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ERYTHROMYCIN BINDING TO HUMAN SERUM*

GERHARD A. DETTE,† HANS KNOTHE and GUNTER HERRMANN
Zentrum der Hygiene der Johann-Wolfgang-Goethe-Universität, Abt. f. Medizinische Mikrobiologie, Paul-Ehrlich-Str. 40, 6000 Frankfurt 70, West Germany

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Abstract—Erythromycin binding to human serum was measured under conditions of binding equilibrium. The binding is sensitive to pH changes, decreasing at acid pH. Over a great range of serum dilution, the bound fraction is semilogarithmically related to serum concentration. Binding is shown to be completely reversible. With increasing erythromycin concentration a specific part of binding is saturable and specifically displaceable by erythromycin in excess, whereas a nonspecific part linearly increases with total concentration. Erythromycin is specifically bound to a single class of noninteracting binding sites with an apparent dissociation constant $K_d = 5.9\ \mu M$ (38°C). The kinetic and thermodynamic parameters at 25° are: $K_d = 8.4\ \mu M$, $\Delta H^\circ = +4.4 \times 10^3$ cal per mole, $\Delta G^\circ = -6.9 \times 10^3$ cal per mole, $\Delta S^\circ = +38$ e.u.

Antibiotics like other drugs bind to serum proteins in different degrees. The assumption that only the unbound part of total antibiotic is the biologically active fraction [1, 2] implies the therapeutic significance of antibiotic binding. Concerning erythromycin, the binding values given in the literature are conflicting. The bound fraction ranges from zero to more than 95% of total erythromycin [3–12]. The present paper now follows erythromycin binding to human serum as a function of erythromycin concentration and describes kinetic and thermodynamic parameters of binding equilibrium.

MATERIALS AND METHODS

Materials. The chemicals used in this study were purchased as follows: unlabelled erythromycin lactobionate, Lot 39 949 AF was from Abbott Laboratories (N. Chicago, IL); [*N*-methyl-^{14}C]erythromycin lactobionate, sp. act. 1.48 GBq/mmole from Amersham International Ltd. (Amersham, U.K.); PPO and POPOP from Merck A.G. (Darmstadt, F.R.G.); Sephadex G 25 fine from Deutsche Pharmacia GmbH (Freiburg, F.R.G.); Bacto Penassay Broth dehydrated (Antiobiotic medium 3) and Bacto agar from Difco (Detroit, MI). All other chemicals were of analytical grade from conventional commercial sources. Normal human serum (total protein 74 g/l) was pooled from healthy individuals. The test strain used in the experiments on biological erythromycin activity was Sarcina lutea ATCC 9341.

Methods. Binding was studied by equilibrium dialysis in a Dianorm® apparatus (Diachema AG, Rüschlikon, Switzerland). The two Teflon cell compartments (working volume 1.0 ml each) were separated by a cellulose hydrate membrane (exclusion limit 5000). The rotation speed during dialysis was 16 rpm in all the experiments. Prior to dialysis the Teflon cells like buffer and serum were allowed to equilibrate with the temperature chosen for experiment. Then 1.0 ml aliquots of human serum were dialyzed against 1.0 ml of 0.1 M phosphate buffer pH 7.2, containing ^{14}C-labelled or, in some experiments, unlabelled erythromycin lactobionate at various concentrations. The final concentrations of free erythromycin ranged from 5×10^{-7} to 2×10^{-5} M. When unlabelled erythromycin was used to inhibit the binding of ^{14}C-labelled erythromycin, it was added to the side of dialysis buffer. The effect of serum protein concentration on total erythromycin bound fraction was studied at 38°. Serum dilutions (pH 7.2) up to 0.025 of native serum were prepared by adding 0.1 M phosphate buffer and then dialyzed against erythromycin (10 μM) as described above. Similarly, the pH influence on erythromycin binding was examined (final erythromycin conc 10 μM), after the pH had been adjusted to 5.0, 5.5, 6.0, 6.5, 6.85, 7.0, 7.2, 8.0, and 8.5, respectively. In parallel, the pH stability of erythromycin was controlled in terms of biological activity. Erythromycin (final concn 200 μg/ml) was incubated at 38° and pH 5.0, 5.5, 6.0, 7.2, and 8.4. After 0, 30, 60, 120, and 180 min of incubation, aliquots were taken and added to 0.1 M phosphate buffer, pH 7.2. The erythromycin contents (final concn 6.0 μg/ml each) of these buffer samples were assayed by the agar plate technique as described below. With adding the erythromycin solutions, in no case was the pH of the phosphate buffer changed. This fact is important concerning the relationship between pH and test strain sensitivity [5, 13].

Controls for evaluation of optimal dialysis time and for determination of binding loss due to erythromycin adsorption by dialysis membranes (or cells) and degradation during dialysis, respectively, were prepared by dialyzing buffer with known erythromycin amounts (^{14}C-labelled and unlabelled) against antibiotic free buffer. After equilibration in experiments with unlabelled erythromycin the buffer side was assayed biologically whereas the labelled eryth-

* Part of this study was work carried out by G. Herrmann for his M.D. thesis.

† Author to whom correspondence should be addressed.

romycin samples (100 μl aliquots), both from the buffer and serum side, were mixed with Bray's solution [14] and counted for radioactivity by liquid scintillation spectrometry.

Unlabelled erythromycin was biologically assayed by the agar plate diffusion technique. Agar (Bacto agar 150 g, Antibiotic medium 3 17.5 g, aqua dest. 1 l) was seeded with the test organism, Sarcina lutea ATCC 9341, and poured on glass plates bordered by rings of 190 mm diameter. Then after cooling with the help of a stencil cylindrical wells (dia. 10 mm) were punched in the agar and filled with sample aliquots (100 μl). Calibration standards were prepared using the same erythromycin stock solution as in dialysis experiments after appropriately diluting with 0.1 M phosphate buffer pH 7.2. Standard and sample aliquots were running in parallel on each agar plate. After 18 hr of incubation at 37° the diameters of inhibition zones were read with an accuracy of 1/10 mm. The linear calibration curve was given by the semilogarithmic plot of standard concentrations (exponentially) versus corresponding diameters (arithmetically) of inhibition zones [15].

Gel filtration experiments were based on the equilibrium method [16]. Ascending chromatographic technique was used. A 0.9 × 30 cm column of Sephadex G 25 fine was equilibrated at 22° with 0.1 M phosphate buffer pH 7.2, containing [^{14}C]erythromycin at a final concentration (5.25×10^{-6} M) close to the serum sample (5.5×10^{-6} M) to be chromatographed. In some experiments the labelled erythromycin in the buffer was omitted. A 1.0 ml aliquot of human serum, prior to chromatography incubated with 5×10^{-6} M [^{14}C]erythromycin at 22° for 1 hr, was applied to the column. The column was loaded and eluted at a constant rate of 13.8 ml/hr and fractions of 0.48 ml were collected. 100 μl aliquots of eluates were counted for radioactivity. Protein was continuously monitored through a quartz microflow cell at 280 nm wave length and was further controlled by external absorbance measurements of eluate fractions.

Protein was determined by the method of Lowry *et al.* [17] with bovine serum albumin as a standard.

RESULTS

The binding of erythromycin to human serum was studied after the binding reaction had reached equilibrium. Binding was not influenced by the use of phosphate buffer, which had been previously dialyzed against serum free of erythromycin. Likewise, no difference in total binding values could be detected, when erythromycin was assayed biologically or by radioactivity counting. This indicates that the labelled and the unlabelled ligand exhibit identical binding properties.

pH and binding

A marked effect of pH was observed (Fig. 1). Binding decreased with increasing acidity of the incubation medium, e.g., at pH 8.5 65.2% of total erythromycin (10 μM) was bound, whereas at pH 5.0 the bound fraction amounted only to 46.8%. To distinguish pH-linked binding effects from binding of erythromycin degradation products (due to acid

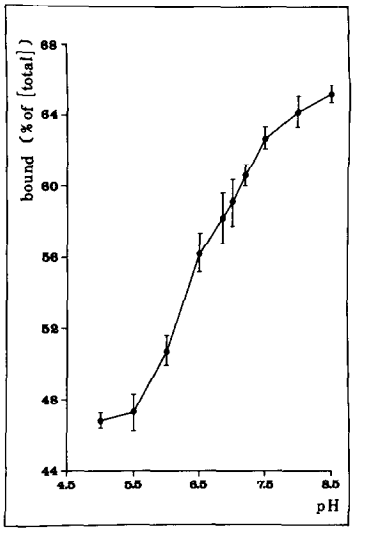

Fig. 1. Binding of [^{14}C]erythromycin to human serum as a function of pH. Serum aliquots (quadruplicates) were dialyzed (38°) against 0.1 M phosphate buffer, [^{14}C]erythromycin (10 μM) at different pH values (abscissa). After the binding reaction had reached equilibrium, the amounts of bound [^{14}C]erythromycin were determined, expressed in % of total erythromycin concentration (ordinate).

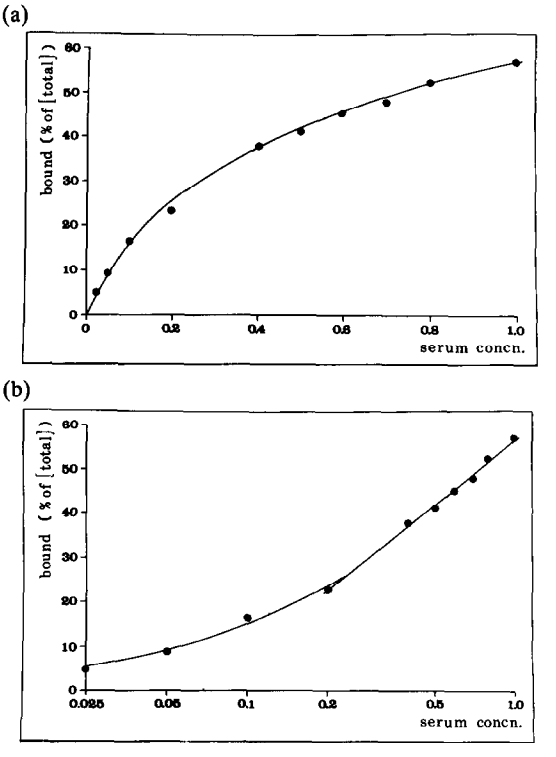

Fig. 2. Binding of [^{14}C]erythromycin to human serum as a function of serum dilution. Serum dilutions prepared with 0.1 M phosphate buffer (quadruplicates) were dialyzed (38°) against 0.1 M phosphate buffer, [^{14}C]erythromycin (10 μM) at pH 7.2 up to binding equilibrium. The amounts of bound [^{14}C]erythromycin (in % of total erythromycin concentration) are plotted against numerical values of serum dilutions (a) and against serum dilutions in a logarithmic scale (b), respectively.

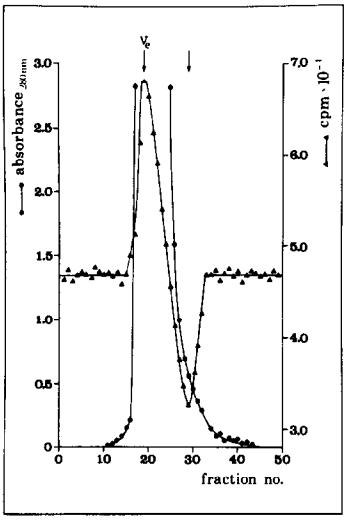

Fig. 3. Binding of [^{14}C]erythromycin to human serum as demonstrated by gel filtration. A serum aliquot (1.0 ml) was equilibrated with [^{14}C]erythromycin (final concn 5.5 μM) and chromatographied on a column of Sephadex G 25 fine, equilibrated with [^{14}C]erythromycin (5.25 μM). Eluent was 0.1 phosphate buffer (pH 7.2), 5.25 μM [^{14}C]erythromycin. Protein (●——●) and [^{14}C]erythromycin (▲——▲) were assayed as described under Methods.

pH), the stability of unlabelled erythromycin at different pH values was tested biologically.

A distinct loss of biological activity was detectable at pH 5.0. At this pH the erythromycin half life time of inactivation was calculated as 4.7 hr and the binding characteristics of native and degradated erythromycin possibly may overlap. All the following experiments were undertaken at pH 7.2.

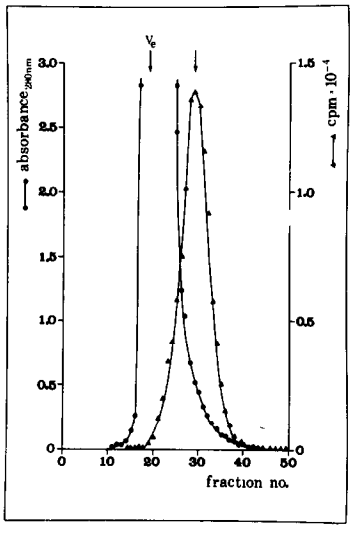

Fig. 4. Reversibility of [^{14}C]erythromycin binding to human serum as demonstrated by gel filtration. A serum aliquot (1.0 ml) was equilibrated with [^{14}C]erythromycin (final concn 5 μM) and chromatographied on the same column as in Fig. 3. But here, the column was not equilibrated with [^{14}C]erythromycin and eluted with buffer not containing [^{14}C]erythromycin. Protein (●——●) and [^{14}C]erythromycin (▲——▲) were assayed as described under Methods.

Protein concentration and binding

The influence of serum protein concentration on erythromycin binding is illustrated by Fig. 2. In the range of high serum dilutions (total protein concn $\ll$3.7 g/l) binding increased almost linearly with increasing protein concentration. At lower serum dilutions approaching normal serum protein concentration, erythromycin binding was not related directly to the dilution steps (Fig. 2 (a)), but was found to fit well ($r = +0.998$) a semilogarithmic relationship (Fig. 2 (b)).

Reversibility of binding

The reversibility of the binding reaction was characterized by gel filtration. In equilibrium experiments like that shown by the elution diagram of Fig. 3, a serum aliquot, the column, and the elution buffer were equilibrated prior to loading the column with erythromycin at final concentrations of 5.5×10^{-6} M (serum) and 5.25×10^{-6} M (column and buffer), respectively.

After the start of gel filtration, the serum protein, running down in the excluded volume (V_e) of the column, was found coincident with a radioactivity peak of [^{14}C]erythromycin. This peak, like the radioactivity trough 'negative' peak below the equilibrium base line) eluted in subsequent fractions, both demonstrate bound erythromycin. As expected from the theory [16], to a certain extent the trough was filled by excess of [^{14}C]erythromycin added together with the serum sample. Conversely, in gel filtration experiments with samples not preincubated with [^{14}C]erythromycin, but then equilibrated with [^{14}C]erythromycin during chromatography, the amount of the ^{14}C-peak area was exceeded by that of the trough area.

The gel filtration was repeated under conditions of disturbed binding equilibrium. The sample aliquot was preincubated with erythromycin 5.5×10^{-6} M, but eluted from the column with buffer not containing erythromycin. As can be seen in Fig. 4, the peaks of erythromycin and protein now were separated. The protein peak was eluted in the void volume, whereas the erythromycin emerged in only one peak, like the trough in Fig. 3 located at the salt volume of the column, indicating a complete dissociation of the erythromycin serum protein complex.

Saturation of binding

Within a low concentration range of total (bound and free) erythromycin, the amount of the bound fraction decreased with increase of erythromycin concentration. At 38° for instance, regarding total erythromycin concentrations of 1.0, 4.0, 6.0, 8.5, 16.5, and 24.0 μg/ml, about 74, 70, 68, 65, 55, and 46% of total erythromycin, respectively, was bound. This binding behaviour was linked to a specific part of binding, following hyperbolic saturation. Specific erythromycin binding was defined as the difference of total binding minus the nonspecific binding, i.e., binding in the presence of 1000-fold excess of unlabelled erythromycin. Nonspecific binding was not

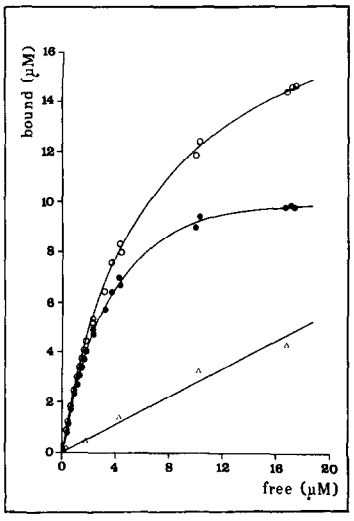

Fig. 5. Binding of [^{14}C]erythromycin to human serum as a function of erythromycin concentration. Serum aliquots (triplicates) were dialyzed (38°) against 0.1 M phosphate buffer (pH 7.2), [^{14}C]erythromycin at various concentrations. The amounts of binding equilibrium represent total binding (○——○). In parallel, aliquots were dialyzed against [^{14}C]erythromycin in presence of unlabelled erythromycin in 1000-fold excess (nonspecific binding, △——△). Specific binding (●——●) was calculated by subtraction of nonspecific from total binding.

saturable within the concentration range tested and increased linearly with increasing erythromycin concentration (Fig. 5).

The saturation of erythromycin binding sites was further characterized by the ability of unlabelled erythromycin to inhibit the formation of [^{14}C]erythromycin serum complex. This competitive displacement of labelled erythromycin by increasing amounts of unlabelled erythromycin is shown in Fig. 6. As calculated from the double logarithmic transformation plot of the typical S-shaped inhibition curve, half maximal displacement of specific [^{14}C]erythromycin binding occurred at about 1.4×10^{-5} M unlabelled erythromycin (IC_{50}).

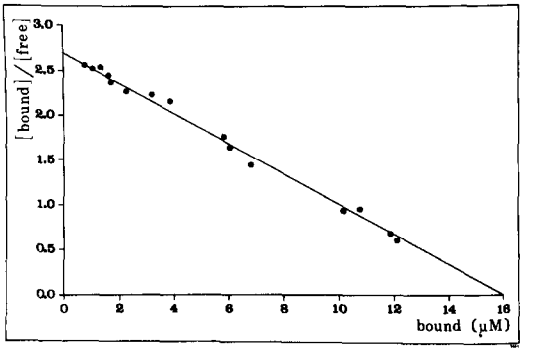

Fig. 7. Scatchard plot of values for specific [^{14}C]erythromycin binding. See legend to Fig. 5.

Kinetics of binding

The data analysis of specific binding as described by Scatchard [18] yielded a straight line over the concentration range studied (Fig. 7), indicating a single class of binding sites. At +38°, the apparent dissociation constant K_d was 5.9×10^{-6} M. No evidence for positive or negative cooperativity in erythromycin binding has been detected. The noncooperative nature is illustrated by the kinetic data rearranged according to Hill [19] with a Hill coefficient of +1.04 (Fig. 8).

Temperature and binding

The kinetic parameters of erythromycin binding were followed as a function of temperature within the temperature range 4–38°C. Figure 9 shows the double reciprocal plot of experimental data according to the method of Lineweaver and Burk [20]. The

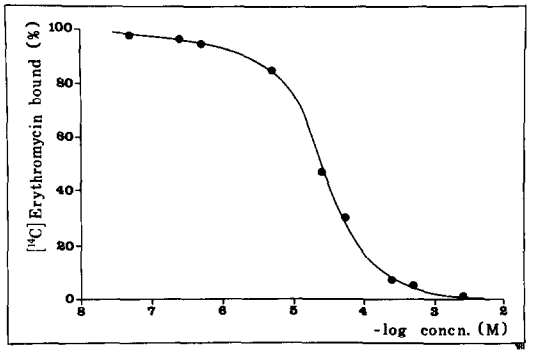

Fig. 6. Displacement of bound [^{14}C]erythromycin by unlabelled erythromycin. Serum aliquots (triplicates) were dialyzed (38°) against 0.1 M phosphate buffer (pH 7.2), [^{14}C]erythromycin (3.4 μM) in presence of various concentrations of unlabelled erythromycin. The amounts of specific [^{14}C]erythromycin binding (ordinate) are given in % of labelled binding at zero concentration of unlabelled erythromycin. The abscissa indicates the logarithms of unlabelled erythromycin concentration.

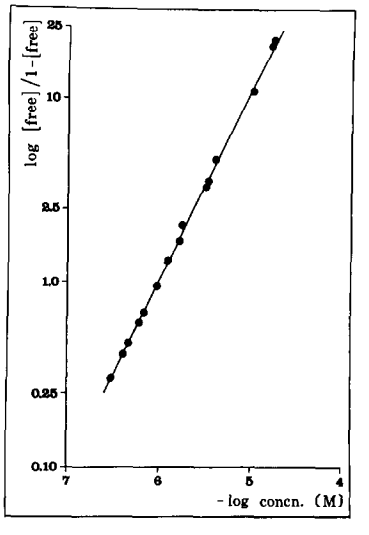

Fig. 8. Hill plot of values for specific [^{14}C]erythromycin binding. See legend to Fig. 5.

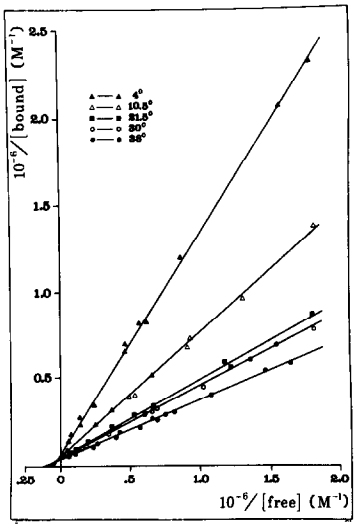

Fig. 9. Specific binding of [^{14}C]erythromycin to human serum as a function of erythromycin concentration at different temperatures. (Lineweaver–Burk plot). See legend to Fig. 5.

specific binding satisfies the characteristics of an endothermic reaction. As can be seen from the Van't Hoff plot [21], the affinity of serum for erythromycin increased with increasing temperature (Fig. 10). At 4.0, 10.5, 21.4, 30.0 and 38.0°, the values of the apparent dissociation constants were found to be 14.9, 11.9, 8.2, 7.4 and 5.9 $\times 10^{-6}$ M, respectively. By use of the Van't Hoff plot the following thermodynamic parameters were calculated (25°): free energy change $\Delta G° = -6.9 \times 10^3$ cal per mole, standard enthalpy $\Delta H° = +4.4 \times 10^3$ cal per mole, and entropy change $\Delta S° = +38$ e.u.

DISCUSSION

The significance of antibiotic binding to serum proteins has been repeatedly reviewed [22–24]. Generally, attention should be payed to the experimental conditions employed, since binding equilibrium may be disturbed by methods of binding determination.

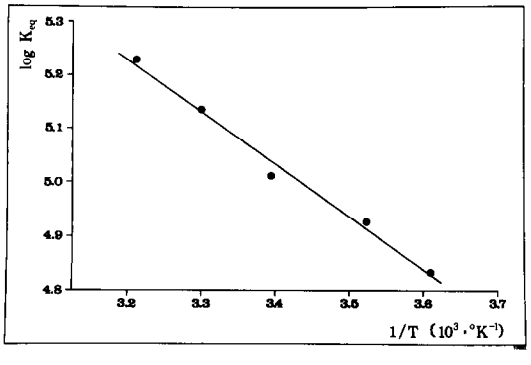

Fig. 10. Dependence of the equilibrium constant of specific [^{14}C]erythromycin binding on temperature (Van't Hoff plot). The data used for deriving the plot are given in the text.

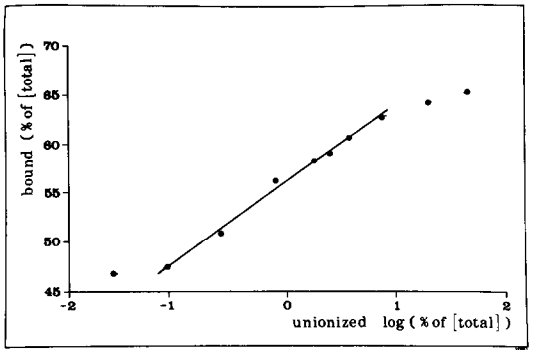

Fig. 11. Binding of [^{14}C]erythromycin to human serum as a function of neutral erythromycin concentration. The amounts of bound [^{14}C]erythromycin (in % of total erythromycin concn), obtained at different pH values as seen in Fig. 1, are plotted against the logarithms of total unionized erythromycin concentration present at the respective pH.

Differing results are not surprising and correspond to the problem of assay method. The values of erythromycin binding to human serum, given in the literature, are mostly non-equilibrium values [3–12].

The classical arguments cited against equilibrium dialysis method, for instance a long dialysis time with consecutive ligand degradation, dilution effects of the protein phase due to large external buffer volumes and considerable nonspecific adsorption effects on to the dialysis membrane are irrelevant regarding the dialysis system used in this study. Nonspecific adsorption of erythromycin was negligible (<5% of total erythromycin) and moreover corrected by separate countings of aliquots from both the buffer and the protein phase. Volume changes of the two dialysis compartments, theoretically excluded by the cell geometry, could not be detected by volume measurements and protein determinations in the protein phase compartment at start and at end of dialysis. The equilibrium was achieved within 5.0 and 2.0 hr at 4° and 37°, respectively. As followed by bioassay, erythromycin, with the exception of acid incubation media, was not inactivated during dialysis period and with respect to binding characteristics, unlabelled and ^{14}C-labelled erythromycin behaved identically.

The erythromycin binding showed a clear cut pH dependency. In the range of pH 5.0 to pH 8.5 the bound part was augmented nearly by half from 46.8% to 65.2% of total erythromycin amount (10 μM). Serum, not freshly taken, with standing at room temperature gets an alkaline pH ($\geq$8.0). Consequently erythromycin binding studies done without noticing this pH shift, give erroneous results. On the other hand, in infected tissue, where the pH becomes acidic (>6.0 <7.0), some bound erythromycin will be liberated as compared with the bound fraction at pH 7.4. The binding changes linked to pH variation may reflect conformational changes of the binding proteins as well as changes in lipophilicity of the erythromycin ligand. The binding of many compounds parallels the lipophilic character. The higher the lipophilicity, the greater is the extent of binding [26]. Erythromycin is a weak base with a pK_a of 8.6

[27]. According to the equation of Henderson and Hasselbalch [28, 29] at each pH the concentration of the neutral, more lipophilic form is given by the ratio [neutral]/[ionized]:

$$pH = pK_a + \log \frac{[neutral]}{[ionized]}.$$

The binding data of Fig. 1 were rearranged and plotted semilogarithmically against the concentration of neutral erythromycin form present at the respective pH values (Fig. 11). From pH 5.5–7.5 this graph gives a linear slope ($r = 0.996$) suggesting that lipophilicity is a prominent component of erythromycin binding, whereas at more alkaline pH values other factors may predominate as indicated by the flattened curve. At pH 5.0 the deviation from the straight line is supposed to be related to erythromycin degradation products as pointed out above.

The extent of erythromycin binding could not be described as a simple linear function of protein concentration. Up to serum dilutions of 0.2, the bound fraction resembled a logarithmic function of serum concentration. Similar semilogarithmic relationships of antiobiotic binding were reported by other workers [8, 30, 31]. This apparent increase in binding affinity with decreasing serum protein concentration does not conform to equilibrium theory of mass action. Speculatively, the phenomenon may be explained by masking of binding sites due to association of protein molecules in concentrated solutions. With extended serum dilution dissociation of associates and unmasking of binding sites takes place. Parallel with proceeding dilution the slope of erythromycin bound versus protein concentration more and more deviates from the semilogarithmic straight line and approaches a numeric direct linear correlation, as indicated at high serum dilutions with concentrations lower than 0.1 of original serum.

The reversibility of erythromycin binding to human serum was tested by gel filtration. Under conditions of equilibrium, erythromycin and serum protein peaks were found in the same fraction eluates. In contrast to this elution pattern, two distinct peaks were found for serum protein and for erythromycin, when gel filtration of serum with erythromycin was done with buffer not containing erythromycin (non equilibrium conditions). In other words, gel filtration is no method of choice for separation of free from bound erythromycin (serum erythromycin complex). During the period of filtration, incessantly erythromycin dissociates from the protein erythromycin complex. This dissociation proceeds very rapidly. So we failed (not published) to separate bound from free erythromycin by charcoal technique [32]. For the same reason others, using gel filtration or agar plate technique, could not demonstrate erythromycin binding [3, 5, 6] or observed binding values much lower as compared with dialysis method [9].

In most cases, at a therapeutic concentration range, antibiotic binding is not a function of final antibiotic concentration [23]. But the exception proves the rule and in the special case of erythromycin binding, the extent of binding decreased with increasing total erythromycin concentration. This binding change was small (<10%) under conditions of oral erythromycin administration (concn range <1–4 μg/ml in serum). In contrast, a concentration rise from 1.0 to 24.0 μg/ml, easily available by intravenous administration, levelled down the binding from about 74 to 46%. Considering the concentration range from 0.5 up to 300 μg/ml used in literature for erythromycin binding determinations, differing binding values will result. Obviously, since only one fixed erythromycin concentration was used, the concentration dependency of binding could not be detected [4, 10, 11, 12].

That binding behaviour of erythromycin has now been explained by a saturable, specific binding component in human serum, predominating at lower erythromycin concentrations but masked at higher concentrations by a non saturable part. The analysis of the specific binding revealed a single class of binding sites, the absence of any cooperativity, and a low apparent dissociation constant ($K_d = 5.9 \times 10^{-6}$ M at +38°). Compared with the few kinetic data published of other antibiotics the binding affinity of erythromycin is rather high [31, 33, 34]. The saturability of specific binding sites was further examined by equilibrium competition experiments with unlabelled erythromycin. Regarding the interdependence of half maximal saturation and half maximal displacement [35] the IC_{50} value of 1.4×10^{-5} M given in the present paper closely correlates with the apparent dissociation constant K_d.

As with findings with only one fixed erythromycin concentration [7, 8], binding over all the concentrations used in the present paper increased with increasing temperature. That behaviour seems to be unique among antibiotics, which were generally found to be bound at higher amounts with decreasing temperature [9, 36]. Thermodynamically, erythromycin binding equals an endothermic reaction. The positive entropy change can be explained by conformational changes of the erythromycin binding protein and above all by the suggested hydrophobic nature of erythromycin binding. Compared with this, other interpretations of the positive entropy change are unlikely, e.g. molar increase due to water molecules initially bound to the serum protein but liberated during the binding reaction with erythromycin.

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