on the guinea pig ileum was measured by known procedures (8). A pA_2 value (9) of 2.2 was determined. While the degree of antagonism observed is much weaker than that observed for atropine [pA_2 = 9.0 (10)], it is significant that these effects have not been previously reported.

The effects of pilocarpine methiodide on eel acetylcholinesterase were studied[1], and weak, uncompetitive inhibition was observed ($K_i = 1.64 \times 10^{-4}$).

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Binding of Sulfonylureas to Serum Albumin

Keyphrases ☐ Protein binding, human and bovine serum albumin—sulfonylureas, dynamic dialysis technique ☐ Sulfonylureas—binding to human and bovine serum albumin, dynamic dialysis technique ☐ Drug–protein binding—sulfonylureas ☐ Dialysis, dynamic—used to study sulfonylurea binding to serum albumin

Sir:

A recent paper (1) concerned the binding of the sulfonylureas, tolbutamide, chlorpropamide, and acetohexamide, to serum proteins. We have studied the binding of tolbutamide and chlorpropamide to serum albumin and find that there are substantial differences between our results and those of Judis (1). In this communication we present some results for comparison and attempt to reconcile the differences.

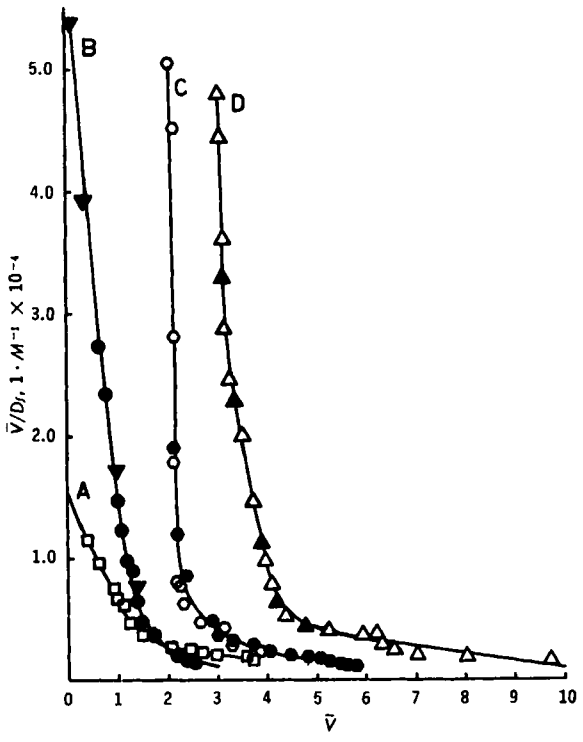

Figure 1—*Scatchard plots for the binding of tolbutamide to serum albumin in 0.067 M buffer at pH 7.4 and 37°. Key: open symbols, 1% albumin; closed symbols, 2% albumin; A, 1% human serum albumin in tromethamine buffer; B, 2% human serum albumin in tromethamine buffer; ●, ▼, Judis' (1) results (40°); C, human serum albumin in phosphate buffer; and D, bovine serum albumin in phosphate buffer. All points are experimental while the solid lines were computed from the binding parameters.*

The drug–protein interaction was characterized using a dynamic dialysis technique. The method and apparatus were based on those described by Meyer and Guttman (2). A concentration of 1 or 2% serum albumin was used, buffered to pH 7.4 with 0.067 *M* phosphate buffer. The volume and frequency of sampling were such that the concentration of drug in the outer compartment never exceeded 5% of the free concentration in the protein compartment to ensure that dialysis proceeded under sink conditions.

Tolbutamide was estimated by two separate methods: (*a*) in buffer, by direct UV spectrophotometry at 228 nm., and (*b*) in solutions containing protein and/or other interfering materials, by a modification of the colorimetric procedure of Alessandro *et al.* (3). Chlorpropamide was estimated spectrophotometrically at 231 nm.

The decline in drug concentration, D_t, within the dialysis bag was followed as a function of time. From a knowledge of the intrinsic dialytic rate constant and the instantaneous dialysis rate at any time, t, the free concentration in the protein compartment was estimated. The instantaneous rate of dialysis was obtained by fitting the plot of D_t *versus* time to an equation of the form:

$$D_t = D_t^0 + at + bt^2 + ct^3 + dt^4 \ldots \quad \text{(Eq. 1)}$$

Table I—Binding Parameters for the Interaction of Chlorpropamide and Tolbutamide with Human and Bovine Serum Albumin at pH 7.4 and 37° in 0.067 *M* Buffer

Drug	Protein	Buffer	n_1	$k_1 \times 10^{-4}$, l. mole^{-1}	n_2	$k_2 \times 10^{-2}$, l. mole^{-1}
Tolbutamide	Human serum albumin, 1 and 2%	Phosphate	2.27	21.86	8.21	1.71
	Human serum albumin, 1%	Tromethamine	1.42	0.97	8.81	1.85
	Human serum albumin, 2%	Tromethamine	1.20	4.95	8.29	1.45
	Human serum albumin, 2%	Tromethamine	1.36[a]	4.06[a]	—	—
	Bovine serum albumin, 1%	Phosphate	2.98	24.82	8.12	3.39
Chlorpropamide	Human serum albumin, 1%	Phosphate	2.19	4.51	8.22	1.71
	Human serum albumin, 2%	Tromethamine	1.64[a]	1.09[a]	—	—
	Bovine serum albumin, 1 and 2%	Phosphate	1.94	4.67	8.93	4.14

[a] Values calculated by Judis (1) (40°).

where D_t^0 is the initial concentration of drug in the protein solution and *a*, *b*, *c*, and *d* are constants. This was achieved by treating the data by polynomial regression to produce four normal equations (4), which were solved by using a program for the solution of a symmetrical matrix and a programmable calculator[1]. Differentiation of Eq. 1 yields the instantaneous rate.

Data for the binding of tolbutamide to both human and bovine serum albumin are shown as Scatchard plots in Fig. 1. The results of Judis (1) determined by equilibrium dialysis and in the presence of tromethamine buffer are included for comparison. It is apparent that the latter results indicate a much weaker interaction than our own. In an attempt to reconcile these differences, binding experiments were repeated using 0.067 *M* tromethamine buffer in place of phosphate in both 1 and 2% human serum albumin. These data are also included in Fig. 1, and our results in 2% human serum albumin are in good agreement with those reported (1). However, these data indicate a much weaker interaction than occurs in phosphate buffer. Furthermore, the degree of interaction in tromethamine buffer shows a marked dependence on the human serum albumin concentration, as evidenced by the large difference between the plots for 1 and 2% protein. No such dependence was observed using phosphate buffer with either human or bovine serum albumin (Fig. 1). This dependence implies that the components of tromethamine buffer itself interact with the protein and compete with tolbutamide for binding sites.

If potentially competitive species are present in the buffer, one might expect quite strong competition because of the high molar concentration of buffer relative to that of tolbutamide. Scatchard plots for the binding of chlorpropamide to human and bovine serum albumin (Fig. 2) show a similar effect. Again the degree of interaction is considerably less in tromethamine buffer than in phosphate.

The Scatchard plots are not linear but curved. Such curvature is generally considered to be indicative of binding to more than one class of sites (5). The curvature results from the combination of two or more linear plots representing the binding to each class of sites. In such cases, simple extrapolation or evaluation of the slope from the linear portion of the curve may give inaccurate estimates of the binding constant for all classes of sites, unless the association constants for the various classes are very widely separated. These aspects have been overlooked by many authors, and linear extrapolation is probably still the means most frequently used to determine binding constants.

Hart (6) reported a mathematical treatment for the estimation of binding constants from curved Scatchard plots. By using Hart's treatment and a multiple linear regression analysis (4) and program similar to that described here, the experimental data were fitted to a model for two classes of sites. Binding parameters for the various systems are shown in Table I. In phosphate buffer, the association constant (k_1) for the binding of tolbutamide to the primary sites (n_1) on human serum

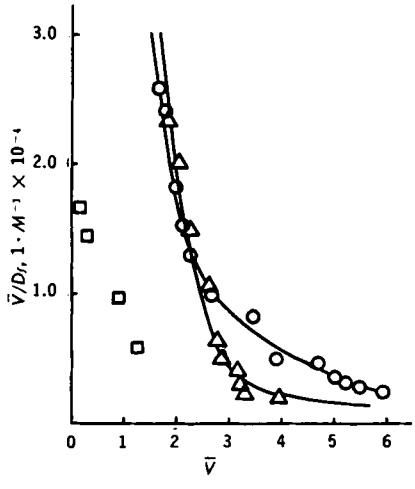

Figure 2—*Scatchard plots for the binding of chlorpropamide to serum albumin in 0.067* M *buffer at pH 7.4 and 37°. Key: △, 1% human serum albumin in phosphate buffer; □, Judis' (1) results for 2% human serum albumin in tromethamine buffer (40°); and ○, 1% bovine serum albumin in phosphate buffer. All points are experimental while the solid lines were computed from the binding parameters.*

[1] Diehl Combitron S.

albumin was four times greater than that using tromethamine buffer and 2% human serum albumin and about 20 times that determined for tromethamine and 1% human serum albumin. Furthermore, the number of sites in the first class (n_1) in phosphate buffer was substantially greater than in tromethamine. In the second class of sites, the association constant (k_2) and the number of sites (n_2) did not appear to show a strong dependence on the buffer used or the protein concentration. This suggests that tromethamine buffer did not interfere with binding in the second class and that binding to these sites possibly occurs by a less specific mechanism.

A comparison of the binding parameters obtained by Judis (1) and ourselves for the binding of tolbutamide to 2% human serum albumin in tromethamine buffer demonstrates the effect of neglecting the existence of the second class of sites. Although both sets of data are in good agreement on the Scatchard plot (Fig. 1), estimation of the primary association constant (k_1) from the slope of the linear portion of the plot does not correct for the slope contribution due to the second class of sites. This leads to an underestimate of the association constant on the order of 20% or 9000 l. mole^{-1} and a larger number of primary binding sites.

In the case of chlorpropamide, determination of binding in the presence of tromethamine buffer leads to a reduction in the number of binding sites and a fourfold reduction in the association constant relative to values obtained in phosphate buffer. Thus, as with tolbutamide, tromethamine strongly suppresses the binding.

These data illustrate the effects that buffers and other materials may have on the extent of protein binding *in vitro*. In such studies it is important to characterize the binding at several buffer or protein concentrations to test that the observed binding is a real effect rather than an artifact dependent on the particular buffer used. This also applies to salts which may be added to suppress the Donnan effect in equilibrium dialysis experiments. These considerations are also relevant to competitive binding studies. When the buffer system itself acts as a competitor and is present in high concentrations relative to the other competitors, the significance of the results is somewhat difficult to assess.

Judis (1) used two graphical methods (7, 8) to present his data, and there is excellent agreement between them. However, this is to be expected since the two methods are closely related. The plot used by Sandberg *et al.* (7) does not normalize the concentrations of bound small molecules for variations in total protein concentration and is for use chiefly when the protein concentration is not precisely known. The method used by Eichman *et al.* (8) is the well-known Scatchard plot, and in this case the results are normalized for variations in protein concentration. The only difference between the two is that, in the method of Sandberg *et al.*, the intercepts on the ordinate and abscissa are nkP_t and nP_t, respectively, where P_t is the concentration of protein. The value of n cannot be estimated without a knowledge of the protein concentration. In all other respects, both plots are essentially mathematically identical. Thus, any variation occurring in the value of n or k must only be the result of the errors expected in deriving information from graphical data.

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Binding of Sulfonylureas to Serum Albumin: A Response

Keyphrases ☐ Sulfonylureas—binding to serum albumin ☐ Protein binding—sulfonylureas to serum albumin ☐ Binding, protein—sulfonylureas to serum albumin ☐ Dialysis, equilibrium and dynamic—explanation of differences found studying sulfonylurea binding to serum albumin

Sir:

Crooks and Brown (1) suggested substantial disagreements between their findings and mine (2). Disagreement between the results is complicated by the fact that a different method was used in each article. Equilibrium dialysis is an old, established method for studying protein binding, and there is a substantial literature of findings with this experimental approach. The new method used by Crooks and Brown, dynamic dialysis, first described by Meyer and Guttman (3) has not been used as extensively as equilibrium dialysis.

Disagreement with the results (2) also may have occurred because Crooks and Brown did not repeat the experiments using equilibrium dialysis or the same methods for analysis, such as radiochemical assay. No data are offered (1) regarding the analytical work, interfering substances, sensitivity of the assays, or specificity of the analyses. Therefore, comparison of data may or may not be valid.

The data in their Fig. 1 (curve B) lead to the conclusion that using tromethamine buffer and 2% human serum albumin results in agreement with our findings.