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The binding of gold to human albumin *in vitro*. Intrinsic association constants at physiological conditions

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Although gold in the form of thio-complexes has been successfully used in the treatment of rheumatoid arthritis for many years, important aspects of their pharmacology and the mode of action of these compounds are still unknown.

The first real quantitative information concerning the binding of gold to plasma proteins *in vitro* were given by Mason [1]. He used ultrafiltration and found that sodium aurothiomalate was bound to human albumin at a single site with a fairly high affinity constant and also at several sites of lower affinity.

In the present study the binding of sodium aurothiosulphate—another gold compound widely used in the treatment of rheumatoid arthritis—to human albumin was investigated at physiological conditions with regard to albumin concentration, pH, temperature and ionic strength. The purpose of determining the binding constants of aurothiosulphate to human albumin was; to compare the degree of binding of this gold compound with that of aurothiomalate; to get more information concerning the binding mechanism of gold to albumin; and to attempt to get a more valuable parameter of monitoring the patients during chrysotherapy. This parameter may well be the nonprotein-bound gold concentration, since it is conceivable, that this concentration correlates with therapeutic as well as toxic effects. In order to calculate the 'free' gold concentration, reliable values for the binding constants are needed.

Aurothiosulphate was found to be strongly bound to the dialysis membrane used. The following experiments were, therefore, performed using an equilibrium dialysis system with the great advantages that partial absorption to the membrane does not influence the results [2].

Materials and methods

Materials. The albumin preparation used was purified, lyophilised human albumin (Behringwerke AG, Marburg, West Germany). The albumin preparation fulfilled the criteria for purity specified in [3]. Crossed-immunoelectrophoresis [4] performed against rabbit antihuman serum (DAKO, Copenhagen, Denmark) showed that no peaks attributable to other proteins than albumin were detectable.

Polyacrylamide gradient gel (PAA 4/30, Pharmacia, Uppsala, Sweden) electrophoresis of a 0.5% albumin solution showed only one distinct band of monomer albumin and one very faint band due to dimer albumin. The sodium aurothiosulphate (Sanocrysin®) was purchased from Ferrosan, Søborg, Denmark. The Visking seamless Cellophane tubing (8/32 inch. Union Carbide Corporation, Chicago, IL) used for dialysis was washed and prepared as described by Pedersen [5]. All initial solutions of albumin, aurothiosulphate (and blanks) are unbuffered solutions prepared in distilled, sterile water containing 0.15 M NaCl; pH was adjusted by addition of 1 M and 0.1 M NaOH, respectively.

Equilibrium reached by association. The binding of aurothiosulphate to human albumin in unbuffered solution at 37°, pH 7.4 and ionic strength 0.15–0.16 M was studied in an equilibrium dialysis system containing initially 1 ml albumin solution of aurothiosulphate outside the membrane using a range of concentrations of total aurothiosulphate from 40 to 2042 μM.

Control tubes, with aurothiosulphate solution on the outside of the dialysis membrane and 0.15 M NaCl on the inside were set up at two different aurothiosulphate concentrations in duplicate to establish that equilibrium was reached at the end of the experiment. After equilibrium was reached, at 37° within 2 days, pH, the concentration of albumin and sodium were measured inside the dialysis membrane and the concentration of gold was measured on each side of the membrane.

Spectrophotometric and electroimmunoassay determination of albumin showed that the albumin solution was stable at 37° in at least 72 hr. No bacterial growth was observed in any of the solutions after dialysis.

Equilibrium reached by dissociation. It is essential to demonstrate that the binding of aurothiosulphate to albumin is reversible in order to allow a later thermodynamic analysis of the binding results. The equilibrium reached by dissociation was, therefore, investigated in an equilibrium dialysis system containing initially 2 ml albumin aurothiosulphate solution inside and 2 ml NaCl solution outside the dialysis membrane under similar experimental conditions with regard to pH, ionic strength, and temperature as described above. The range of concentrations of total aurothiosulphate was (285–4490 μM).

To establish that equilibrium was reached at the end of the experiment, control tubes, with aurothiosulphate solution on the inside and 0.15 M NaCl on the outside of the dialysis membrane were set up at two different aurothiosulphate concentrations in duplicate. An equilibrium was found within 2 days. Then pH, the concentration of albumin and sodium were measured on the inside and the concentration of gold on each side of the dialysis membrane.

pH. It was found that during dialysis pH decreased by 0.10–0.15 pH units. In order to obtain pH = 7.4 at equilibrium pH was adjusted to 7.50–7.55 in each initial solution.

Ionic strength. In preliminary experiments it was found that the ionic strength in each albumin solution in the concentration range of total aurothiosulphate from 40 to 4490 μM was 0.15–0.16 M, when albumin and aurothiosulphate were dissolved in distilled, sterile water containing 0.15 M NaCl. According to general practice the contribution of albumin to ionic strength was ignored.

Measurements. All pH measurements were performed at 37°. For albumin determinations a quantitative electroimmunoassay technique was used according to the principles of Laurell [6] (Albumin standard: Standard-Human-Serum, Behringwerke AG). Gold was determined with a flameless atomic absorption spectrophotometer (Beckman model 485 fitted with a Masmann cuvette model 1268) as described by Pedersen and Graabæk [7].

Results

Treatment and presentation of experimental data. A number of methods for treating and presenting the results of studies concerned with macromolecule–small molecule interactions at equilibrium involve determination of C and $\bar{\nu}$ where C is the molar concentration of unbound small molecule and $\bar{\nu}$ is the average number of small molecules bound to each macromolecule, see for example Refs. [8–10].

In the present study total concentration of gold was determined on both sides of the dialysis membrane when equilibrium was reached. The measurement on the inside yields the sum of protein- and nonprotein-bound gold. That on the other side is a measure of nonprotein-bound gold. The protein-bound gold was then calculated as the difference between total gold and nonprotein-bound gold; and $\bar{\nu}$ evaluated by the equation,

$$\bar{\nu} = \frac{[\text{albumin-bound gold}]}{[\text{albumin}]} \quad (1)$$

A molecular weight for albumin of 67,000 was assumed in the calculation of the albumin concentrations [3].

Although both the gold or gold-complex and the albumin are probably present as ions no correction for the Donnan effect was made as the concentration of added salt (0.15 M NaCl) is sufficiently high to suppress the Donnan effect.

A convenient way to present binding data is to plot $\bar{\nu}$ versus log C. This plot is shown in Fig. 1. The experimental data $\{\bar{\nu}, C\}$ was fitted as closely as possible to the equation,

$$\bar{\nu} = \sum_{i=1}^{n} \frac{k_i C}{1 + k_i C} \quad (2)$$

by a non-linear least-square curve fitting procedure. The relationship given by equation (2) implies a specific binding mechanism, i.e. gold (single or as gold-complex) binds to n independent sites on the albumin molecule; the summation is over all sites and k_i is the intrinsic association constant for site i.

The experimental data and the best fits using $n = 3$, 4 or 5 are shown in Fig. 1. Although the binding data $\{\bar{\nu}, C\}$ for small gold concentrations (indicated by: (▲) or (●) in the figures) were reproducible they were not considered accurate and therefore not included in the analysis. The reason for this inaccuracy at small gold concentrations is under further investigation. However, if these binding data were included the intrinsic association constant k_1 was

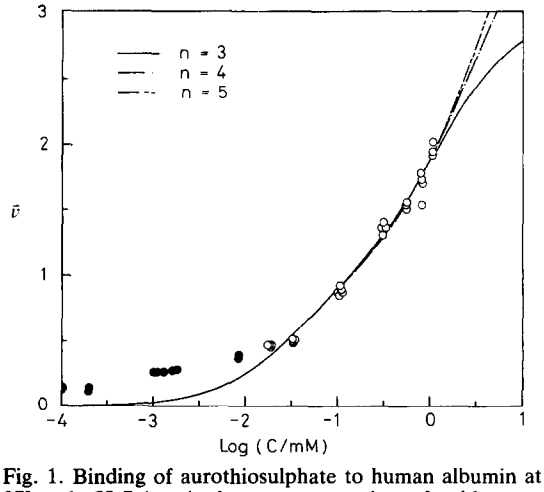

Fig. 1. Binding of aurothiosulphate to human albumin at 37° and pH 7.4. $\bar{\nu}$ is the average number of gold atoms bound per albumin molecule and C is the concentration of nonprotein-bound gold; the experimental points represent 5 equivalent but strictly independent sets of experiments; the lines represent the best fit to equation (2) for the indicated values of n; the points indicated by (●) are not included in the analysis.

Table 1. Intrinsic association constants for binding of aurothiosulphate to human albumin* at $T = 37°$, pH = 7.40 ± 0.03 (1 S.D.), and ionic strength 0.15 M

No. of experiments†	No. of sites (n)‡	$k_1 \times 10^{-3}\,M^{-1}$§	$k_2 \times 10^{-3}\,M^{-1}$	$k_3 \times 10^{-3}\,M^{-1}$	$k_4 \times 10^{-3}\,M^{-1}$	$k_5 \times 10^{-3}\,M^{-1}$	r.m.s. × 10²‖
5	3	30.0 ± 2.0 (30.1)	0.95 ± 0.14 (0.83)	0.73 ± 0.17 (0.83)			6.0 ± 1.6 (1 S.D.) (6.7)
5	4	30.8 ± 1.0 (31.1)	0.97 ± 0.47 (0.99)	0.30 ± 0.15 (0.27)	0.30 ± 0.15 (0.27)		5.7 ± 1.5 (1 S.D.) (6.6)
5	5	30.9 ± 0.8 (31.0)	1.00 ± 0.51 (1.09)	0.23 ± 0.17 (0.15)	0.20 ± 0.11 (0.15)	0.13 ± 0.11 (0.15)	5.7 ± 1.5 (1 S.D.) (6.6)

* Albumin concentration in each solution was 0.53 ± 0.02 (1 S.D.) mM.
† Each of the independent experiments contains 6 or 7 experimental points which cover the experimental range.
‡ Number of combining sites per molecule of albumin.
§ The value of k is the mean value ±(1 S.D.) of the calculated intrinsic association constants for each of the 5 sets of experimental data. The numbers in parentheses are the corresponding values obtained by considering all experimental points as belonging to a single data set.
‖ The r.m.s. value is the standard deviation of $\{\bar{\nu}\}$ on C from the least square fit to equation (2).

increased by 50% or less and the other constants remained virtually unchanged.

Table 1 summarises the binding results obtained for the five independent sets of experimental data considered separately or as a single set. The results demonstrate that there is at least two classes of binding sites. One class with a single binding site and an intrinsic association constant $k_1 = 3.0 \times 10^4\,M^{-1}$ and one or two classes with a smaller intrinsic association constants of the order of $10^3\,M^{-1}$. The best fit with 2 or 3 binding sites had higher r.m.s. values than that with 4. The possibility that more than 4 binding sites exist cannot be excluded, but the fit was not improved significantly by increasing the number of sites beyond 4. It is not possible to conclude whether the intrinsic association constants for sites 2 to 4 are identical. The results do not depend on whether the 5 sets of experimental data are analysed separately or as a single data set.

Figure 2 shows the experimental points $\{\bar{\nu}, C\}$ obtained when equilibrium was reached respectively by dissociation or by association, and the closest fits according to equation (2) using $n = 4$. The figure and the calculated dissociation and association constants (e.g. k_1 (diss.) $= 2.8 \times 10^4\,M^{-1}$, k_1 (ass.) $= 2.9 \times 10^4\,M^{-1}$) showed that the binding of aurothiosulphate to albumin was reversible.

Discussion

The binding of aurothiosulphate to human albumin under simulated physiological conditions was studied in an equilibrium dialysis system, a system with great advantages as partial adsorption to the membrane or to the glass surface does not influence the results [2].

It was found that aurothiosulphate was bound reversibly to human serum albumin at a single site with the intrinsic association constant $k_1 = 3.0 \times 10^4\,M^{-1}$, and at three or more sites with intrinsic association constants of the order of $10^3\,M^{-1}$. The number of binding sites and the values of the intrinsic association constants might be important in an attempt to identify the combining sites and understand the mode of action of the compound.

For clinical purposes it is the determination of the thermodynamic equilibrium constant K_1 for the first consecutive reaction scheme given below which is of interest:

$$\mathrm{Au} + \mathrm{Alb} \rightleftharpoons \mathrm{AuAlb} \qquad (3)$$

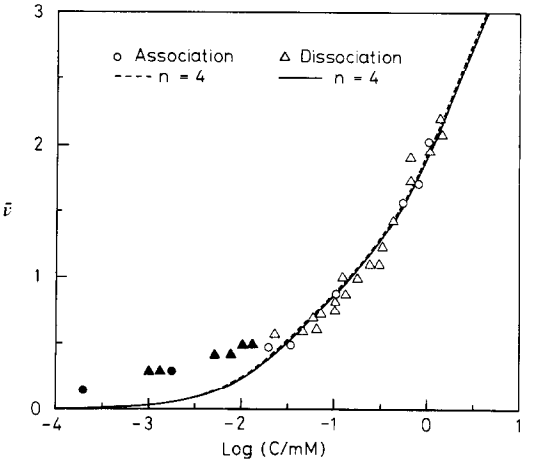

Fig. 2. Comparison of binding data of aurothiosulphate to human albumin obtained from equilibria reached by dissociation (△) or by association (○), at 37°, at pH 7.3 and 7.4 and albumin 0.58 mM and 0.53 mM, respectively; the ionic strength in each solution was 0.15–0.16 M; the curves are the best fits to equation (2) using $n = 4$; the points indicated by (▲) and (●) are not included in the analysis.

$$K_1 = \frac{[AuAlb]}{[Alb][Au]} \quad (4)$$

The K_1 value is equal to the sum of the calculated intrinsic association constants ($\Sigma_{i=1}^{n} k_i$) and was found to be $3.3 \times 10^4\ M^{-1}$ using $n = 4$. The range of K_1 values was $3.18 \times 10^4\ M^{-1}$ to $3.26 \times 10^4\ M^{-1}$ using $n = 3$ to $n = 8$. The value of k_1 corresponds to 95–96% binding of gold to human albumin under physiological conditions.

In the study by Mason [1] the binding parameters for binding of aurothiomalate to albumin were found to be $n_1 = 1.0$, $k_1 = 6.10 \times 10^3\ M^{-1}$ and $n_2 = 6.6$, $k_2 = 2.35 \times 10^2\ M^{-1}$. These results correspond to a thermodynamic constant $K_1 = 7.65 \times 10^3\ M^{-1}$. The higher degree of binding of aurothiosulphate to albumin found in this investigation might be an observation of practical importance as the two compounds on empirical base are administrated in equivalent doses with respect to gold content. Lederer [11] studied chromatographic properties of colloidal gold sulphide (Autosulfa Lab. G. Manzoni, Milan, Italy) and aurothiosulphate. He found that the two compounds had radically different properties in almost all systems, and thus presumably different transport properties in biological systems. As aurothiosulphate and aurothiomalate are respectively inorganic and organic gold compounds, the found differences in binding to albumin might conceivably be explained by different physical–chemical properties of the two compounds. However, the differences might also be due to the problems of drug–membrane binding in the ultrafiltration method and to the buffered solutions employed in the study by Mason [1]. Therefore, any conclusion obtained by comparison of the results of the two methods is uncertain.

In summary, the binding of aurothiosulphate to human serum albumin was studied by equilibrium dialysis at 37° in unbuffered solutions with pH 7.4 and ionic strength 0.15–0.16 M. At constant albumin concentration (in the *in vivo* range) and various gold concentrations aurothiosulphate was bound reversibly to human serum albumin at a single site with an intrinsic association constant of $3.0 \times 10^4\ M^{-1}$ and at 3 or more sites with intrinsic association constants of the order of $10^3\ M^{-1}$.

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Dependence of glucuronidation rate on UDP-glucuronic acid levels in isolated hepatocytes

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Glucuronidation is a major pathway in the biotransformation of foreign and endogenous compounds [1]. The reaction is catalysed by membrane-bound UDP-glucuronosyltransferases (GT) (EC 2.4.1.17) [2, 3] and requires uridine-diphosphoglucuronic acid (UDP-GA) as co-factor [1]. While there are some indications that the cellular UDP-GA level may be a determinant of glucuronidation rate *in vivo* [4, 5], studies performed with native microsomes show a relative independence of GT activity of UDP-GA at physiological and lower concentration [6]. Isolated hepatocytes offer the possibility to study the role of co-factor levels on GT activity under controlled *in vivo* conditions in the intact cell.

UDP-GA levels were modulated in this system by addition of various amounts of D-galactosamine [7], which has been shown to lower the concentration of the co-factor by trapping UTP [8] and inhibiting UDP-glucose dehydrogenase [9]. Glucuronidation activity of the intact cells was determined with 3-hydroxybenzo(*a*)pyrene (3-OH-BP) [10] which is a typical substrate for the late foetal and 3-methylcholanthrene-inducible GT form recently classified by the planar substrates 1-naphthol, 4-nitrophenol and *N*-hydroxy-2-naphthylamine [2, 11]. In order to minimise possible effects on the rate of glucuronide formation by sulphation of 3-OH-BP, an alternative pathway of metabolism for the phenol [12], experiments were performed in sulphate-free medium in which sulphate conjugation is decreased by about 80% [7, 13].