# A comment on estimating binding constants at variable protein concentrations

LEON J. AARONS, *Pharmacy Department, University of Manchester, Manchester M13 9PL, U.K.*

Recently Romer & Bickel published an article in this Journal on the determination of binding constants of ligands to a protein at low saturation of the protein using variable protein concentrations (Romer & Bickel 1978). Their arguments are based on the statistical and experimental problems of determining binding constants at low saturation by the more conventional method of varying the ligand concentration. I would like to take this opportunity to point out several possible shortcomings in their method.

The binding isotherms for the binding of a ligand L to n pre-existing identical noninteracting sites on a protein B is given by the usual equation

$$\frac{L_b}{B_o} = \frac{n.K.L_f}{1 + K.L_f} \quad .. \quad .. \quad (1)$$

where $L_f$ is the concentration of free (unbound) ligand, $L_b$ is the concentration of bound ligand, $B_o$ is the total concentration of protein and K is the association constant. n is given by

$$n = \lim_{L_f \to \infty} \frac{L_b}{B_o}$$

Subsituting for $L_o$, the total concentration of ligand, equation 1 becomes

$$\frac{L_b}{B_o} = \frac{nK(L_o - L_b)}{1 + K(L_o - L_b)} \quad .. \quad (2)$$

It is interesting to compare this exact equation with the approximate equation derived by Romer & Bickel (equation 10 of their paper) viz:

$$\frac{L_b}{B_o} = KL_{b\,max} - KL_b \quad .. \quad (3)$$

where n is assumed to be 1. Equation 2, with n equal to 1, has the following intercepts

$$\lim_{L_b \to O} \frac{L_b}{B_o} = \frac{KL_o}{1 + KL_o}$$

$$\lim_{(L_b/B_o) \to 0} L_b = L_o$$

and slope

$$\frac{d(L_b/B_o)}{dL_b} = \frac{-K}{(1 + K(L_o - L_b))^2} \quad .. \quad (4)$$

In the limit $L_b \to L_o$ the slope of equation (2) approaches $-K$ and the intercept on the abscissa is $L_o$ which is the same as for equation (3). In fact as long as

$$(L_o - L_b) \ll 1/K \quad .. \quad .. \quad (5)$$

equation (3) is a good approximation to equation (2). This was true for the case of chlorpromazine and imipramine (5 $\mu$M) discussed by Romer & Bickel. However the criteria that equation (3) approximates to equation (2) is not that $L_o \ll B_o$ as stated by Romer & Bickel but that the inequality given by equation (5) holds. The breakdown of this criterion is seen when the chlorpromazine experiment is done at 150 $\mu$M and the observed value of the slope falls, as would be predicted by equation (4). Furthermore as n in the development of equation (1) is the number of pre-existing sites it is not justifiable to assume it is unity at low protein saturation.

Romer & Bickel go on to point out that the advantage of their method is that it is only necessary to measure $L_b$. However this is misleading from an experimental and statistical point of view. Firstly, since the authors used equilibrium dialysis to determine the extent of binding, because of ligand movement across the membrane, the ligand concentration on the protein side of the membrane after dialysis is not equal to the original concentration. In fact if the original ligand concentration was $L_o$ and $\alpha$ is the fraction of the ligand which is unbound at equilibrium then the concentration of ligand on the protein side of the membrane, at equilibrium, will be approximately ($L'_o$)

$$L'_o = \frac{L_o}{1 + \alpha}$$

Now if $\alpha$ is small then $L_o$ in equation (2) is approximately constant but for weaker binding this approximation is no longer valid and so $L_o$ cannot be assumed to be constant. Also the protein concentration, $B_o$, is subject to a slight dilution in the dialysis experiment, due to water movement, and again the final protein concentration cannot be assumed to be the same as the orginal concentration. Secondly in obtaining an estimate of K, Romer & Bickel plot $L_b/B_o$ against $L_b$ and claim that statistical errors are smaller because only one experimentally measured quantity is involved. However, statistically this is incorrect as the errors in the dependent variable ($L_b/B_o$) are heavily correlated with the errors in the independent variable ($L_b$) which invalidate the use of the method of least squares (Draper & Smith 1966).

Finally it is worth pointing out that there is a growing body of evidence to suggest that the binding results obtained by varying the drug concentration are not the same as those obtained by varying the protein concentration. In particular several Scatchard plots with

positive slopes have been observed when the protein concentration has been varied (Bowmer & Lindup 1978). No satisfactory explanation for this phenomenon has as yet been put forward. However, care should be exercised in extrapolating from binding results obtained by varying the protein concentration to the situation in which the drug concentration is varied.

In conclusion the approach of Romer & Bickel, although it works for the examples chosen by them, should be viewed with caution for the several reasons listed above, which could invalidate it.

March 23, 1979

REFERENCES

Bowmer, C. J., Lindup, W. E. (1978) J. Pharm. Sci. 67: 1193–1195

Draper, N. R., Smith, H. (1966) Applied Regression Analysis, Wiley: New York

Romer, J., Bickel, M. H. (1978) J. Pharm. Pharmacol. 31: 7–11

# Variation in the heats of reaction of drugs and albumin with the source and pretreatment of the albumin

G. E. HARDEE, M. OTAGIRI, J. H. PERRIN*, *College of Pharmacy, University of Florida, Gainesville, Florida 32610, U.S.A.*

It has been shown previously that microcalorimetry can be used to investigate the interaction of drugs with macromolecules (Otagiri et al 1978) including those with human serum albumin (HSA). Primary binding constants, heats of reaction and entropy changes following the binding phenomena were reported. Recent investigations of the binding of salicylic acid to HSA have shown the heat of reaction but not the free energy, for the binding of the first salicylate molecule, to be greatly dependent upon the source and pretreatment of the albumin (Table 1). The measurements were made in an LKB flow microcalorimeter using the serial dilution technique (Hardee et al 1978) and using an iterative least squares technique to calculate the first binding constant and the heat of reaction at the first binding site.

Table 1. Derived parameters for salicylic acid albumin interaction.

| HSA Source | ΔG (J mol$^{-1}$) | ΔH (J mol$^{-1}$)* | ΔS (J mol$^{-1}$ deg$^{-1}$) | n** |
|---|---|---|---|---|
| Fraction V | −30 000 | −35 000 ± 400 | −18 | 15 |
| Deionized Fraction Five | −32 000 | −37 000 ± 400 | −17 | 15 |
| Fraction V + 0·075 M NaCl | −33 000 | −24 000 ± 600 | +30 | 14 |
| Crystalline | −30 000 | −24 000 ± 500 | +20 | 13 |
| Deionized crystalline | −25 000 | −40 000 ± 600 | −50 | 20 |
| Deionized crystalline + 0·075 M NaCl | −28 000 | −22 000 ± 300 | +18 | 16 |
| Fatty acid free | −36 000 | −40 000 ± 500 | −14 | 14 |
| Plasma | −21 000 | −55 000 ± 2600 | −112 | 13 |
| Bovine serum albumin | −32 000 | −37 000 ± 1000 | −17 | 15 |

All measurements are made at 25·0 °C, in 0·1 M phosphate buffers of pH 7·40 using the LKB Model 2107–121.
*Standard error estimate from asymptotic correlation $\alpha = 0{\cdot}05$.
**n = number of data points.

The three albumins were from normal commercial sources and gave similar optical densities at 210 and 278 nm (Perrin & Vallner 1975) and at 615 nm following the interaction with bromocresol green (Rodkey 1965). The albumins also gave similar intrinsic optical activities (Perrin & Vallner 1975). L. H. M. Jansen & T. H. A. Nelen (submitted for publication) have recently shown that sulphaethidole displaces chloride ion from bovine serum albumin and unpublished observations in the same laboratory have shown that warfarin displaces chloride ion from HSA.

An explanation of the data in Table 1 is that the albumins contain a small amount of inorganic ions, possibly chloride, and the low affinity of these ions for HSA compared to the salicylate results in little change in the derived binding constant but the displacement of the chloride by the drug results in a large effect on the heat of reaction. The deionization was carried out using Amberlite IR120 and IRA400 as recommended by Jansen & Nelen and seems to be an essential pretreatment of HSA before use in any binding studies, particularly if heats of reactions are to be measured. Gel electrophoresis of the three HSA samples showed that all had the same bands present, however the fraction of the total albumin in the minor bands increased as the number of purification steps was increased. The defatted material seems to be far less pure than the fraction V. Defatting would appear to be an unnecessary complication in binding studies, particularly after the deionization treatment. The binding constant in plasma was significantly lower than in the albumin solutions, but the heat evolution was higher. This may be due to competition between the drug and plasma components for the binding site on albumin as well as the binding of the salicylate to other proteins in the plasma. Although the data presented represent the interaction of salicylate with HSA, similar results have been obtained for the interaction of sulphaethidole with HSA.

January 8, 1979

REFERENCES

Hardee, G. E., Otagiri, M., Perrin, J. H. (1978) Acta Pharm. Sue. 15: 188–199

Rodkey, F. L. (1965) Clin. Chem. 11: 478–487

Otagiri, M., Hardee, G. E., Perrin, J. H. (1978) Biochem Pharmacol. 27: 1401–1404

Perrin, J. H., Vallner, J. J. (1975) J. Pharm. Sci. 64: 1508–1513

* Correspondence.