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A COMPARISON OF THE VISKING MEMBRANE AND BATCH SEPHADEX METHODS OF EQUILIBRIUM DIALYSIS

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SUMMARY

A comparison of the batch Sephadex method of equilibrium dialysis with the classical Visking membrane method reveals that binding affinity values for the former are 20–30% lower than for the latter under a variety of experimental conditions in the interaction of testosterone with three batches of crystalline human serum albumin. This discrepancy is in fact in the opposite direction to what might have been anticipated on the basis of a demonstrated influence of protein on the partition factor, K', for gel steroid interaction. It has also been shown that the alcohols ethanol and Tris exert an inhibitory effect on testosterone human albumin interaction.

INTRODUCTION

The determination of binding isotherms for ligand-protein interaction is an important procedure in experimental biochemistry. The most direct method involves measurement of unbound and total ligand concentration on opposite sides of a semipermeable membrane, such as Visking sausage casing, after allowing enough time and agitation for equilibrium to be established. Modern high flux membranes make a rapid ultrafiltration variant of this possible [1, 2] and, in addition, direct techniques such as organic solvent-aqueous phase partition [3], classical ultrafiltration [4], frontal analysis [5] on columns and the more recently introduced membrane-less aqueous two-phase partition system [6] may be used. Substitution of the interface between an insoluble polymer gel (Sephadex) and aqueous solution for the Visking or other membrane, as in the method of Pearlman and Crépy[7] for studying steroid–protein interaction, provides a very rapid means of attaining equilibrium between free and bound ligand, as well as between phases internal and external to the gel, and an equally rapid means of separating phases while maintaining equilibrium. In addition to many advantages of simplicity and economy in materials, it suffers from the disadvantage that only total (bound plus free) ligand is directly determined. Free ligand concentration is derived with the aid of a separately determined partition factor, K', for distribution of unbound ligand between gel and aqueous phase. The latter is determined in the absence of protein and is assumed to be unaffected by presence of protein. Thus if A is moles of ligand added to a tube containing a known amount of Sephadex and aqueous solution and Y is the total moles in the external phase at equilibrium, then $K' = Y/(A - Y)$. If there is no binding of the ligand to the gel, this number will be equal to the ratio of the volumes of external and internal phases. Having had reason to use this procedure for study of steroid-protein interaction [8, 9] and having encountered imprecision for data in unbuffered solutions [10] we have been led to test both the validity of such results, in relation to what is obtainable by Visking equilibrium dialysis, under different experimental conditions employed in our work and the assumption that K' is uninfluenced by protein. The validity of results obtainable by the Sephadex method has heretofore rested on comparison with published values of binding parameters.

MATERIALS AND METHODS

Unlabeled testosterone was obtained from Schwartz-Mann and Steraloids Inc. and purity was checked by melting point and thin-layer chromatography. [^{14}C]-Testosterone was obtained from New England Nuclear Corp. and was used within 2 months. Radiochemical purity was checked occasionally and found to be better than 97%. Crystalline lot 31, 32 and 33 human serum albumin were obtained from Miles Laboratories. Some binding and other data involving two of these batches, under experimental conditions different from those reported here, have been described elsewhere [11]. Deionization of the protein was performed as described previously [11].

Measurement of bound steroid

1. *The batch Sephadex* equilibrium dialysis method of Pearlman and Crépy[7] was performed in the manner described previously [8, 9] using triplicate tubes for each steroid concentration. Thus, 400 mg amounts of G-25 Sephadex were equilibrated overnight with 2 ml of either 100 mM NaCl or Tris chlor-

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ide buffer pH 8.5, I = 0.1, so as to yield 1 ml internal phase and 1 ml external phase. Then, 2 ml of a solution of protein and steroid was added such that the protein concentration in the 3 ml external phase is 90 μM. After shaking in a water bath at 25°C for 2 h the tubes are allowed to stand vertically for 1 h and duplicate 100 μl aliquots are sampled for counting in 4 ml scintillator at 2% standard error and 75% efficiency in a Nuclear Chicago Mark II Counter. Measurements of K' are made using the same procedure but omitting protein. Some measurements of K' have been made (see below) in the presence of protein. The internal volume of gel has been found to be fairly constant over all of the conditions used here and there is no evidence of binding of albumin to the gel.

2. *Visking membrane* equilibrium dialysis was performed as previously described [2, 11]. Dialysis bags containing 4 ml of 90 μM deionized protein and two glass beads were immersed in flasks (50 ml Erlenmeyer with elongated necks) containing 15 ml of a solution of labeled testosterone in either 100 mM NaCl or Tris chloride buffer, pH 8.5, I = 0.1, containing 0.19 mg streptomycin sulphate and in the absence or presence of 1% ethanol. They were then placed in a shaking water-bath for 48 h at 25°C. Duplicate flasks were used for each steroid concentration. After the 48 h period, duplicate 500 μl or 100 μl samples from the inside and outside contents were counted as above.

Values of K′ *in presence of protein*

These can be obtained using a variant of the Visking method in which Sephadex is included in the dialysis bag. It is necessary to maximize the percentage of total steroid which is bound to the Sephadex in competition with the protein, so that the volume external to the bag must be at a minimum. Hence 600 mg of Sephadex, 5 ml of buffer solution containing protein and [^{14}C]-testosterone were placed in a dialysis bag containing two glass beads and the bag was tied. After hydration of the Sephadex, this resulted in a volume of 1.5 ml internal to the gel and 4.5 ml external. The bag was immersed in a Pyrex test tube (i.d. 20 mm, length 175 mm) containing 6 ml of a solution of [^{14}C]-testosterone and the tube was sealed with parafilm. Duplicate tubes were used for each concentration of steroid and protein. Measurements were made at 0, 30, 50, 70, 90 μM protein and using approximately saturated and half-saturated testosterone solutions. The tubes were mounted on a rotator (Rugged Rotator, Kraft Corp) at a 15° angle and equilibration allowed to take place at room temp. for a 48 h-period. The bags were removed, the contents emptied and, after allowing the gel to settle, duplicate 500 μl aliquots of the aqueous phase inside and outside the bag were sampled and counted in 20 ml of scintillator solution to 2% standard error at 75% efficiency in a Nuclear Chicago Mark II Counter.

Pipetting

The precision of analysis is dependent on the precision of pipetting. For sampling 500 μl aliquots we have generally used either glass transfer pipets (Fisher Scientific) or Eppendorf polyethylene tipped pipets. The latter are claimed to yield a precision of $\pm 0.6\%$ and $\pm 1\%$ respectively. In the case of 100 μl aliquots used in some of the work reported here we have found it is possible to obtain sampling precision and accuracy of $\pm 0.25\%$ using Micropettor (Scientific Manufacturing Industries, Emeryville, CA) pipets, using a single glass capillary for all sampling, as well as rapid rinsing with the solution being measured and calibrating the pipet with a ^{14}C solution of known concentration. The precision thus attainable is reflected in some of the values for K' reported here.

RESULTS

Data are presented in Table 1 summarizing the results of many experiments on the value and precision of the K' for reversible binding of testosterone to G-25 Sephadex over the full range of steroid concentrations in aqueous solution. Each value represents means from duplicate or triplicate aliquots obtained from each tube and duplicate or triplicate tubes were used for each concentration. It can be seen that a high order of repeatability is obtained and that the values are independent of testosterone concentration. This is in support of previous measurements [8] and contrary to the slight dependency observed by Pearlman and Crépy[7]. The data suggest that the gel has a very large, low affinity, capacity for reversible testosterone binding. If there were no such binding, the K' would have a value of 3. These values are comparable to those we have routinely found for Tris buffer [9, 11].

The values in Table 2 represent measurements of K' in the presence of increasing concentration of protein. Because the uncertainty in such values for K' is inversely related to the percentage of total steroid bound to the gel, and the latter decreases within increasing protein concentration, meaningful values can only be obtained for 30 and 50 μM protein. Thus, it can be shown that if X is mol unbound steroid in the external phase, A total steroid in the tube and S is the standard error of sampling then, for small values of S, the possible standard error in K' is approximately $A/(A - X)\,S$. It may be seen from Table 2 that there is excellent agreement for values in the absence of protein with those determined by the tube method. There is furthermore a marked 32% increase in the value of K' in presence of 30 μM protein and a slightly greater (41%) increase for 50 μM protein. The trend is also obvious at higher protein concentrations, but uncertainty in values is much greater. The good duplication at low protein concentration and in absence of protein is a reflection of the precision of pipetting. The data in Table 2 would thus

Table 1. Values for gel–buffer partition factor (K')* in the binding of testosterone to G-25 Sephadex in 100 mM NaCl/1.25% ethanol in absence of protein

	23.1 μM Testosterone	46.2 μM Testosterone	69.3 μM Testosterone	92.4 μM Testosterone
Expt. 1	1.26 (6)†	1.24 (6)	1.30 (6)	1.34 (6)
Expt. 2	1.31 (3)	1.34 (3)	1.34 (3)	1.34 (3)
	1.27 (3)	1.32 (3)	1.30 (3)	1.33 (3)
	1.29 (3)	1.33 (3)	1.27 (3)	1.26 (3)
Expt. 3		1.24 (2)		1.23 (2)
		1.24 (2)		1.21 (2)
		1.26 (2)		1.26 (2)
Expt. 4		1.23 (3)		1.31 (6)
		1.27 (6)		1.37 (6)
		1.23 (6)		1.33 (6)
		1.25 (6)		1.34 (6)
		1.23 (6)		1.36 (6)
Expt. 5		1.29 (3)		
		1.21 (3)		
		1.27 (3)		
		1.25 (3)		

* Measured as the ratio of [^{14}C]-steroid in 3 ml external phase to that in 1 ml internal phase.

† Values given are means for each tube; figure in brackets refers to number of aliquots of external phase samples per tube.

suggest that the erroneous assumption that K' is unaffected by the presence of protein should lead to some overestimate of the values of nk in measurements of binding parameters by this method. It is impossible to know what the actual K' value might be at 90 μM protein. Extrapolation from the reasonable values at 30 and 50 μM protein suggest it could be as high as 2.1, a 63% increase.

The data of Table 3 presents a comparison of the Sephadex and Visking membrane method for a variety of experimental conditions, using three lots of human serum albumin. The K' value used in all calculations for the Sephadex method is that measured in the absence of protein. The variation in binding values manifested by different preparations of crystalline albumin under identical conditions, as seen in this data, has been observed previously. It may be noted first that the data obtained in buffer solution by the Visking method indicates that the presence of 1% ethanol (line 2, Table 3) lowers the binding affinity by about 25% in the case of all three batches of albumin. This is greater than what we had previously inferred from a comparison of data with published values [9]. When measurements are made in unbuffered 100 mM NaCl/1% ethanol, pH 8.5, (conditions we have used in pH Stat studies of this interaction [10]) using the Visking method, there is an apparent further drop (line 3, Table 3) in binding affinity. However, these lower values are due to the fact that such prolonged exposure in unbuffered 100 mM NaCl solutions leads to a marked drop in pH (to 7.0). When 95 mM NaCl/1% ethanol solutions which are lightly buffered with 5 mM Tris chloride, pH 8.5 are used, this change in pH is avoided and a much higher binding value (lot 33) is obtained (line 5, Table 3). In fact it is only slightly lower than the value for Tris chloride in the absence of ethanol (line 1, Table 3) and is surprisingly much higher than that for Tris chloride/1% ethanol (line 2, Table 3). The highest binding affinity values were obtained for measurements made in lightly buffered NaCl in the absence of ethanol (line 4, Table 3). This was also true for the Sephadex method (see below). It thus seems that Tris exerts an inhibitory effect on testosterone binding. The reciprocal plots for the results for both the lightly buffered and unbuffered solutions (Fig. 1) are as good as those for buffered solution (Fig. 1).

Table 2. Values for gel–buffer partition factor (K')* for binding of testosterone to G-25 Sephadex in absence and presence of human serum albumin in Tris chloride pH 8.0, I = 0.1

Albumin Conc. (μM)	46 μM Testosterone	92 μM Testosterone	$\frac{A}{A-X}$‡
0	1.25†	1.30†	3.6
	1.28	1.20	
	1.28		
30	1.67	1.71	6
	1.58	1.67	
50	1.69	1.84	7.5
	1.79	1.80	
70	2.33	2.15	11
	5.29	3.0	
90	11.87	—	32
	3.95	—	

* Based on measurements made using Visking membrane technique. See Methods.

† Each value is derived from means of triplicate sampling of inside and outside dialysis bag contents.

‡ Factor by which standard error in K' values may increase over standard error in sampling. See Results.

Table 3. Comparison of values of binding parameters (nk, n) for the interaction of testosterone and 90 μM deionized human albumin, measured by equilibrium dialysis using Visking membrane and batch Sephadex technique at pH 8.5, I = 0.1

	Experimental conditions	Visking membrane						Batch Sephadex†					
		Lot 31		Lot 32		Lot 33		Lot 31		Lot 32		Lot 33	
		nk	n	nk	n	nk	n	nk	n	nk	n	nk	n
(1)	Tris chloride	5.3	11	4.95	11	5.02	7	—	—		—	4.2	5
(2)	Tris chloride 1% ethanol	4.4	8	3.5	10	3.56	11	3.48 3.49	6 6	3.1	3	2.59	5.5
(3)	100 mM NaCl 1% ethanol	3.34	5	3.62	3	2.39 2.54	5.0 5.0	3.0 4.72 5.5 3.62	10 20 5 2	3.8* 3.4* 3.57	3 5 14	3.69	5.6
(4)	95 mM NaCl 5 mM Tris Cl	—	—	—	—	6.69	6.0	—	—	—	—	6.0	1.7
(5)	95 mM NaCl 5 mM Tris Cl 1% ethanol	—	—	—	—	4.89	9	—	—	—	—	3.79	6
(6)	100 mM NaCl 1% ethanol 10 mM $CaCl_2$	—	—	—	—	—	—	—	—	—	—	2.16	5
(7)	95 mM NaCl 5 mM Tris Cl 1% ethanol 10 mM $CaCl_2$	—	—	—	—	2.68	11	—	—	—	—	2.22	20

* The steroid protein mixtures were allowed to stand for 24 h before equilibration with gel in the case of the lower of the two values.

† The K' for the Sephadex method was that in absence of protein.

When measurements are made by means of the Sephadex method in Tris chloride buffer, values are obtained for lot 33 albumin (line 1, Table 3) which are 16% lower than those for the Visking method while in the presence of 1% ethanol the discrepancy is of the order of 25% for all three batches (line 2, Table 3). The observation referred to above suggests that one might anticipate higher values—not lower—when using the Sephadex method. Similarly when measurements were made in 95 mM NaCl/5 mM Tris chloride/1% ethanol, pH 8.5, values were obtained for lot 33 albumin which are only 77% of those obtained when employing similar conditions with the Visking method (line 5, Table 3). These Sephadex values were in fact almost identical to those obtained in 100 mM NaCl/1% ethanol in the absence of Tris (line 3, Table 3), since in contrast to measurements with the Visking method, there is only slight change in pH of unbuffered NaCl solutions over the time period involved in the Sephadex method. It will be noted that the Sephadex value for lot 33 protein in unbuffered NaCl is very close to the corresponding ones for lot 32. In the case of the latter, allowing the protein mixtures to stand for 24 h before equilibrating with the Sephadex caused a 10% drop in binding affinity. The Sephadex values for lot 31 were quite variable but nevertheless the reciprocal plots for individual experiments were surprisingly good. This variability may have been due to a lack of adequate control of the time in some of these experiments (see below). Similarly, comparison of the Sephadex and Visking membrane data in 95 mM NaCl/5 mM Tris chloride/1% ethanol/10 mM $CaCl_2$ reveals not only the marked inhibitory effect of Ca^{2+} on testosterone binding previously reported by us [9] but that the Sephadex values are only 82% of those for the Visking method. However, lot 33 albumin is much more sensitive to Ca^{2+} than lot 24 or 25 [9]. Furthermore, the Sephadex value in 95 mM NaCl/5 mM Tris chloride in the absence of ethanol is lower than the corresponding values for the Visking method. While there

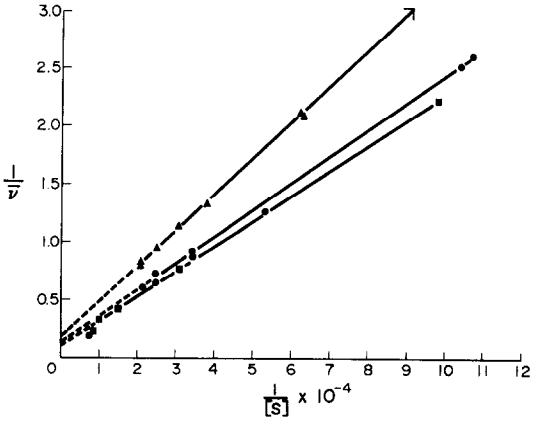

Fig. 1. Reciprocal plots of data obtained by Visking membrane equilibrium dialysis for interaction of testosterone with human serum albumin at pH 8.5, 25°C. (●——●) In Tris chloride/1% ethanol, I = 0.1; lot 31; (▲——▲) in 100 mM NaCl/1% ethanol; lot 31; (■——■) 95 mM NaCl/5 mM Tris chloride/1% ethanol; lot 33.

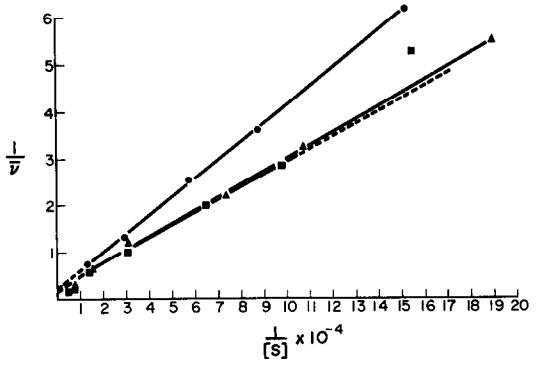

Fig. 2. Reciprocal plots of binding data obtained by batch Sephadex equilibrium dialysis for interaction of testosterone with lot 33 human serum albumin at pH 8.5, 25°. (●——●) Tris chloride/1% ethanol, I = 0.1; (▲——▲) 100 mM NaCl/1% ethanol; (■——■) 95 mM NaCl/5 mM Tris chloride/1% ethanol.

is some variability and anomaly in the extrapolation values of n, the Sephadex values are generally lower than those for the Visking method.

Earlier results [10], containing considerable imprecision, had led to the suggestion that apparent deviations from linearity in the reciprocal plots at high values of $\bar{\nu}$ might be due to ligand-induced conformational change. More extensive results reported here for data obtained both by the Sephadex and Visking methods, not only in strongly buffered Tris, but lightly buffered Tris/NaCl/ethanol, or unbuffered NaCl/ethanol support this suggestion. While the reciprocal plots do not show as much deviation at the high binding levels (see Figs. 1 and 2) as observed in previous measurements, Hill plots (not shown) of such data yield straight lines with a slope of 1.0 up to a level of $\bar{\nu} \simeq 2$. Above this level the plots deviate in the manner indicated previously [10], suggestive of an induced conformation change driven by the binding energy of the altered form. That such an altered state of the protein may be unstable in NaCl is seen in the results of an experiment in which two stock solutions of albumin–testosterone complex in 100 mM NaCl, with $\bar{\nu} = 0.2$ and 2.4 respectively, were kept at room temperature for 72 h and measurements of $\bar{\nu}$ were made using the Sephadex method at different time intervals. From data in Table 4 it can be seen that even in unbuffered NaCl the values of $\bar{\nu}$ are unchanging up to 4 h. At 24 h the low value decreases by 5% and the high value by 15%, whereas at 72 h the former has decreased by 8% but the latter by 46%. However, the pH was not monitored in this experiment. In lightly buffered solutions, however, the values remain constant over the 72 h period at both binding levels.

DISCUSSION

In the above data it may be seen that the assumption that the K' used in measurements with the batch Sephadex method is independent of the presence of protein is invalid. However, the discrepancy between results obtained in parallel experiments using both methods is in the opposite direction to what might be anticipated on this basis, the Sephadex values being about 20–30% lower. Results of studies in progress lead us to believe that this may be due to a related, opposite, but overcompensating effect arising out of an influence of protein on solubility of unbound steroid. The latter is known to vary with experimental conditions in the absence of protein [12]. We will report elsewhere on the influence of protein. Nevertheless these investigations provide evidence, not hitherto available, that the batch Sephadex method can yield data for measurements in buffered solution which are analytically as precise as those of the Visking method in spite of the systematic discrepancy in the binding affinity values of the order of 20–30%.

Variations in binding affinity for steroid albumin interaction for different batches of crystalline albumin have been referred to previously [9, 11, 13]. It is evident from the data here that this may depend on the conditions of measurement. Thus the Visking data

Table 4. Stability of testosterone–albumin complex in 100 mM NaCl/1% ethanol and 95 mM NaCl/5 mM Tris chloride/1% ethanol at pH 8.5, 25°C

	100 mM NaCl/1% ethanol				95 mM NaCl/5 mM Tris/1% ethanol			
Time	Solution I		Solution II		Solution III		Solution IV	
(h)	$\bar{\nu}$	%	$\bar{\nu}$	%	$\bar{\nu}$	%	$\bar{\nu}$	%
0	0.203	100	2.39	100	0.302	100	2.24	100
2	0.199	98	2.4	100	0.293	96	2.31	103
4	0.195	96	2.36	99	0.291	96	2.31	103
21.5	0.195	96	—	—	—	—	—	—
22	—	—	2.1	88	—	—	—	—
24	0.192	94.5	—	—	0.299	99	2.27	101
26	—	—	2.04	85	—	—	—	—
48	—	—	—	—	0.304	100	2.37	105
71	—	—	1.26	54	—	—	—	—
72	—	—	—	—	0.303	100	2.33	104
98	0.187	92	—	—	—	—	—	—

for Tris chloride indicate identical *nk* values for lot 32 and lot 33 albumin which are only 6% lower than that for lot 31. However, in Tris chloride/1% ethanol the discrepancy is 25% both in the Visking and Sephadex data. Furthermore, in the equilibrium dialysis data of lot 33 protein, the values for 95 mM NaCl/5 mM Tris chloride are much higher than those for Tris chloride I = 0.1 by both methods in the presence and absence of ethanol. This is also true for all of the data of the Sephadex method for lot 32, 33, both in the presence and absence of ethanol. It is thus only possible to make comparisons of binding data for steroid albumin interaction using identical conditions and (preferably) a single albumin preparation.

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