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SERUM ALBUMIN AND LIPOPROTEINS AS THE QUINIDINE BINDING MOLECULES IN NORMAL HUMAN SERA

ODD G. NILSEN
Institute of Pharmacology, University of Oslo, Norway

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Abstract To substantiate the binding of quinidine in human sera and predict variations of binding, dissociation constants and number of binding sites were determined for separate serum proteins. Human sera were fractionated by gel filtration and ultracentrifugation, and binding was evaluated by equilibrium dialysis at pH 7·30 at 20° and 37° in a Krebs–Ringer phosphate buffer. Quinidine was bound to all serum lipoproteins and to serum albumin. The binding was influenced by the buffer composition. In sodium phosphate buffer there were two separate binding sites for quinidine on LDL and HDL, while there was only one detectable binding site on VLDL and HDL in a Krebs–Ringer phosphate buffer. On LDL also there appeared to be one binding site but it exhibited a positive cooperative binding effect at lower concentrations of quinidine. This effect was assumed to be caused by inorganic ions of the Krebs–Ringer phosphate buffer. At a therapeutic level of quinidine in normal human serum the concentration of quinidine bound to serum proteins was $1{\cdot}062 \times 10^{-5}$ M. Calculated from the evaluated binding parameters VLDL contributed with $0{\cdot}101 \times 10^{-5}$ M of this binding, LDL with $0{\cdot}143 \times 10^{-5}$ M, HDL with $0{\cdot}083 \times 10^{-5}$ M and albumin with $0{\cdot}699 \times 10^{-5}$ M.

When evaluating the interaction between ligand and macromolecules the results can be influenced by the method, pH, electrolytes, protein conformation and concentration. To state that experimental binding with isolated protein preparations has direct relevance for native serum, the total binding produced by the separate protein fractions should be compared with the binding obtained with whole serum.

Serum protein binding of the antiarrhythmic agent quinidine exhibits great interindividual variability [1, 2]. The variability of binding seems not to be related to the concentration of serum albumin present. The causes of the variable binding are unknown. To approach this problem, the binding of quinidine to some of the different serum proteins was investigated qualitatively. From these observations serum albumin [3, 4] and high and low density serum lipoproteins [4] appeared to be responsible for the greater part, but not the total of quinidine bound in human serum, when estimated in a sodium phosphate buffer. Earlier observations indicated that very low density lipoproteins may also be important quinidine binding molecules in human serum [4] as well as in rat serum [5]. However, the binding parameters of the quinidine–albumin complex differ depending on the buffer and the albumin preparation used [6].

The aim of this investigation was to quantitate the binding of quinidine to human serum albumin, chylomicrons, very low, low and high density lipoproteins in a more physiological buffer, the Krebs Ringer phosphate buffer, to substantiate that these serum proteins actually explain the total binding of quinidine in serum at therapeutic concentrations, and that the choice of buffer may also influence the binding of quinidine to lipoproteins.

MATERIALS AND METHODS

Serum. Serum was obtained from fasting healthy males, 27–35 yr of age. Blood was allowed to clot at room temperature (20°) for 2 hr. Serum was obtained by centrifugation at 1100 *g* for 30 min at room temperature. Three different sera were pooled.

Chemicals. Quinidine hydrochloride of a purity of 96·7% was supplied by the Norwegian Drug Monopoly, Oslo [5]. ^{3}H-labelled quinidine (sp. act. about 500 mCi/m-mole) was supplied by Buchler & Co., Braunschweig, W. Germany. The tritiated quinidine had identical R_F values with the main spot of unlabelled quinidine when chromatographed in three separate solvent systems.

Equilibrium dialysis. Equilibrium dialysis was performed in Perspex® cells with two chambers separated by semipermeable membranes (Visking dialysis membranes 20/32). The serum or protein solution (500 μl) and the drug were added to one chamber and the buffer (500 μl) to the other. Serum was dialyzed against Krebs–Ringer bicarbonate buffer, pH 7·30 in an atmosphere of 5% (v/v) carbon dioxide in air, and the protein fractions against Krebs Ringer phosphate buffer, pH 7·30. The dialysis was usually run for 18 hr at 20° under standardized rotary shaking, although equilibrium was achieved within 6 hr and remained unaltered during the next 12 hr for all fractions involved; the lipoproteins, albumin and serum. The pH and protein concentration were determined before and after dialysis. The concentrations of quinidine were measured in both compartments after dialysis was completed. The protein concentration measured in serum after equilibrium dialysis was equal to that in the original serum, because the gassing of serum

with carbon dioxide prior to the equilibrium dialysis caused a slight evaporation of water which was balanced by the dilution during equilibrium dialysis.

Protein binding calculation. To make a direct comparison of the binding of quinidine to different proteins, the extent of binding should be related to the same concentration of unbound drug [5]. This could not be achieved in the dialysis cells because of the relatively small volume of buffer used. Therefore, the quinidine binding was expressed as $[B]/[F]$ and $[B]\,[F] \times [P]$; $[B]$, $[F]$ and $[P]$ being the molar concentration of bound drug, unbound drug and protein, respectively. Number of binding sites and dissociation constants for the drug–protein complexes were obtained by plotting the data according to Scatchard [7] as described earlier [6]. The concentration of quinidine used was in the range $1{\cdot}25 \times 10^{-6}$ to $4{\cdot}0 \times 10^{-4}$ M.

The protein binding of quinidine in a solution of protein molecules may be evaluated on the basis of the actual free concentration $[F]$ of quinidine in two ways. One possibility is to use the number of binding sites (n) on the protein molecule and the dissociation constant (K) in the following equation [8],

$$[B] = \frac{n \times [P] \times [F]}{K + [F]}$$

giving the molar concentration of quinidine bound. The molar concentration of quinidine bound may also be evaluated directly from the binding curve. The actual concentration of free quinidine may be presented as a straight line through the origin in the Scatchard plot with the free concentration given as the slope (value of abscissa/value of ordinate) of the line [9]. The value on the abscissa corresponding to the intercept of the binding curve and the straight line representing the fixed concentration of free quinidine gives the number of quinidine molecules bound per protein molecule. The molar concentration of quinidine bound in the solution was obtained by multiplying by the molar concentration of protein.

Protein determination. Protein content was determined by the method described by Lowry *et al.* [10], using bovine serum albumin as a standard. The concentration of lipoproteins was calculated on the assumption that the protein part of the lipoprotein (apoprotein) occupies 4·3% (w/w) of the chylomicron, 8·3% (w/w) of the very low density lipoprotein, 22·7% (w/w) of the low density lipoprotein and 58·1% (w/w) of the high density lipoprotein molecules [11].

Quinidine determination. The activity of the ^{3}H-isotope was determined by liquid scintillation in a Packard Tri-Carb spectrometer model 3330 operated at 5 °, 50 µl buffer or protein solution was added to 10 ml scintillation liquid as described earlier [5]. The counting efficiency was 28·5 per cent and was the same in the buffer and in the protein solution.

Isolation of chylomicrons. Serum was centrifuged at 12,200 rev/min (9800 g) and 4 ° for 30 min in a L2-65 Beckman ultracentrifuge using a Ti-50 rotor. The floating chylomicrons were aspirated and layered under an equal volume of 0·15 M sodium chloride and recentrifuged. The floating chylomicrons were then removed and emulsified in 0·15 M sodium chloride and recentrifuged. The last washing procedure was carried out until the infranatant was free from turbidity.

Isolation of very low density lipoproteins (VLDL). After the removal of chylomicrons, the serum was centrifuged at 40,000 rev/min (105,000 g) and 4 ° for 18 hr using a Ti-50 rotor. The VLDL present in the supernatant were removed and recentrifuged (105,000 g) at a density of 1·006 g/ml for 18 hr, to remove the remaining LDL. The VLDL fraction was then layered under an equal volume of 0·15 M sodium chloride and centrifuged at 9800 g in the Ti-50 rotor for 30 min; remaining chylomicrons floated and were excluded.

Isolation of low density lipoproteins (LDL). Solid potassium bromide (18·39 mg/ml serum) was added to the serum from which chylomicrons and VLDL had been removed, to achieve a density of 1·019 g/ml. This solution was centrifuged for 18 hr as described earlier. The floating proteins, consisting mainly of VLDL, were excluded. 64·37 mg potassium bromide per ml was added to the remaining infranatant to increase the density to 1·063 g/ml. The solution was centrifuged at 40,000 rev/min and 4 ° for 22 hr using the same equipment as before and the floating LDL were removed.

Isolation of high density lipoproteins (HDL). The serum remaining after removal of the LDL was adjusted to a density of 1·195 g/ml by adding 211·14 mg potassium bromide per ml. This solution was centrifuged at 40,000 rev/min and 4 ° for 45 hr using the Ti-50 rotor, and the floating HDL fraction was removed. After centrifugation the individual lipoprotein fractions (about 20 ml) were dialyzed against three changes of 1000 ml Krebs Ringer phosphate buffer pH 7·30 at 4 ° for 24 hr.

Isolation of serum albumin. The remaining serum protein solution (20 ml) with density higher than 1·195 g/ml was dialyzed against three changes of 1000 ml Krebs Ringer phosphate buffer pH 7·30, and applied on a Sephadex G-200 column (100 × 5 cm, A.B. Pharmacia, Uppsala, Sweden), and the serum albumin was eluted at 4 ° with Krebs Ringer phosphate buffer, pH 7·30. The albumin preparation was obtained from the last eluted protein peak as described earlier [6], where the initial part of the protein peak was discarded to obtain an albumin preparation relatively free from other proteins. The purity of albumin was determined by polyacrylamide gel electrophoresis. Polymers of albumin were not assignable, but traces of pre-albumin and transferrin were observed [5].

Solubility experiment. Three ml Krebs Ringer phosphate buffer pH 7·30, containing ^{3}H-labelled and unlabelled quinidine in concentrations ranging from $1{\cdot}25 \times 10^{-6}$ to 4×10^{-4} M, was added to an equal volume of chloroform in stoppered glass tubes. After mechanical shaking for 30 min at 20 °, the two phases were allowed to separate for 60 min. 50 µl of each phase was taken for liquid scintillation counting. The chloroform phase was evaporated prior to addition of scintillation liquid (10 ml) to avoid quenching.

Buffers. Krebs Ringer phosphate buffer [12] pH 7·30 or Krebs Ringer bicarbonate buffer [12] pH 7·30 was used as noted for the separate experiments. When Krebs–Ringer bicarbonate buffer was used, dialysis was performed in air with 5% (v/v) carbon dioxide.

Table 1. Binding of quinidine* to isolated human serum lipoproteins, chylomicrons and albumin

Protein	Protein and lipoprotein concentration (g/l)	Concentration of free quinidine ($M \times 10^{-5}$)	Concentration of bound quinidine ($M \times 10^{-5}$)	Binding ratio† ($[B]/[F]$)
Chylomicrons	3·0	0·96	0·04	0·041 (0·032 0·060)
VLDL	5·2	0·62	0·73	1·177 (1·158 1·201)
LDL	9·9	0·67	0·64	0·955 (0·947 0·960)
HDL	5·9	0·83	0·34	0·410 (0·397 0·432)
Albumin	22·3	0·72	0·55	0·764 (0·761 0·768)

* Initial concentration of 1×10^{-5} M.
† Mean values and range of 3 experiments.

RESULTS

Binding of quinidine to chylomicrons, lipoproteins and albumin. Serum lipoproteins, chylomicrons and albumin were isolated as described in Methods. These protein preparations were used to estimate the binding of quinidine by adding 1×10^{-8} moles to the dialysis cells, giving an initial concentration of 1×10^{-5} M. All proteins were dialyzed against a Krebs-Ringer phosphate buffer pH 7·30 prior to the equilibrium dialysis which was performed with the same buffer. The concentration of lipoproteins used was approximately twice that found in normal human sera [13] and albumin was present in a concentration half that in serum. As can be seen from Table 1, the extent of binding to lipoproteins was greater than to albumin when lipoprotein and albumin concentrations were taken into account. The chylomicrons did not seem to be of any importance as quinidine binding molecules.

The binding of quinidine to lipoproteins. The interaction of quinidine with the three classes of human serum lipoproteins, VLDL, LDL and HDL was determined by equilibrium dialysis in a Krebs Ringer phosphate buffer pH 7·30 with concentrations of quinidine ranging from $1{\cdot}25 \times 10^{-6}$ to $4{\cdot}0 \times 10^{-4}$ M and the results are shown in Figs. 1, 2 and 3, plotted according to Scatchard [7]. Assuming one group of binding sites, the dissociation constants and number of binding sites per molecule were calculated from these plots (Table 2). All the lipoproteins appeared to have one group of binding sites for quinidine in the concentration range investigated. The average molecular weights of VLDL, LDL and HDL were estimated to be $5{\cdot}2 \times 10^6$, $2{\cdot}3 \times 10^6$ and $2{\cdot}5 \times 10^5$, respectively [11]. The binding site of LDL exhibited a positive cooperative binding effect at lower concentrations of quinidine and the binding parameters given for quinidine in Table 2 are valid only when the concentration of free quinidine is more than $2{\cdot}5 \times 10^{-5}$ M as can be seen from Fig. 2. To evaluate whether a physical distribution of quinidine to the lipid phase of the lipoproteins produced a similar curve as those observed, the distribution of quinidine

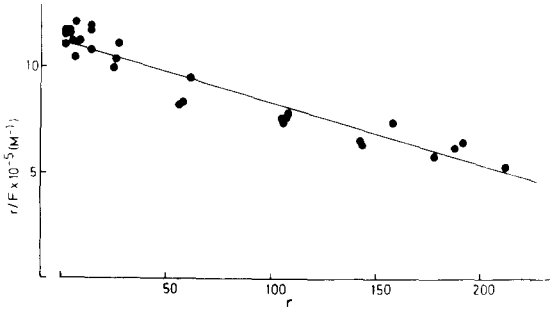

Fig. 1. Scatchard plot showing the binding of quinidine to human serum very low density lipoproteins (VLDL) at 20° in a Krebs-Ringer phosphate buffer pH 7·30, r being moles of quinidine bound per mole of lipoprotein and F the molar concentrations of unbound quinidine. Each point represents the mean value of duplicate experiments. The continuous line represents a computer plot drawn as a least square regression line with equal weight on each point. The concentration of VLDL after equilibrium dialysis was in the range (9·89–9·99) $\times 10^{-7}$ M.

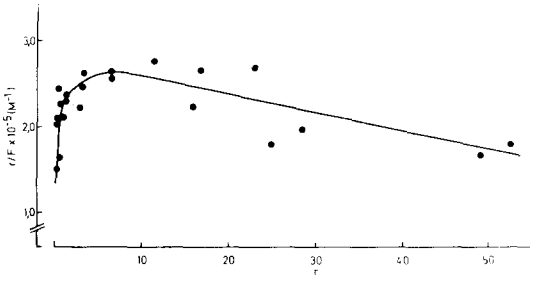

Fig. 2. Scatchard plot showing the binding of quinidine to human serum low density lipoproteins (LDL). Each point represents the mean value of duplicate experiments. Conditions and designations are the same as those in Fig. 1. The concentration of LDL after equilibrium dialysis was in the range (4·29–4·60) $\times 10^{-6}$ M.

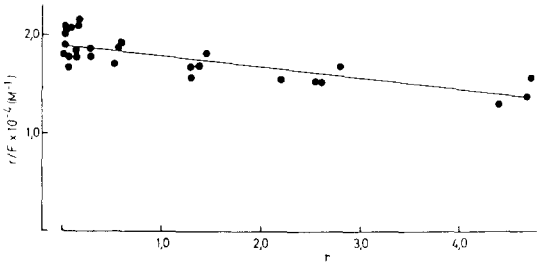

Fig. 3. Scatchard plot showing the binding of quinidine to human serum high density lipoproteins (HDL). Each point represents the mean value of duplicate experiments. Conditions and designations are the same as in Fig. 1. The concentration of HDL after equilibrium dialysis was in the range (2·34–2·51) $\times 10^{-5}$ M.

Table 2. Binding characteristics of quinidine with human serum lipoproteins and albumin*

Protein	Number of binding sites per molecule	Dissociation constant (M)
VLDL (4)	399·3 ± 27·9	(3·55 ± 0·2) × 10^{-4}
LDL (3)	128·5 ± 31·5	(4·55 ± 1·1) × 10^{-4}
HDL (4)	18·5 ± 3·6	(9·75 ± 1·9) × 10^{-4}
Albumin (3)†		
First binding site	0·011 ± 0·001	(6·94 ± 0·9) × 10^{-6}
Second binding site	1·29 ± 0·12	(6·80 ± 0·6) × 10^{-4}

* Mean values ± S.E.M. The number in parentheses is the number of separate experiments.

† The experimental results for albumin are taken from a previous investigation [6].

between chloroform and Krebs Ringer phosphate buffer pH 7·30 was examined. The result indicated a proportional distribution of quinidine between the two phases in this concentration range.

Theoretical calculation. The concentration of bound quinidine in pooled normal human sera identical to those from which the protein fractions were separated, was determined to be $1{\cdot}062 \times 10^{-5}$ M at a total quinidine concentration in serum close to the accepted therapeutic range [15] ($1{\cdot}43 \times 10^{-5}$ M ≈ 5 μg per ml). On the basis of the actual free concentration of quinidine in this serum pool ($3{\cdot}66 \times 10^{-6}$ M ≈ 1·25 μg per ml) the binding of quinidine to the separate proteins were calculated from the evaluated binding parameters for VLDL, HDL and albumin while the binding of quinidine to LDL was evaluated directly from the binding curve for LDL as described in Methods. The binding of quinidine to the pooled sera was determined experimentally in a Krebs Ringer bicarbonate buffer pH 7·30. The experimental and calculated data are presented in Table 3.

Influence of temperature. The effect of temperature on the binding of quinidine to human serum, lipoproteins and albumin was investigated at 20° and 37°. The equilibrium dialysis was run for 9 hr only, because the lipoprotein fraction exhibited a marked decrease in pH from 7·30 to 7·00 when the dialysis was continued for 18 hr at 37°. A slight fall in pH from 7·30 to 7·25 was still observed after only 9 hr of dialysis. The pH of serum and albumin remained constant during the equilibrium dialysis at both temperatures. Table 4 demonstrates that an increase in temperature decreased the binding ratio of quinidine to all protein fractions. All fractions, except the lipoproteins, demonstrated also a decreased binding ratio with increased concentration of quinidine in accordance with the law of mass action.

DISCUSSION

It has been shown previously that human high and low density lipoproteins bind quinidine extensively [4]. The present experiments establish that serum

Table 3. Theoretical calculations of the binding of quinidine to lipoproteins and albumin from normal human sera

Protein	Protein concentration in serum* (M)	Unbound quinidine (M)	Bound quinidine Experimental (M)	Bound quinidine Calculated (M)
VLDL	$2{\cdot}48 \times 10^{-7}$	$3{\cdot}66 \times 10^{-6}$		$0{\cdot}101 \times 10^{-5}$
LDL	$1{\cdot}91 \times 10^{-6}$	$3{\cdot}66 \times 10^{-6}$		$0{\cdot}143 \times 10^{-5}$
HDL	$1{\cdot}20 \times 10^{-5}$	$3{\cdot}66 \times 10^{-6}$		$0{\cdot}083 \times 10^{-5}$
Albumin	$6{\cdot}52 \times 10^{-4}$	$3{\cdot}66 \times 10^{-6}$		
First binding site				$0{\cdot}248 \times 10^{-5}$
Second binding site				$0{\cdot}451 \times 10^{-5}$
All protein fractions				$1{\cdot}026 \times 10^{-5}$
Serum		$3{\cdot}66 \times 10^{-6}$	$1{\cdot}062 \times 10^{-5}$	

* Mean values of the serum protein concentrations in healthy males [13].

Table 4. The influence of temperature on the protein binding of quinidine*

Protein	Concentration of protein‡ (g l)	Concentration of quinidine $1{\cdot}25 \times 10^{-6}$ M 20	$1{\cdot}25 \times 10^{-6}$ M 37	$1{\cdot}00 \times 10^{-5}$ M 20	$1{\cdot}00 \times 10^{-5}$ M 37	$4{\cdot}00 \times 10^{-4}$ M 20	$4{\cdot}00 \times 10^{-4}$ M 37
Serum	58·2	4·03(3·98 4·10)	3·37(3·35 3·39)	2·30(2·28 2·33)	2·12(2·10 2·15)	0·97(0·95 0·98)	0·91(0·89 0·92)
Lipoproteins†	4·5	0·92(0·90 0·93)	0·82(0·81 0·83)	1·01(1·00 1·02)	0·85(0·83 0·86)	0·89(0·85 0·91)	0·74(0·73 0·75)
Albumin	22·1	0·98(0·96 0·99)	0·86(0·84 0·87)	0·83(0·82 0·85)	0·75(0·74 0·76)	0·50(0·48 0·51)	0·46(0·45 0·48)

* The binding to albumin and lipoproteins was performed in Krebs Ringer phosphate buffer pH 7·30, while the binding to serum was performed in Krebs Ringer bicarbonate buffer of the same pH. Binding is presented as the ratio [B]/[F]. Results are given as mean values and range of three experiments.

† The lipoprotein fraction consist of lipoproteins with density < 1·195 g/ml.

‡ The lipoprotein concentration is related to the concentration of apoprotein.

very low density lipoproteins also participate in the binding of quinidine, while the chylomicrons are of less importance for the total binding. The albumin together with the serum lipoproteins are responsible for the quinidine that is bound in human serum.

The binding parameters obtained with Krebs Ringer phosphate buffer should be considered to be more adequate when human serum is used as this buffer has an ion composition close to that of serum. Krebs Ringer phosphate and bicarbonate buffers of the same pH produced identical binding of quinidine when measured by equilibrium dialysis. The present experiments with different serum proteins were performed with Krebs Ringer phosphate buffer pH 7·30, while the binding parameters for high and low density lipoproteins were formerly [4] obtained with a 0·15 M sodium phosphate buffer at the same pH. The binding parameters for the lipoproteins determined in these two investigations, were similar with respect to the low affinity binding sites, but the high affinity sites for quinidine were not detectable when Krebs Ringer phosphate buffer was used. This observation may be explained by an association between buffer ions, possibly the chloride ions [6], present in the Krebs Ringer phosphate buffer and the lipoproteins, blocking binding sites for quinidine or producing an allosteric effect on the macromolecules. A similar modification of the binding parameters produced by inorganic anions and buffer was observed for the interaction between quinidine and albumin [6]. Furthermore the binding of acetyl-L-tryptophan and skatol with albumin was also reported to be influenced by halide or halide-like ions, the binding of the former being inhibited and the latter favoured [16]. The ions mainly affected the primary binding site for skatol.

The binding parameters for the lipoproteins were calculated on the assumption of identical multiple binding sites for molecules within each group, and a possible difference in binding of subgroups [17–19] has not been examined.

Low density lipoproteins exhibited an unexpected pattern, exhibiting increased binding with rising quinidine concentrations in the lower concentration range. A similar effect was reported [14] for the interaction between tetracycline and serum lipoproteins. However, this interaction was assumed to be a result of a partition of tetracycline into the lipophilic portion of the lipoprotein molecule, rather than an association with binding sites. This is probably not valid for the quinidine low density lipoprotein interaction, because the distribution ratio for quinidine was constant in the chloroform buffer system in contrast to that observed for tetracycline. The present observation for quinidine could more likely be explained by a cooperative effect on the low density lipoprotein produced by quinidine in Krebs Ringer phosphate buffer or by the buffer alone. A similar cooperativity is known for the binding of succinate to the native enzyme aspartic transcarbamylase (ATCase) [20]. It is also reported [21] that if the progesterone–AAG (α-acid glycoprotein) complexes are exposed to increasing concentrations of sodium chloride, the association constant rises proportionally to the salt concentration. The interaction of progesteron–AAG is anticipated to be favoured energetically by sodium chloride which produces a more ordered conformation of the protein [22]. With this background and the fact that this cooperativity was not observed with the low density lipoproteins in sodium phosphate buffer [4], it can be assumed that the additional ions in a Krebs–Ringer phosphate buffer, possibly mainly the chloride ions, may produce similar conformational alterations of the low density lipoproteins making the binding of quinidine cooperative at low concentrations. Figure 2 demonstrates that the therapeutic level of quinidine in serum is within the range where cooperativity is present.

An increase in temperature resulted in a fall in protein binding of quinidine to serum, albumin and lipoproteins. Our results were obtained at 20° and differ consequently from the ideal conditions at 37°. However, the effect of temperature on the binding of quinidine to the isolated albumin and lipoprotein fractions seemed to be about equal to the effect observed in serum. For this reason a comparison of quinidine binding to isolated protein fractions and serum at 20° seems to be adequate even though the *in vivo* temperature is 37°.

An interesting observation in Table 4 is that while serum and albumin obey the law of mass action, the lipoprotein fraction ($d < 1{\cdot}195$ g/ml) does not. They actually demonstrated an increased binding when the concentration of quinidine was raised from $1{\cdot}25 \times 10^{-6}$ M to $1{\cdot}00 \times 10^{-5}$ M. This is in accordance with the observation made on the isolated low density lipoproteins. Being the most important quinidine binding molecules among the lipoproteins, they also bind quinidine with a cooperative effect at low concentrations of quinidine. This cooperative binding effect seems consequently to be dominant in the total lipoprotein fraction while the albumin, following the law of mass action, probably blurs this effect of the lipoproteins in serum.

The albumin molecule possessed less than one primary binding site per molecule. This may be due to endogenous substances such as free fatty acids or bilirubin tightly bound to the albumin molecule partially blocking the primary binding site. The ability of these substances to interact with the binding of several drugs to albumin [23–28] is well known.

The calculated values for the binding of quinidine in serum are based on the assumption that the binding parameters obtained for the isolated proteins are valid for the different proteins when present in serum [4]. The lipoproteins contribute with 30·8 per cent and albumin with 65·8 per cent of the total protein binding in a normal human serum. This indicates that only 3·4 per cent of the quinidine in serum is bound to other serum proteins. The binding to lipoproteins may contribute even more to the total binding in common types of hyperlipoproteininemia where the concentration of VLDL and LDL are considerably increased. Even the normal values of plasma lipids and lipoproteins are known to vary depending on age, sex, environment, food and race [29]. When the total binding of quinidine to human serum proteins is to be evaluated, the plasma lipids and lipoproteins must be taken into consideration in addition to albumin. In general the binding of other compounds to lipoproteins should be considered when serum protein binding is investigated.

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