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THE BINDING PROTEIN OF ERYTHROMYCIN IN HUMAN SERUM

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Abstract—Erythromycin binding to human serum albumin and to α_1-acid glycoprotein was measured under conditions of binding equilibrium. At therapeutical concentrations of erythromycin the binding to albumin is not saturable. The fraction of total erythromycin bound to α_1-acid glycoprotein is proportionally related to the protein concentration and is bound to a single class of binding sites with an apparent association constant $K_a = 0.16 \times 10^6\ M^{-1}$ (38°). About one mole of erythromycin is bound per mole of α_1-acid glycoprotein. The binding affinity can be enhanced and vice versa lowered by increasing the concentrations of NaCl and urea, respectively. The semilogarithmic plot of bound/free ratios vs log concentration of NaCl or urea exhibits linear relationships. Erythromycin binding can be competitively inhibited by mersalyl (K_i = 11–16 μM) but not by other SH-reagents or by neuraminidase treatment. A marked reduction of erythromycin binding to α_1-acid glycoprotein is seen with dithiothreitol. α_1-acid glycoprotein is the main erythromycin binding protein in human serum.

In a previous study [1] the binding behaviour of erythromycin to human serum could be explained by a saturable, specific binding component which predominates at low erythromycin concentrations but is masked at higher concentrations by a non saturable part. The present paper now describes the distribution of binding sites in serum, the chemical nature and binding characteristics of the saturable binding component, and the kinetics of binding equilibrium.

MATERIALS AND METHODS

Materials. The chemicals and reagents used in this study were purchased as follows: unlabelled erythromycin lactobionate (Abbott Laboratories, N. Chicago, IL), [*N*-methyl-^{14}C]erythromycin lactobionate, sp. act. 1.48 GBq/mmole (Amersham International Ltd., Amersham, U.K.), *N*-acetyl-neuraminic acid, neuraminidase (acylneuraminyl hydrolase, EC 3.2.1.18) from *Clostridium perfringens* (Boehringer Mannheim GmbH, Mannheim, F.R.G.), *N*-ethylmaleiimide, 4-hydroxymercuribenzoate ("pCMB", sodium salt), iodoacetamide, monoiodoacetate (sodium salt) (Fluka AG, Buchs, Switzerland), Folin-Ciocalteus phenol reagent, PPO, POPOP (E. Merck, Darmstadt, F.R.G.), *o*-iodosobenzoic acid, 2-thiobarbituric acid p.a. (Serva Feinbiochemica GmbH & Co., Heidelberg, F.R.G.), *p*-chloromercuriphenylsulfonic acid (sodium salt), mersalyl (Sigma Chemical Co., St. Louis, MO), LC-partigen, α_1-acid glycoprotein, M-partigen (-albumin, α_1-acid glycoprotein, -α_2-macroglobulin, -β-lipoprotein), Tri-partigen (IgG, IgA, IgM) (Behringwerke AG, Marburg, F.R.G.), Sephacryl S 300 superfine, Sephadex G25 fine (Deutsche Pharmacia GmbH, Freiburg, F.R.G.). All other chemicals were of analytical grade from conventional commercial sources.

Pooled serum was freshly prepared from young, healthy volunteers. α_1-acid glycoprotein (human) and protein standard serum LC-A (human) were purchased from Behringwerke AG, Marburg, F.R.G. and human albumin (100%, cryst.) and bovine albumin were from Fluka AG, Buchs, Switzerland and from Serva Feinbiochemica, Heidelberg, F.R.G., respectively.

Methods. Procedures for binding studies (Dianorm® dialysis apparatus, from Diachema AG, Rüschlikon, Switzerland) have been described previously [1]. Briefly, prior to dialysis against ^{14}C-erythromycin (final concn. 3.5×10^{-7} M) α_1-acid glycoprotein (0.5 g/l phosphate buffer, 0.1 M, pH 7.2) was preincubated for 1.5 hr at 38° in the presence and absence of SH-group reagents (final concn. 5.0 mM) or the protein was pretreated at 4° for 72 hr with dithiothreitol at a final 50-fold molar excess. The influence of mersalyl on erythromycin binding was tested at constant and at changed concentrations of mersalyl (2.5×10^{-5} M and 2.5×10^{-6} to 1×10^{-4} M) with ^{14}C-erythromycin in the concentration range 1.5×10^{-6} to 1.2×10^{-5} M and at two constant concentrations, 1.2 and 6.2×10^{-6} M, respectively. In some dialysis experiments NaCl (final concn. 1.0, 2.0, 3.0, 3.75 M) and urea (final concn. 1.0, 2.0, 3.0, 4.0, 6.0 M) were present.

After the dialysis equilibrium was attained, the samples (100 μl aliquots) both from the buffer and protein side were mixed with Bray's solution [2] and counted for radioactivity by liquid scintillation spectrometry.

Human serum was incubated at 37°, pH 5.5 (acetate buffer 0.1 M), for 3 hr with neuraminidase (final concn. 0.25 u/ml). In preliminary tests the liberation

of neuraminic acid was studied as a function of incubation time at the pH values 7.2 (0.1 M phosphate buffer) and 5.5 (0.1 M acetate buffer). Serum was separated from the splitted neuraminic acid by gel filtration (Sephadex G fine, gel bed dimensions: 51 × 0.9 cm column, eluant: phosphate buffered saline, pH 7.2) and was subsequently concentrated by ultrafiltration (Minicon B15, Amicon Corp., Danvers, MA) up to a final protein concentration of 73.0 g/l. Control samples were treated in the same way but without neuraminidase.

Neuraminic acid was assayed according to Aminoff [3] with *N*-acetylneuraminic acid as a standard. The total content of neuraminic acid in serum was determined after incubation of serum aliquots (0.1 ml) with 0.05 M sulphuric acid (9.9 ml) at 80° for 1 hr. For estimation of the distribution of erythromycin binding sites human serum was gel-filtrated (sephacryl 300 fine, eluant: 0.1 M phosphate buffer, pH 7.2, with ^{14}C-erythromycin, final concn. 2.4×10^{-6} M).

Total protein was estimated by the method of Lowry *et al.* [4] with bovine serum albumin as a

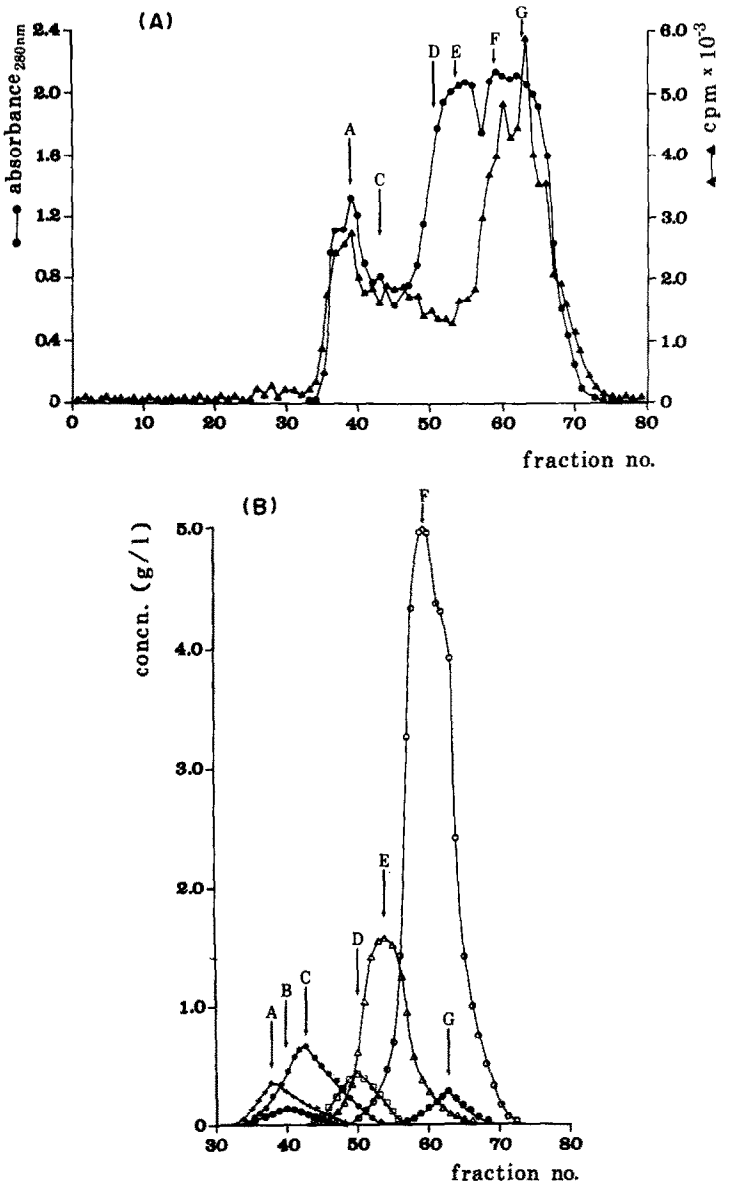

Fig. 1. (A) Distribution of erythromycin binding in human serum. Serum was gel filtrated by a column preequilibrated and eluted with [^{14}C]erythromycin (2.4 μM). Abscissa: number of eluate fraction; ordinates: absorbance at $\lambda = 280$ nm (left) and radioactivity in the fractions (right). ●—● protein, ▲—▲ [^{14}C]erythromycin. (B) Distribution of serum proteins in the eluate fractions. Protein was estimated by radial immunodiffusion. Number of eluate fraction (abscissa) vs protein concentration (see (A)) A, IgM; B, Apolipoprotein B; C, α_2-macroglobulin; D, IgA; E, IgG; F, albumin; G, α_1-acid glycoprotein.

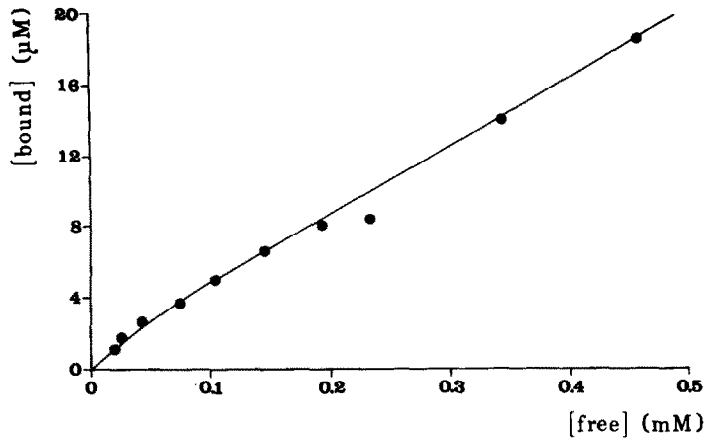

Fig. 2. [^{14}C]erythromycin binding to human albumin as a function of erythromycin concentration. Dialysis of albumin (0.58 mM) at 38° against 0.1 M phosphate buffer, pH 7.2, in the presence of different concentrations of erythromycin. Concentrations of free (abscissa) vs. bound (ordinate) erythromycin.

standard. The concentrations of α_1-acid glycoprotein, albumin, α_2-macroglobulin, β-lipoprotein, IgG, IgA, and IgM were assayed by radial immunodiffusion [5] with partigen plates using a calibration curve obtained with a serum standard. The plates were read after incubation at room temperature for 72 hr.

RESULTS

Distribution of binding sites in serum

Figure 1A shows the separation of the serum proteins and the distribution of radioactivity. The identification and the concentrations of the protein in the respective fractions can be read from Fig. 1B. The radioactivity associated with the serum proteins was minimal in the IgA and IgG fractions and high in the albumin fraction. The highest radioactivity was found in the fraction of α_1-acid glycoprotein.

Binding to albumin

The binding of erythromycin to albumin (5.8×10^{-4} M) was estimated at different antibiotic concentrations. Over the concentration range tested (1.0 to 50.0×10^{-5} M) was estimated at different antibiotic concentrations. Over the concentration range tested (1.0 to 50.0×10^{-5} M) no saturation of binding sites was observed (Fig. 2).

α_1-Acid glycoprotein and binding

Erythromycin binding was studied at a constant concentration of erythromycin (2.5×10^{-6} M) and at various concentrations of α_1-acid glycoprotein. Binding in terms of b/f ratio increased proportionally with increasing concentration (range 0.9 to 19.5×10^{-6} M) of α_1-acid glycoprotein. The Scatchard plot exhibits a negative slope (Fig. 3). The association constant, $K_a = 0.14 \times 10^6\,M^{-1}$, and the

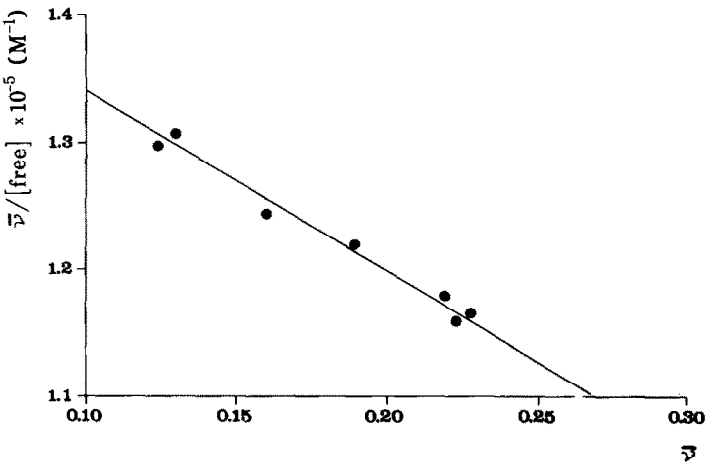

Fig. 3. [^{14}C]erythromycin binding to α_1-acid glycoprotein as a function of α_1-acid glycoprotein concentration. Dialysis of erythromycin (2.5 μM) in 0.1 M phosphate buffer, pH 7.2, against α_1-acid glycoprotein at different concentrations. Scatchard-plot. $\bar{\nu}$ = moles of erythromycin bound per mole of protein (abscissa) vs $\bar{\nu}$ (mole/mole)/f ratio of erythromycin (ordinate).

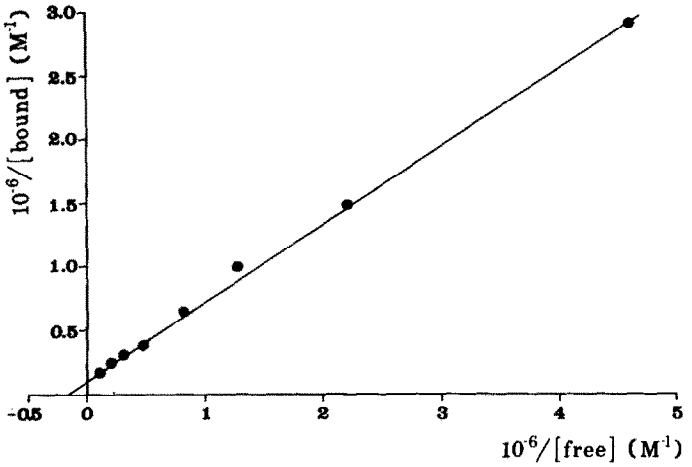

Fig. 4. [^{14}C]erythromycin binding to α_1-acid glycoprotein as a function of erythromycin concentration. Dialysis of α_1-acid glycoprotein (10.6 μM) against 0.1 M phosphate buffer, pH 7.2, in the presence of different concentrations of erythromycin. Lineweaver–Burk plot; reciprocals of concentrations of free (abscissa) and bound (ordinate) erythromycin.

number of binding sites, N = 1.03 moles bound per mole of protein, are both in close agreement with the data obtained when the protein concentration was held constant in the presence of different erythromycin concentrations (see below).

Kinetics of binding

With increasing concentrations of erythromycin (range 5.0×10^{-7} M to 1.4×10^{-5} M, α_1-acid glycoprotein 1.06×10^{-5} M) in contrast to the binding properties shown by albumin a clear cut saturation phenomenon was detected. The data plotted according to Lineweaver and Burk [6] and Scatchard [7] are given in the Figs. 4 and 5. The apparent affinity constants are $K_a = 0.17 \times 10^6\,M^{-1}$ (out from $K_d = 5.8 \times 10^{-6}$ M, Lineweaver–Burk) and $0.16 \times 10^6\,M^{-1}$ (Scatchard). The Scatchard graph shows a straight line (Fig. 5), indicating a single class of binding sites. The number of binding sites per molecule of α_1-acid glycoprotein is 0.96.

NaCl *and binding*

Erythromycin binding was studied in the presence of NaCl (concentration range 0–4.0 M). In accordance with experiments previously done with whole serum (not published) erythromycin binding to α_1-acid glycoprotein was enhanced proportionally with increasing NaCl concentration as shown by the semi-log plot of Fig. 6. In further experiments the nature of this NaCl dependent modulation of erythromycin binding was elucidated. The variation of erythromycin concentration in the presence and absence of NaCl (2.0 M) yielded the pattern of a competitive inhibition, i.e. binding inhibition in the absence of NaCl (Fig. 7). Whereas the concentration of binding sites was nearly unchanged (10.7×10^{-6} vs

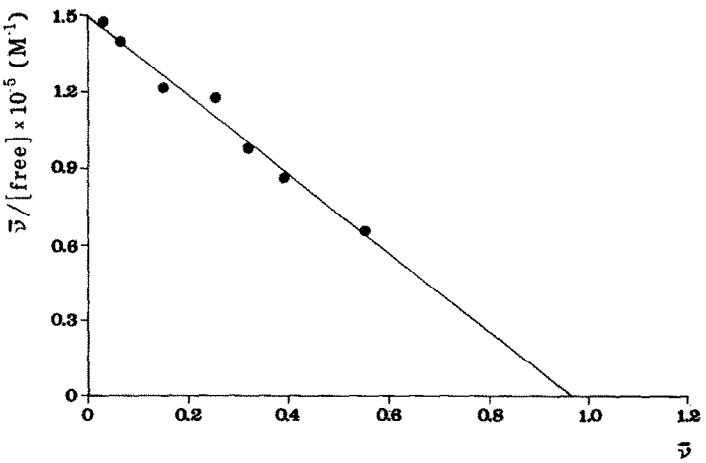

Fig. 5. [^{14}C]erythromycin binding to α_1-acid glycoprotein as a function of erythromycin concentration (vid. Fig. 4). Scatchard plot. $\bar{\nu}$ = moles of erythromycin bound per mole protein (abscissa) vs $\bar{\nu}$ (mole/mole)/f ratio of erythromycin (ordinate).

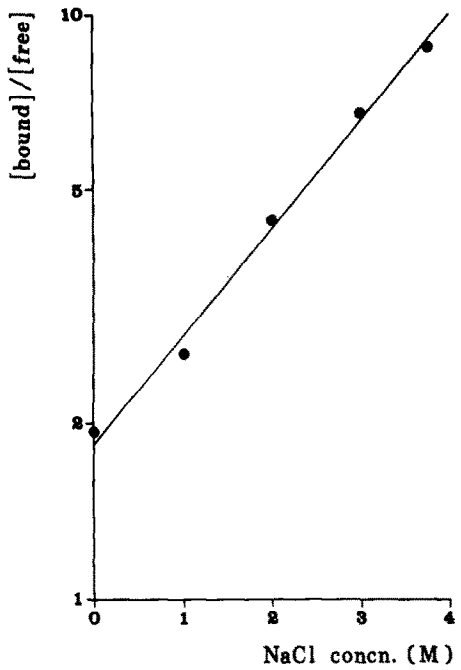

Fig. 6. Effect of NaCl on [^{14}C]erythromycin binding to α_1-acid glycoprotein. Dialysis of α_1-acid glycoprotein at 38° against 0.1 M phosphate buffer, pH 7.2, erythromycin 2.4 μM, in the presence of different NaCl concentrations. Abscissa: NaCl concentrations; ordinate: logarithm of b/f erythromycin concentration ratio.

10.4×10^{-6} M in the presence of NaCl) the apparent affinity constant K_a was increased by NaCl from 0.16 up to 0.48×10^6 M^{-1}.

Urea and binding

In contrast to the results obtained with NaCl, erythromycin binding was reduced in the presence of urea. In the semi-log plot (Fig. 8) the b/f ratio increased proportionally to urea in the concentration range from 0 to 4.0 M. Higher urea concentrations up to 6.0 caused a rapid decline and deviation from the curve of semilogarithmic relationship.

SH-group reagents and binding

Neither oxidizing (e.g. *o*-iodosobenzoic acid) nor alkylating reagents (e.g. iodoacetamide, monoiodoacetate) had any influence on erythromycin binding reaction (Table 1). Some effect was seen with *N*-ethylmaleiimide, which represents a special case of alkylation by addition of a double bond. Out of the mercaptide producers only mersalyl inhibited erythromycin binding markedly. *p*-Chloromercuriphenylsulfonate and 4-hydroxymercuribenzoate ("p-CMB") both had no effect.

The binding inhibition due to mersalyl was further analysed. Mersalyl did not impair the number of erythromycin binding sites but increased the erythromycin concentration necessary for half maximal saturation of the binding sites. From the data analysis as described by Lineweaver and Burk [6] and Dixon [8] follows a competitive type of binding inhibition with apparent K_i-values of 1.1×10^{-5} M (Lineweaver–Burk) and 1.6×10^{-5} M (Dixon), respectively (Figs. 9 and 10).

Dithiothreitol and binding

Erythromycin binding to α_1-acid glycoprotein, which was pretreated with dithiothreitol, was reduced to 56.2% (±9.8, N = 4) of the binding in controls.

Neuraminidase and binding

Human serum was incubated at 37° with neuraminidase (0.25 u/ml) in 0.1 M phosphate buffer, pH 7.2, and in 0.1 M acetate buffer, pH 5.5, respectively. As compared with the amount of neuraminic acid, splitted off by sulphuric acid in the controls (8.06 mg/g protein), about 50% were enzymatically liberated within 50 min at pH 7.2 and more than 90% at pH 5.5. The residual content of neuraminic acid in the latter samples was 0.41 mg/g protein. The concentration of free, unbound neuraminic acid in serum was 9.7 ± 1.1 mg/l ($31.6 \pm 3.7 \times 10^{-6}$ M).

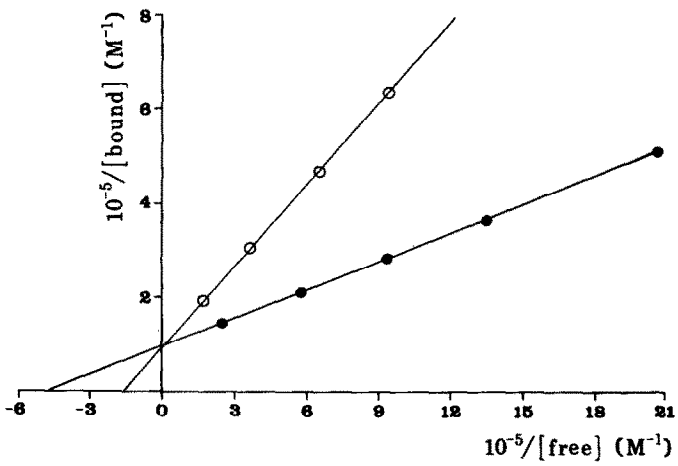

Fig. 7. Effect of NaCl on [^{14}C]erythromycin binding to α_1-acid glycoprotein as a function of erythromycin concentration (see Fig. 6). Lineweaver–Burk plot; O—O control, ●—● in the presence of NaCl (2.0 M).

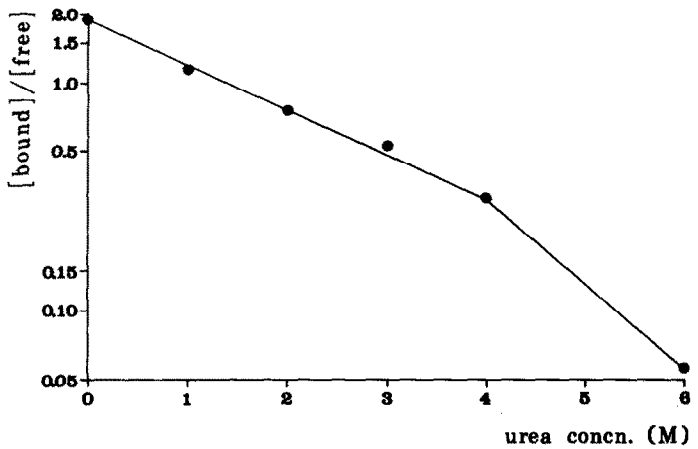

Fig. 8. Effect of urea on [^{14}C]erythromycin binding to α_1-acid glycoprotein. Dialysis of α-$_1$-acid glycoprotein at 38° against 0.1 M phosphate buffer, pH 7.2, erythromycin 2.4 μM, in the presence of different urea concentrations. Abscissa: urea concentrations, ordinate: logarithm of b/f erythromycin concentration ratio.

The removal of neuraminic acid from human serum did not significantly influence the fraction of bound erythromycin, which amounted to 96.6 ± 3.4% of the controls without neuraminidase.

DISCUSSION

The literature concerning the binding sites of erythromycin in human serum is conflicting. Some authors reported binding to the α_1-globulin fraction, to β- and α_2-globulin but not to γ-globulins [9, 10], whereas others have observed binding mainly to albumin with some contribution of α_1-, α_2-, β-, and γ-globulins [11]. In our previous paper [1], we have explained the total erythromycin binding in serum by a saturable binding component which consists of one class of binding sites and acts predominantly at lower erythromycin concentrations, but is masked at higher concentrations by a non-saturable part. In the present study the binding to α_1-acid glycoprotein could be characterized by a saturation phenomenon and a high affinity. The apparent affinity constant, $K_a = 0.16$ to $0.17 \times 10^6\,M^{-1}$, closely resembles the respective value ($K_a = 0.16 \times 10^6\,M^{-1}$ from $K_d = 5.9 \times 10^{-6}\,M$) of the high affinity part of binding estimated previously in whole serum [1]. Approximately one mole of erythromycin was bound per mole of α_1-acid glycoprotein.

Table 1. Effects of sulfhydrylgroup reagents on erythromycin binding to α_1-acid glycoprotein

Inhibitor (5.0 mM)	Residual binding (%)*
p-Chloromercuriphenyl sulfonic acid	81.7 ± 2.6
N-ethylmaleiimide	71.5 ± 3.1
4-Hydroxymercuribenzoate ("p-CMB")	90.7 ± 5.2
Iodoacetamide	102.0 ± 5.5
o-Iodosobenzoic acid	100.7 ± 9.4
Mersalyl	3.3 ± 0.3
Monoiodoacetate	92.9 ± 4.4

* ± S.D. (N = 5).

In contrast to α_1-acid glycoprotein, the binding to albumin was not saturable over the tested concentration range. Drug binding to albumin is usually characterized by more than one class of binding sites, of which at least one exhibits an affinity constant K_a in the range of 10^4 to $10^8\,M^{-1}$ [12]. The even lower erythromycin binding affinity is in accordance with the suggested absence of specific binding sites and generally it agrees with the well-known weak binding of positively charged ligands to albumin. In whole serum the extent of total erythromycin binding was linearly related to the logarithm of serum dilution [1]. The Scatchard plot yields a positive slope, which indicates a rise in binding affinity and/or an augmentation of binding capacity in parallel with decreasing protein concentration. But the respective plot of α_1-acid glycoprotein binding data is characterized by a negative slope. Both the binding affinity of the α_1-acid glycoprotein and the number of binding sites are independent from the protein concentration. In total serum, however, the semilogarithmic binding function describes a superposition of the directly proportional part (i.e. α_1-acid glycoprotein) and a second one, probably mainly albumin, which fits well a logarithmic relationship.

Neither oxidizing nor alkylating SH-reagents affected erythromycin binding to α_1-acid glycoprotein. The action of *N*-ethylmaleiimide may be explained by reaction with amino groups [13], e.g. the ε-amino groups of the lysinyl residues present in the α_1-acid glycoprotein molecule which form *N*-ε-succino-2-yl-lysinyl residues. Likewise, the marked competitive inhibition due to mersalyl is not caused by reaction with SH-groups. The unexpected competitive type of inhibition is a phenomenon often observed with mercurial inhibitors [14]. Dithiothreitol (Cleland's reagent) cleaves the disulfid bonds which are present between the positions 5 and 147 and 72 and 164 of the aminoacid backbone of the α_1-acid glycoprotein [15]. Conformational changes of

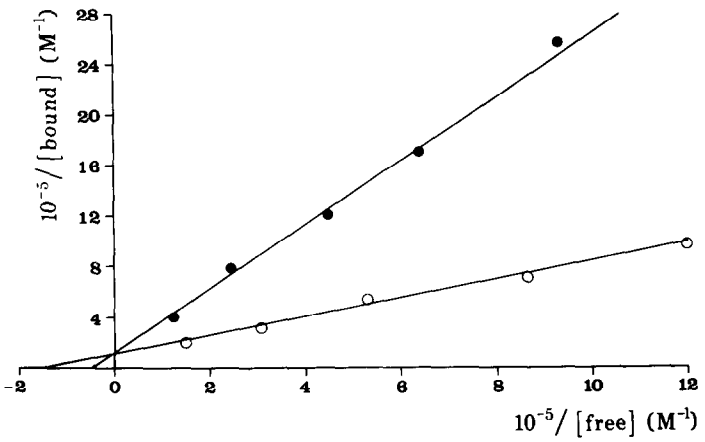

Fig. 9. Effect of mersalyl on erythromycin binding to α_1-acid glycoprotein as a function of erythromycin concentration. Lineweaver–Burk plot; ○—○ control, ●—● binding in the presence of mersalyl (25 μM). K_i = 11 μM.

the protein—probably thereby induced—result in the observed distinct decrease of erythromycin binding. The semilogarithmic relationship found between erythromycin binding and NaCl concentration is not caused by an increase (e.g. demasking) of binding sites, but is related to an enhancement of the binding affinity. This effect can be explained by stabilization of the conformation of the α_1-acid glycoprotein [16]. Conversely, the binding of erythromycin was decreased by urea, which produces an unfolding of the β-helix of α_1-acid glycoprotein [17]. At this place it should be mentioned that erythromycin binding has many features in common with the binding of Δ^4-3-ketosteroids [16, 18] but the binding sites are different. The residual binding of erythromycin in the presence of progesterone was 98.6% (±5.6, N = 6) of the controls (Dette *et al.*, not publ.).

The stability of erythromycin binding in the presence of a high ionic strength (i.e. NaCl) as well as the negligible alteration caused by the removal of extremely acid groups (i.e. neuraminic acid) both confirm the concept of the predominating hydrophobic nature of the binding reaction [1]. The retained binding affinity of desialylated binding proteins is no unique feature. For instance, the high affinity binding of serotonin to nerve ending membranes is not affected by neuraminidase treatment [19] and the binding of the α-blocking agent nicergoline, like erythromycin, to α_1-acid glycoprotein is unaltered by desialylation [20]. Desialylated α_1-acid glycoprotein exhibited a modest decrease in the affinity constant for propranolol, whereas the binding capacity did not change [21]. These examples may show that neuraminic acid, although it contributes strong ionic charges, is probably not substantially involved in the structural requirements of the specific binding sites. Enzymatic hydrolysis of neuraminic acid residues from the five carbohydrate units, which

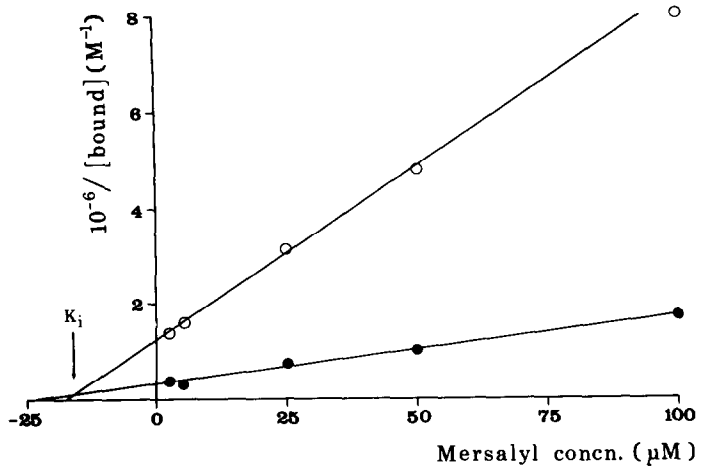

Fig. 10. Effect of mersalyl on erythromycin binding to α_1-acid glycoprotein. Dixon-plot; concentrations of mersalyl (abscissa) vs reciprocals of concentrations of bound erythromycin (ordinate) at total concentrations of 1.2, ●—●, and 6.2 μM, ○—○.

are *N*-glycosidically linked to the aminoacid chain of α_1-acid glycoprotein, almost does not change the conformation of the protein [17, 22].

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