation is kept higher than 2 min.

At low enzyme concentrations (not shown in the figure) the range of proportionality extends below 20 pM peroxidase. The enzyme is remarkably stable even at these extreme dilutions.

Competitive effect of drugs. The competitive effect of 20 substances was examined. The method proved applicable in all cases. Two cationic drugs, gentamicin and polymyxin B, precipitated bilirubin in the absence of albumin. These substances did not change the rate of oxidation of bilirubin when albumin was present, and it was accordingly possible to ascertain their noncompetitive nature. No other complications were encountered.

15 of the drugs showed measurable competition with bilirubin. In all cases a linear relationship was obtained for the velocity as a function of drug concentration (Fig. 4), in agreement with the proposed model.

It is concluded that certain drugs decrease the binding of bilirubin to its high-affinity site on human serum

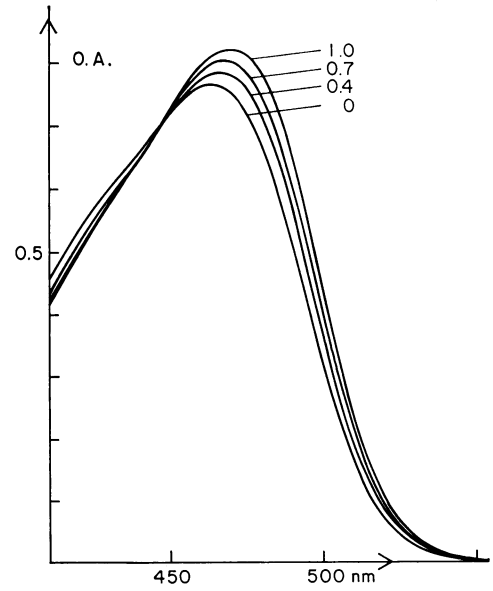

FIGURE 5 Light absorption spectra of bilirubin-albumin, 15/30 μM, in phosphate buffer, pH 7.4, in the presence of polymyxin B, concentrations 0, 0.4, 0.7, and 1.0 mM. Cell, 1 cm.

albumin. The increase of free bilirubin concentration may be described in terms of competitive binding of the drug to this site. The binding constants are seen in Table I.

Binding of drugs to other sites. Certain ligands may bind to albumin without interfering with bilirubin binding. *N*-acetyl-L-tryptophan binds to one site on human serum albumin at pH 7.4, with the association constant 3×10^4 M^{-1} (29). The affinity for the bilirubin site is low, $K_D = 1.1 \times 10^8$ M^{-1} (Table I). This seems to indicate that the first molecule of acetyltryptophan is bound to a locus other than the first site for bilirubin.

Shifts of the spectrum of bilirubin-albumin were seen with most of the drugs tested, even when the free bilirubin concentration remained constant. An example of this is shown in Fig. 5. The spectrum of a bilirubin-albumin solution, 15 and 30 μM, is gradually changed by addition of polymyxin B, 0.4–1 mM. The shoulder at 420 nm is decreased and the maximum at 460 nm is increased. Some drugs (salicylate and related substances) caused a shift in the opposite direction. Polymyxin B and several other drugs apparently are bound to albumin at sites other than the high-affinity site for bilirubin. It is interesting to note that binding of polymyxin B to one site influences the light absorption spectrum of bilirubin, bound to a different site, without causing a change in the affinity of the bilirubin-albumin bond.

Besides the competitive binding of drugs demonstrated above, another type of liganding of some drugs takes place at sites distinct from the first site for bilirubin. This does not seem to influence the affinity of bilirubin for the first site. A third, hypothetical, type of liganding, in which a drug bound to one site decreases or increases the affinity of bilirubin on another site, would give curved or sigmoid-shaped relationships and has not been demonstrated in the present material.

DISCUSSION

The experimental principle. Evaluation of kernicterus-promoting effects of drugs, as caused by interaction with binding of bilirubin to albumin, cannot be done directly on newborn humans, and the use of experimental animals is complicated by species variation of binding patterns for drugs and bilirubin to serum albumin. In vitro studies of binding interactions must be relied upon and the effects in the fetus or newborn child has to be inferred from the in vitro observations. For this purpose, knowledge of the mechanism of interaction, whether reversible, competitive, etc., is essential. Furthermore, a quantitative description of the interaction is necessary.

A drug not bound to albumin cannot function as a competitor. Binding is a necessary but not a sufficient condition for a bilirubin-displacing effect, since bilirubin and drug may ligand to distinct sites on the albumin molecule. This seems in fact to take place with some drugs. It is consequently necessary to study the shifts of binding equilibrium caused by the drug, and this again means that knowledge of the physical state of bilirubin in solution is prerequisite.

At a pH of about 8–9, bilirubin is soluble in water and is present as a monomer anion in equilibrium with its dimer (25). The dissociation constant is of the order of 10^{-4} M, independent of pH, indicating that bilirubin in the micro- or nanomolar range is predominantly monomer. At pH 7.4, the solubility is very low and colloid aggregation tends to take place at concentrations higher than about 0.1 μM (24), although higher concentrations may remain in a metastable, supersaturated solution for some time. In the presence of serum albumin, reversible binding of bilirubin takes place to one high-affinity and a few weaker sites (17, 23). According to general concepts of binding equilibrium, this should result in a certain concentration of unbound bilirubin in the solution. At a ratio 1:2 of bilirubin to albumin, the free bilirubin concentration is very low, probably of the order of 10 nM (17), and in any case very small compared to the concentration of bound pigment. Under these circumstances it is not possible to measure the free bilirubin concentration by conventional, spectroscopic means, and evidence for its existence can only be obtained indirectly. The reality of such small, free, monomer bilirubin concentrations is supported by the findings in the present paper of predicted oxidation velocity relations to the concentration of albumin (Fig. 2) and competing drugs (Fig. 4).

Various experimental principles have been used for demonstration of the bilirubin-displacing effect of drugs. A shift of light absorption spectrum takes place when bilirubin is bound to albumin. This, however, cannot be used as a basis for studying binding equilibria at the first site, due to the very low ratio of free to bound pigment. For the same reason, it is also not possible to utilize the fact that bound bilirubin shows a fluorescence while the free does not. The same is true for the optical rotation, seen with bound bilirubin, but not with the free. All these principles would be further complicated by shifts of the spectra that occur on binding of new ligands, drugs, to other sites on the bilirubin-albumin complex (Fig. 5).

Separation of the free bilirubin by ultrafiltration, equilibrium dialysis, or ultracentrifugation (30), followed by spectrophotometric determination, gives disappointing results due to instability of bilirubin in very dilute solutions and a tendency of the pigment to adhere to membranes and glass surfaces. Attempts were made in the author's laboratory to use ^{14}C-labeled bilirubin in an ultracentrifugation technique. It was found that a small, nearly

constant, fraction of the radioactivity was present in the protein-free supernate. This could not be identified as bilirubin and was thought to originate from decomposition, probably due to light (26).

Observation of bilirubin displacement to an adsorbent, cholestyramine (9, 28) or Sephadex (12, 19, 31, 32), or to erythrocytes (7, 8, 33, 34) has yielded valuable information. Displacement by several drugs has been demonstrated, but these methods have not been used for quantitative determination of the binding constants.

The peroxidase method is based on an analogous principle. Two proteins, peroxidase and albumin, compete for the bilirubin. Added drugs displace the ligand from albumin to the enzyme, resulting in an increased rate of oxidation. Several advantages are obtained by use of peroxidase as the competing binder, rather than an adsorbent like Sephadex. First, the fraction of the total bilirubin bound to the enzyme is extremely small so that the equilibrium between bilirubin, drug, and albumin, is undisturbed (in the initial moment of the oxidation process). Second, the amount of bilirubin bound to peroxidase is proportional to the free bilirubin concentration and is easily quantified by measuring the initial rate of oxidation. Finally, it is possible to examine whether the drug competes with bilirubin for binding to the enzyme by testing the influence of the drug on the oxidation velocity of bilirubin in the absence of albumin.

The mechanism of displacement of bilirubin with the drugs tested here seems to be sufficiently described by the model of competitive binding. The experimental results of the present work may be interpreted in terms of simple competition of one molecule of drug with one of bilirubin for the high-affinity bilirubin site on the albumin molecule. Other stoichiometric relations, such as displacement of one molecule of bilirubin by two molecules of the drug, would give curved lines for the variation of oxidation velocity with drug concentration.

Site-to-site inhibition or cooperativity of binding, effected through conformational changes, would likewise result in curved functions. Such effects have been observed when long-chain fatty acids (35), steroids (36), detergents (37), or calcium ions (36) are bound to serum albumin. It is, accordingly, necessary with each displacing substance to examine whether a linear relationship of oxidation velocity and drug concentration exists at levels of the latter considerably higher than that of albumin, to establish a simple, competitive mechanism of displacement.

Description of the mechanism of displacement as a simple competition between one molecule of bilirubin and one of the drug for a specific site on the albumin molecule opens the possibility of assessing the effect of each drug by its binding constant, K_D, to this locus. Knowledge of this constant and of the free drug concentration in plasma defines the relative increase of free bilirubin concentration caused by the drug, according to Eq. 1. It is not necessary to know the affinity of bilirubin for binding to albumin.

Validity of the approximative assumptions. The experimental determination of K_D by the peroxidase method is based on several approximations, namely: (*a*) The free bilirubin concentration is very small compared to the total;(*b*) The free concentration of the competing drug is equal to the total; and (*c*) Bilirubin is bound to one site only.

The errors caused hereby in determination of K_D are discussed in the following:

(*a*) In a solution containing 30 μM albumin and 15 μM bilirubin, the free concentration of the latter is equal to $1/K_B$, i.e., probably around 10^{-6} M. With a strong competitor at high concentration, it might appear possible to displace a major fraction of the bilirubin. This would result in smaller increases of oxidation velocity at high drug concentrations. The straight lines obtained in all cases, with three of the drugs up to free bilirubin concentrations as high as 50 times the original (Fig. 4, methyl *p*-hydroxygenzoate, propyl *p*-hydroxybenzoate, phenylbutazone), confirm the validity of the assumption that the free bilirubin concentration is very small compared to the bound within this range of increase. (*b*) Eq. 1 is thus valid in all cases and may be written:

$$\frac{b}{b_0} = K_D \frac{d}{D} D + 1. \qquad (6)$$

At high drug levels, the free concentration, d, approaches the total, D, Eq. 2 becomes valid, and the slope of the line approaches K_D. At the other extreme, when D is small compared to the albumin concentration, the slope is smaller, $K_D d/D$.

Let us first consider the deviation if the drug is bound strongly to one or a few sites. A curve is then obtained (Fig. 6*a*), merging into a straight line with the slope K_D at high drug concentrations. This straight line is shifted along the abscissa away from the ideal, by a certain concentration, e. The value of e is equal to the maximal concentration, $nP - B$, of bound drug, obtained at full saturation of all sites, where n is the total number of drug-binding sites, since the concentration, B, of these is occupied by bilirubin. A straight line, fitted through (0, 1) and the experimental points scattered along the curve, may be drawn through a point on the straight portion of the curve around the abscissa $D_{max}/2$, if D_{max} is the highest concentration of the drug plotted in the graph. (Fig. 6*a*, dotted line). The slope of this line is not K_D, but

$$\left(1 - \frac{2(nP - B)}{D_{max}}\right) K_D,$$

This makes it possible to estimate the error of the determination of K_D. The greatest error is obtained with a high value of n and low D_{max}, relative to the albumin concentration. Among the 20 drugs tested (Fig. 4), diphenylhydantoin would be likely to show an especially large error. At a guessed n value of 4, K_D would be determined as low as 0.475 times the true value. It is unlikely, however, that a corresponding curvature of the line should be masked by experimental scatter of the points. Actual errors are probably smaller. In case of doubt, the determination could be repeated with one-fifth the concentrations of albumin and bilirubin, reducing the error to one fifth. This was done in case of diphenylhydantoin, but gave no significant change of the slope, (Fig. 7). Typical errors in this category range about 1–10%.

If the drug is bound with low affinity to a high number of loci, in addition to the bilirubin site, it becomes impossible to attain drug concentrations high enough to fulfill the condition $d \simeq D$. This would be likely to result in a curved line, except in the extreme case when all sites remain largely unoccupied but are present in a high number, so that the total amount of bound drug becomes significant. The ratio of free to bound drug would then remain constant at all concentrations, and a straight line would ensue with the slope $K_D d/D$, resulting in a corresponding error of K_D. A 50% error would be caused by binding to 170 sites, each occupied to one tenth, if the highest drug concentration is 1 mM, and with 30 μM albumin. With 6 μM albumin, 850 sites would be needed. Errors of the order of 1–10% are more likely.

(*c*) Secondary binding sites for bilirubin are present on the albumin molecule (17, 23), and may influence the determination of K_D. The effect of a competing drug under the circumstances of the in vitro experiment may be described as a change of the binding constant for bilirubin to a lower, apparent value,

$$K_{B(app)} = K_B/(K_D d + 1), \qquad (7)$$

derived in analogy with the apparent Michaelis constant of an enzyme-substrate in the presence of a competitive inhibitor. Corresponding relations hold for the secondary sites. If K_D values of all sites are equal, it is seen that the free bilirubin concentration will vary with that of the drug in accordance with Eq. 1. The true value of K_D is then obtained from the slope of the line. Problems arise when the K_D of the primary site is much higher than that of the secondary, i.e. when the drug competes mainly or exclusively for the primary site. In this case, the apparent K_B of the primary site may approach the values of the secondary loci, or the apparent affinities may even be reversed, with the result that major amounts of bilirubin are displaced to the

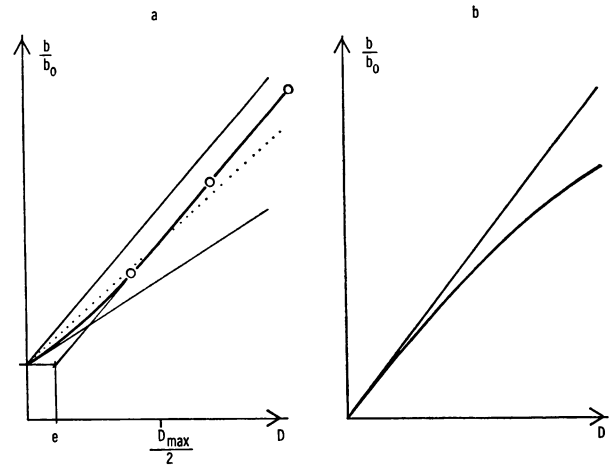

FIGURE 6 Deviations from the straight linear course of the relation of free bilirubin to added drug concentration. In *a*, a curvature has resulted from binding of the drug to one or several strong sites on the albumin molecule. *b* shows the type of deviation expected if substantial amounts of bilirubin are displaced by the drug to weaker sites.

secondary sites. Each site will then carry a smaller amount of bilirubin, and the free bilirubin concentration will be lower than predicted from Eq. 1. At low drug concentrations, the slope of the line will be close to K_D for the primary site, provided that nearly all bilirubin is bound to this in the absence of a drug. At very high drug levels, the slope will approach the lowest K_D value of any site, since all bilirubin finally will be displaced to the site where the drug has the least affinity (Fig. 6*b*). The slope may theoretically approach zero if one or more secondary sites do not bind the drug at all. Curvatures of this type are best observed if the examinations are extended to high drug concentrations, as permitted by solubility, etc. During the work with the limited number of drugs in the present paper, no such observations were made. The

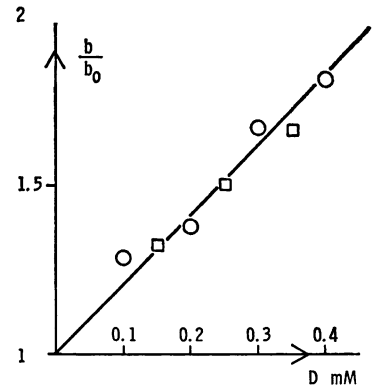

FIGURE 7 Detail of Fig. 4: diphenylhydantoin. Results obtained with 6 and with 30 μM albumin are identical.

TABLE II

Calculated Total and Free Bilirubin Concentration in Blood Plasma in the Presence of a Competing Drug, and the Resultant Displacement of Bilirubin from Albumin to a Tissue Receptor

Drug Free concn. in plasma	Bilirubin						
	Concn. in plasma (drug added in vitro)			After equilibration with tissues			
				Concn. in plasma		Amount bound to nonalbumin receptor	Amount displaced
	Bound		Free	Bound	Free		
mM	*mg/100 ml*	*μM*	*μM*	*μM*	*μM*	*μmol*	*μmol*
0	17.5200	300.000	0.043	300.0	0.043	0.1	0
0.01	17.5197	299.996	0.047	299.9	0.047	0.11	0.01
0.1	17.5174	299.957	0.086	299	0.086	0.2	0.1
1	17.4923	299.527	0.47	290	0.45	1.1	1
10	17.27	295.7	4.3	ca 200	ca 4	ca 10	ca 10

Total plasma albumin concn. 400 μM, plasma volume 100 ml, $K_B = 7 \times 10^7\ M^{-1}$, $K_D = 1.0 \times 10^4\ M^{-1}$. Displacement to or from reservoirs other than plasma albumin and an arbitrarily chosen receptor is neglected.

reason for this may be that the affinity of bilirubin for the secondary sites is very much less than for the first. It is possible, however, that curvatures of the types shown in Fig. 6*a* and *b* may partly cancel each other under suitable circumstances. If, therefore, a drug shows a degree of competition, estimated to be of clinical importance (see below), further studies of binding to all sites on albumin should be conducted to evaluate the possible errors. It should be noticed that all sources of error tend to give too low K_D values.

Attempted calculation of maximal displacement in vivo. The binding constants for drugs to the high-affinity bilirubin site fall in the same range as binding constants for most drugs to albumin, $0\text{–}10^5\ M^{-1}$, as known from studies of equilibrium dialysis, etc. (38), and are several orders of magnitude lower than that of bilirubin, about $10^8\ M^{-1}$. Generally the affinities are unfavorable for displacement of bilirubin with drugs. Accordingly, addition of a drug to blood plasma in vitro does not result in release of a major fraction of the bound bilirubin. The relative increase of the free bilirubin concentration may, however, be high. This is illustrated in Table II, by calculated values of bound and free bilirubin concentrations in plasma with varying concentrations of a competing drug. At zero drug concentration, 300 μM albumin of the 400 present is occupied by bilirubin, leaving 100 μM free albumin. The free bilirubin concentration is calculated from the equilibrium equation, $300 \times 10^{-6}/(b \times 100 \times 10^{-6}) = 7 \times 10^7$. With added drug, the free bilirubin concentrations are calculated from Eq. 1.

From the left-hand half of Table II, it is obvious that the effect of the drug in vitro is not a gross displacement of bilirubin from binding to albumin but consists of occupation of available sites, whereby the free bilirubin concentration is increased in the same proportion as the amount of vacant sites is decreased. The concentration of bound or total bilirubin is not decreased measurably in the absence of nonalbumin receptors that can take over the bilirubin.

Displacement of a large fraction of the bilirubin may result in vivo. According to Odell (39), free bilirubin in the blood plasma is diffusible and in equilibrium with receptors outside the circulation. An increase of the free bilirubin concentration in the plasma results in an increased extravascular binding. Numerical evaluation of the displacement is possible in certain conditions, as follows:

Let P^* denote the molar concentration of a tissue acceptor site and d^* etc. signify the other parameters of the tissue phase, in analogy with the symbols used for blood plasma. The free bilirubin concentration in the plasma may be obtained from the former equations as

$$b = \frac{1}{K_B} \times \frac{[BP]}{P - [BP]} (K_D d + 1).$$

In equilibrium, the same free bilirubin concentration prevails in the tissue, and this is found from the analogous expression

$$b = \frac{1}{K_B^*} \times \frac{[BP^*]}{P^* - [BP^*]} (K_D^* d^* + 1)$$

and in the absence of a drug,

$$b_o = \frac{1}{K_B^*} \times \frac{[BP^*]_o}{P^* - [BP^*]_o}.$$

From the latter two equations we derive,

$$\frac{[BP^*]}{[BP^*]_o} = \frac{b}{b_o} \times \frac{K_B^* b_o + 1}{K_D^* d^* + K_B^* b + 1},$$

which is equal to the relative increase of bilirubin, bound to the acceptor, and is a measure of the displacement. It is seen that the maximal value of this is obtained if $K_D^* << 1/d^*$ and $b << 1/K_B^*$, i.e., if the drug does not displace bilirubin from the tissue acceptor, and if the acceptor is far from saturated with bilirubin. In this case, we have, using Eq. 1, and $b_o < b$,

$$\left(\frac{[BP^*]}{[BP^*]_o}\right)_{max} = K_D d + 1.$$

This maximal displacement factor is a quantitative measure of the risk of bilirubin displacement incurred by the use of a drug at a certain free concentration in the plasma. It may be convenient to signify this figure by δ and to express it by the total plasma concentration D of the drug,

$$\delta = K_D d + 1 = K_D(1 - q)D + 1, \qquad (8)$$

when q is the fraction of the drug bound to plasma proteins.

The numerical value may be calculated if K_D is known and the free drug concentration needed for therapeutic effect or the total plasma concentration and degree of protein binding are specified.

The binding constants obtained for the four sulfonamides (Table I) indicate an increasing competition with bilirubin in the order sulfanilamide < sulfadiazine < sulfamethizole < sulfisoxazole. Bratlid (7) has examined the binding of bilirubin to erythrocytes in solutions with albumin after addition of these sulfonamides and has found that it is increased in the same order of efficiency. Sulfisoxazole at usual dosage gives a plasma level about 0.5 mM, 85% of which is protein bound. The maximal displacement factor, calculated from Eq. 8, is 2.4. At this blood plasma concentration, sulfisoxazole may thus cause an increase by a factor of 2.4 of the amount of bilirubin bound to a critical receptor, such as an area of the brain. These findings are consistent with the clinical experience of a displacing effect of sulfisoxazole (1, 2, 5).

The mechanism of displacement with salicylate. Sodium salicylate is a fairly potent competitor, as seen from its K_D value, 0.9×10^4 M^{-1}. The degree of protein binding, however, is high at moderate plasma concentrations. According to Rudman, Bixler, and Del Rio (35), two molecules of salicylate are bound to human serum albumin with a binding constant of 10^5 M^{-1}. Half-saturation of these sites is produced by moderate doses and gives a free plasma concentration of 10^{-5} M of salicylate. The maximal displacement factor is 1.09, in agreement with the fact that clinical displacement has not been reported with this drug. Presumably, the first two molecules of salicylate are bound to human serum albumin without interfering with the bilirubin site. A following molecule of the drug, bound in a subordinate class of sites, competitively displaces bilirubin. Maximal dosage, giving a drug/albumin ratio of 3 or 4, would be expected to result in displacement. A decrease of bilirubin concentration in blood plasma has in fact been demonstrated after large doses of salicylate in the Gunn rat (9, 10) and in man (8).

Thaler and Schmid (14) refer to the permanent acetylation of albumin with acetylsalicylic acid, demonstrated by Hawkins, Pinckard, Crawford, and Farr (15), and warn against use of this drug in patients with unconjugated hyperbilirubinemia. This is based on the fact that acetylation of albumin decreases the affinity for certain anions. Jacobsen (40) has found, however, that acetylation of human serum albumin in vitro with acetylsalicylic acid did not change the affinity for bilirubin. One specific lysyl-amino group is attacked by acetylsalicylic acid and seems to be involved in the binding of fatty acids and tryptophan (41), whereas bilirubin is bound to a different site. The competitive effect of acetylsalicylic acid as such is slight (Table I), and large doses would be needed to produce displacing amounts of salicylate.

Clinical experience and observations from animal experiments and from kinetic studies in vitro can be explained by a simple, competitive mechanism of bilirubin displacement by drugs.

As stressed by Stern (42), any drug used in the newborn should be examined for its ability to displace bilirubin from albumin. Even during pregnancy, a displacing drug may reach the fetus via the placenta and exert its effect in case of fetal hyperbilirubinemia. Similarly, passage of pharmaca with breast milk should be considered. At present, such testing is not required by official authorities but ought to be carried out for all drugs in current use and as a routine before licensing of new substances.

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