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PROTEIN BINDING OF COHERIN AND OTHER SMALL MOLECULES BY THIN-LAYER GEL CHROMATOGRAPHY

IRVING GOODMAN, RONALD D. UNTERMAN, STEVEN M. SOLOMON and ROBERT B. HIATT

Columbia University, College of Physicians and Surgeons, Departments of Biochemistry and Surgery, New York, N.Y. 10032 (U.S.A.)

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SUMMARY

The protein binding stoichiometry of small molecules is here determined on a nanomole scale by a simplified procedure utilizing chromatography on thin layers of cross-linked dextran gels. New data are presented on the thin layer chromatographic properties of representative ligands, including α-amino acids, peptides, dyes and fluorigenic reagents, in relation to their molecular weights, polar characteristics, gel water regain values and denaturants, providing criteria for the general application of this method to studies of ligand binding with large as well as small molecules. By this procedure coherin peptides, A_1 and B^{1-4}, respectively, bind to coherin C in the molar ratio, 2:1, with a binding constant of about $10^5\ M^{-1}$. Coherin C is believed to act as a carrier peptide.

INTRODUCTION

Column gel filtration techniques have been applied extensively to the determination of protein binding characteristics of small molecules[5–7]. Thin-layer gel chromatography (TLGC) has been applied to similar studies but to a far lesser extent[8]. The differences in molecular weight between protein bound and unbound ligands are reflected in corresponding differences in their rates of elution[9–11].

Using these principles the present simplified procedure was developed for the determination of ligand binding stoichiometry on thin layers of cross-linked dextran gels over wide ranges of ligand or protein molecular weights. The logarithmic expression relating molecular weights of protein macromolecules to their migration rates on thin layers of Sephadex gels[12,13] is here found applicable, under specified conditions, to peptides and to other compounds of low molecular weight. Molar binding ratios are derived from TLGC experiments and by independent methods using fluorescence and UV absorbance.

The influence on binding of agents which induce dissociation of non-covalent protein structure is determined by incorporation of denaturing solutes into the thin-layer gel system. Multiple binding experiments may be performed on a single 20 × 20

cm thin-layer plate in periods from 30 min to 3 h by adjusting concentrations in a manner similar to a series of stoichiometric titrations .Equivalence points are located on the wet gel plates and their migration rates are recorded by exact copies on diazotized paper or by photography. The TLGC procedure is demonstrated and binding constants are estimated using as model systems the interaction of bromophenol blue (BPB) and of 8-anilinonaphthalene-1-sulphonic acid (ANS), respectively with bovine serum albumin (BSA). This procedure is particularly useful in the binding study of coherin (Coh), an enteroactive peptide complex of the neurohypophysis which dissociates upon continuous electrophoresis to yield at least four peptide subfractions[2–4,14]; for in this case Coh C, with low biological activity, has a molecular weight (MW) of about 3000, only 3 to 4 fold greater than that of the highly enteroactive peptides, Coh A and B (Table I). Thus, whether Coh A and/or Coh B may be bound to C was a matter of theoretical as well as practical interest.

MATERIALS AND METHODS

Chemical reagents and materials

All chemical compounds (reagent grade) were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.), with the following exceptions: Sephadex and Blue Dextran were products of Pharmacia (Uppsala, Sweden); fluorescamine (Flu) is a product of Hoffmann-La Roche (Basel, Switzerland). Sources of compounds used in protein binding and MW estimations are indicated in Table I. Coherin peptides were prepared in our laboratories[15]. Diazotized paper was Helios No. 21-1115, Keuffel and Esser (Morristown, N. J., U.S.A.).

Molar binding ratios and MW estimation by TLGC

The procedure for TLGC is a modification of those previously described[12,16] but differs from them in significant details. Using the applicator S-II of Brinkman Instruments, glass plates (20 × 20 cm) were spread at a thickness of 0.5 mm with suspensions (9%) of cross-linked dextran gels: Sephadex G-200, G-100, G-50, G-25 or G-10. All gels were of superfine particle size*, previously swelled in sodium acetate or in tris–trihydroxymethylaminomethane (Tris) buffer**, ionic strength 0.05, pH 7.5.

For Sephadex G-50 this application leaves a coat of about 2 g (dry weight) per plate. After application of the gel, plates were exposed to air at 25° for about 10 min or until the gel layer formed a dull surface. The plates were then transferred to a humidity chamber and left to equilibrate for 1.5 h. Samples for chromatography were dissolved in the above Tris buffer, Tris urea, Tris guanidine, acetic acid, or sodium acetate solvents, and volumes of 3 μl per sample were applied to the horizontal moist TLGC plate by micro pipette at intervals of 2 cm along a line 3 cm from the top of the plate. Small deformations in the gel surface at the point of application do not interfere with chromatography. The plate was then immediately placed at an angle of

* Sephadex G-10 superfine is not commercially available but was prepared in our own laboratory from medium grade Sephadex G-10 by molecular mill.

** To avoid the high background fluorescence of Tris, especially for location of samples after reaction with 8-anilino-1-naphthalene sulfonic acid (ANS) or Flu and to yield good sensitivity in locating samples by exposure to I_2, sodium acetate may be substituted for Tris without significant change in R_{Bac} (= 100·migration distance of sample from origin/migration distance of bacitracin A).

TABLE I

MIGRATION RATIOS (R_{Bac}) ON THIN LAYERS OF CROSS-LINKED DEXTRAN GELS

Mean migration ratios. S.D. = ±5.2%.

Compound (manufacturer)	*MW*	*G-10**	*G-25*	*G-50*	*G-100*	*G-200*
1 ACTH (Cortosyn)[a] (Organon)	2934 (refs. 32–34)		125	117		
2 8-Anilino-1-naphthalene-sulphonic acid, Na (Eastman Organic Chemicals)	321 (ref. 32)	0	23	58	89	100
3 Bacitracin A (Pfizer)	1411 (refs. 32–34)	100	100	100	100	100
4 Bromophenol blue (Eastman Organic Chemicals)	670 (ref. 32)	0	23	52	72	80
5 Bovine serum albumin (Sigma)	$6.5 \cdot 10^4$ (ref. 33)	123	169	208	185	193
6 Chymotrypsinogen A (bovine) (Pharmacia Fine Chemicals)**	$2.5 \cdot 10^4$ (ref. 33)	113	153	180	144	142
7 Cytochrome *C* (Sigma) (horse heart)	$1.2 \cdot 10^4$ (ref. 33)		154	165	133	113
8 Dextran Blue, 2000 (Pharmacia Fine Chemicals)**	$2 \cdot 10^6$	102	134	177	229	230
9 Fuchsin (basic) (Eastman Organic Chemicals)	338 (ref. 32)	0	5	34	68	82
10 γ-Globulin (bovine) (Sigma)	$1.6 \cdot 10^5$ (ref. 33)	120	157	205	231	258
11 Methionine (Sigma)	149 (refs. 32–34)	70	74	90	95	114
12 Methyl orange (Eastman Organic Chemicals)	327 (ref. 32)	5	20	52	100	
13 Ovalbumin (Pharmacia Fine Chemicals)**	$4.5 \cdot 10^4$ (ref. 33)	125	162	205	170	164
14 Pantethine (Parke Davis & Co.)	554 (refs. 32–34)	75	115	72		
15 Phlorizin (Sigma)	436 (ref. 33)		27	63	71	
16 Ribonuclease (bovine pancreas) (Pharmacia Fine Chemicals)**	$1.36 \cdot 10^4$ (ref. 33)	110	164	172	135	121
17 Tryptophan (Sigma)	204 (ref. 32)	27	45	70	71	86
18 Vitamin B_{12} (E. Merck)	1357 (refs. 32–34)	80	94	99	116	105
19 Coherin A_1 (Goodman and Hiatt)	668***	60	73	81	87	

(Continued on p. 128)

TABLE I *(continued)*

Compound (manufacturer)	*MW*	*G-10**	*G-25*	*G-50*	*G-100*	*G-200*
20 Coherin A_2 (Goodman and Hiatt)	325***	78		77		
21 Coherin B (Goodman and Hiatt)	1156***	92	75	93		74
22 Coherin C (Goodman and Hiatt)	2908***	88	87	121	119	81
23 Gly-Ser-Tyr (Wyeth Labs.)	325	70	75	83	94	
24 Gly-Tyr-Ser (Wyeth Labs.)	325	68	76	88	96	

* Categories of cross-linked dextrans (Sephadex) are designated by numbers with the Prefix G referring to water regain values ($G_{H_2O}/G_{Sephadex} \times 10$).

** Reference compounds for molecular weight determination.

*** Estimated on the basis of amino acid analysis and TLGC (ref. 15).

20° in the chromatogram jar or in the Pharmacia TLG apparatus previously saturated with water vapor, and wicks of Whatman 3 MM filter paper, saturated with buffer were affixed at top and bottom with light pressure to assure contact with the gel as previously described[12,16–18]. A period of prerun was found unnecessary. After development for $\frac{1}{2}$–2 h at 25° (the time depending upon the specific gel used) the plate was removed from the chromatogram jar, placed horizontally in a jar (10 × 26 × 30 cm) saturated with water vapor and containing a few crystals of iodine. To locate the applied samples, plates were examined while wet before and after exposure to iodine vapor under UV light in the Chromato-Vue-Cabinet (Ultra-Violet Products) at 2537A and 3660A. Plates were not allowed to dry and were kept in a horizontal position during exposure to iodine and while making azo copies to avoid dislocation of samples. After 30 min in the iodine chamber the plate was removed. The iodinated wet TLGC plates were copied directly on diazotized paper and migration ratios calculated from the azo copies (Fig. 1A). Migration distances were measured from the point of origin. A reference compound, generally bacitracin A (Bac), because of its molecular weight, and its ease of visualization, was included routinely on each plate; migration distances were expressed as a ratio (R_{Bac}) relative to that of Bac as the standard. Determinations of migration rates were repeated over 10 times.

To determine concentrations of bound and unbound ligands, sets of 4 plates (5 × 20 cm) were coated with Sephadex G-50 and were developed in groups of 4 as a single TLGC plate. After development, one was removed for location of ligands. Corresponding bands on the remaining 3 plates were scraped off, eluted and quantified by UV absorption or by fluorimetry. Alternatively, 9 or more aliquots of a single sample were quantified in a similar way on a single plate (20 × 20 cm).

Denaturants

Urea and guanidine. Sephadex G-50 superfine, was suspended in Tris buffer, ionic strength 0.05, pH 7.50 containing urea (8 *M*) or guanidine hydrochloride (8 *M*) and was kept at 25° for 24 h before spreading on glass plates at a thickness of 0.5 mm. Samples of proteins or polypeptides were equilibrated for at least 24 h in the same buffer solutions containing urea or guanidine before application to the thin-layer plates.

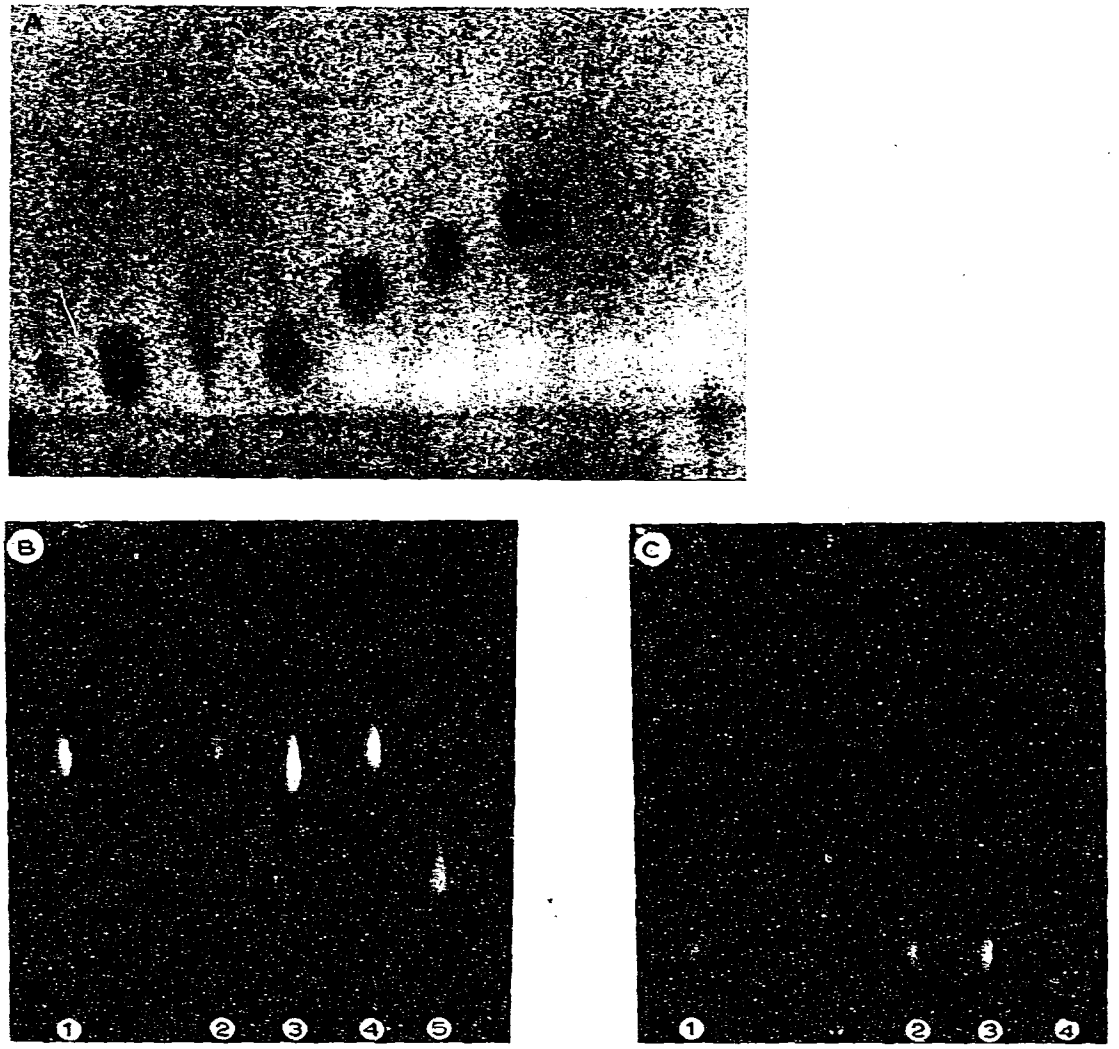

Fig. 1. TLGC of unbound coherin peptides compared with reference compounds and with ANS–bound Coh C. (A) Diazo copy of 20 × 20 cm glass plate coated with a layer of Sephadex G-50 superfine, 0.5 mm thick, developed in 0.05 *M* sodium acetate, pH 7.5, and iodinated. Samples (20 μg; of each) are (from left): 1, Coherin A_1; 2, Coh B; 3, Coh C; 4, bacitracin; 5, ribonuclease; 6, chymotrypsinogen A; 7, ovalbumin and 8, BSA.

The azo copy was made by placing the iodinated plate on a sheet of diazotized paper and illuminating with UV light (four parallel fluorescent lamps, 15 W, G.E. 15T8BL) at a distance of 24 cm for 15 sec. Development was in the NH_3 chamber for 2 min. (B) A TLGC plate of Sephadex G-100 developed as in (A) but with no iodination, containing BSA and ANS. Fluorescence was induced by UV excitation at 366 nm. Samples (from left): 1, BSA (0.3 n*M*) + ANS (0.15 n*M*); 2, BSA (0.3 n*M*) + ANS (0.3 n*M*); 3, BSA (0.3 n*M*) + ANS (0.6 n*M*); 4, BSA (0.3 n*M*) + ANS (0.9 n*M*); 5, Bac (20 n*M*). (C) TLGC of ANS–bound Coh C on Sephadex G-50. Solvent, development and UV excitation as in (B). Samples (from left): 1, ANS (3 n*M*); 2, ANS (0.15 n*M*) + Coh C (0.75 n*M*); 3, ANS (0.3 n*M*) + Coh C (0.75 n*M*); 4, Bac (20 n*M*).

Sodium dodecylsulphate, dimethyl sulphoxide and dimethyl formamide. A solution of sodium dodecylsulphate, 0.1 *M* was prepared in Tris buffer, pH 7.50 0.05 *M*. Protein or peptide samples were dissolved in this solution (10 mg per ml) and allowed to stand at 25° for a minimum of 16 h before application to the gel plate. Similar solutions of test compounds were prepared containing dimethyl sulfoxide (2.82 *M*) and dimethylformamide (3.34 *M*), respectively. Higher concentrations of these reagents caused samples to precipitate.

Performic acid oxidation[19]

BSA (50 mg) was dissolved in formic acid, 1.0 ml, 88%. To this solution was added H_2O_2, 1.0 ml, 30%. After 16 h at 25° the solution was concentrated to dryness *in vacuo*. The dry residue was twice suspended in 3 ml 50% ethanol and concentrated to dryness *in vacuo*. The residue was dissolved in 2.50 ml Tris buffer, pH 7.50. Samples of these solutions were applied to thin-layer plates as described above.

Protein binding

For each binding experiment solutions were prepared containing a ligand (*A*) of low molecular weight and a protein (*P*). Molar concentration ratios of the two reactants (*A*):(*P*) were adjusted to cover the range from 0.1 to 10. Mixtures were maintained at 25 or 38° for periods up to 24 h before TLC. There was no significant difference in binding ratios after longer periods of incubation. Low MW ligands used in the present study include: Coh A_1, B, C, ANS (Na or Mg salt), BPB and Flu.

An experiment on the binding stoichiometry of ANS to BSA illustrates the general method: to each of 14 tubes containing 100 μl of BSA, $3.0 \cdot 10^{-4}$ *M* in Tris buffer, pH 7.50, 0.05 *M*, is added ANS ($2 \cdot 10^{-3}$ *M*) in Tris buffer to contain molar ratios of ANS:BSA from 1.0 to 10. Tris buffer is added to make a final volume of 300 μl per tube. After 1 h at 25°, aliquots of 3 μl from each tube are applied to plates coated with Sephadex G-50, superfine for chromatography as described above. After developing, samples are visualized on the TLGC plate by photocopy, UV light or by iodination, and samples of maximum fluorescent emission or UV absorption are identified. For quantification, plates are prepared containing samples in triplicate. The desired spots containing bound and unbound ligands are scraped off while wet and eluted with tris buffer (2×0.5 ml per spot) for determination of UV absorbance or for fluorimetry. The binding of BPB to BSA was used as a model system in parallel experiments (Table II).

To study the binding properties of Coh peptides, mixtures of Coh A with C, and B with C, respectively were incubated and then applied to TLCG plates as described above. Similarly mixtures of coherin peptides were prepared with ANS, BPB, BSA and with Flu, respectively (Table II).

For reaction with Flu[20] solutions of Coh A_1, B and C were prepared in graded concentrations from 10^{-5} to 10^{-6} *M* in 0.05 *M* sodium acetate, pH 8.5. A 10-μl volume of fluorescamine solution (0.33 mg/ml in acetone) was added to 100 μl of each Coh sample. After 1 h at 25° aliquots of 3 μl of each solution were applied to TLGC plates as described above. Fluorimetric titrations were recorded using 20 μl of each solution with excitation 368 nm and fluorescent emission, 475 nm (Fig. 2).

UV and visible absorption spectra were determined with the Cary Spectrophotometer, Model 11. Fluorescent characteristics were determined by means of the Fluorispec, Model SF-1 (Baird-Atomic).

TABLE II

BINDING OF COHERIN PEPTIDES COMPARED WITH BSA

Areas of Sephadex containing bound or unbound ligands were scraped from the wet TLGC plate, and eluted with 0.1 *M* acetic acid. Quantification was by: (a) fluorimetry; activation, 368 nm; emission, 475 nm. (b) Fluorimetry; activation, 380 nm; emission, 470 nm (ref. 21). (c) Absorbance, 590 nm. Apparent binding constants were estimated by Scatchard plots.

Reactants	*r*	$K_b \cdot 10^5\ M^{-1}$	R_{Bac}
Coherin peptides			
A_1 + C (a)	2.1	0.2	96
A_1 + Flu			93
B + C (a)	1.8	0.1	101
B + Flu			93
C + ANS (b)	1.2	6.1	60
C + BPB (c)	1.1	3.4	50
C + Flu			123
BSA + ANS (b)	2.2	18.	213
BSA + BPB (c)	1.1	8.6	210

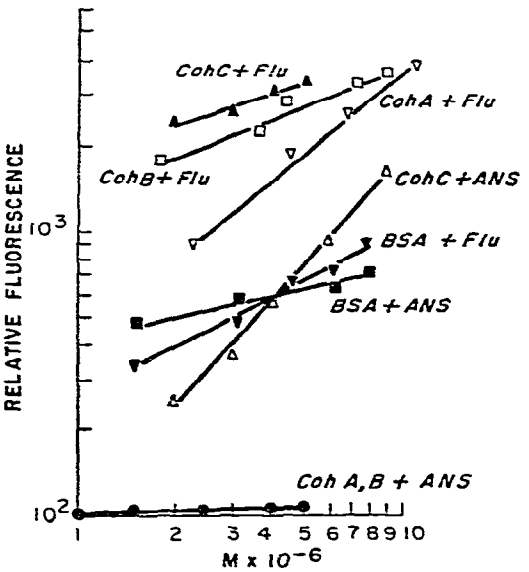

Fig. 2. Log–log plot of fluorescence intensity *vs.* concentration of coherin peptides and BSA upon binding to ANS and to Flu, respectively.

RESULTS

Molecular weights

Using compounds of known molecular weight (Table I), migration distances relative to Bac were determined at pH 7.50. Migration ratios (R_{Bac}) for each of the compounds used in this study were essentially constant between pH 6.0 and 9.0. In some cases R_{Bac} values were altered at pH below 6 and solute precipitation frequently occurred below pH 4. When using Sephadex G-50, migration ratios of known refer-

ence compounds plotted against $\log_{10}$ MW takes the form of a straight line ($y = k \log_{10}$ MW) (Fig. 3). MW values fall on the standard curve for a substantial number of compounds studied between MW 300 and 60,000. Similar logarithmic relationships were obtained using superfine Sephadex G-100 and G-25. Results using Sephadex superfine G-10 and G-200 were more erratic. The slope varied with each class of gel. Departures from the standard curve were numerous for all categories of Sephadex.

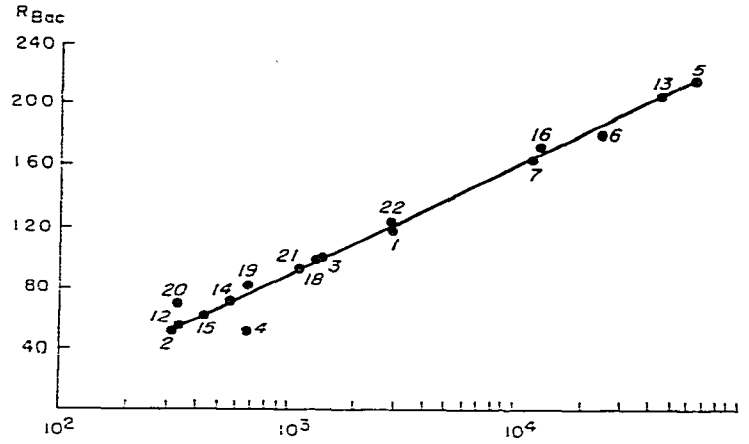

Fig 3. Semi-log plot of migration ratios (R_{Bac}) *vs.* $\log_{10}$ MW of coherin peptides and reference compounds on thin layers of Sephadex G-50. Numbers refer to compounds listed in Table I. Mean standard deviations are less than $\pm 5\%$.

In attempting to select the most suitable gel for binding studies by TLGC the migration ratios of known compounds were compared using each of the five Sephadex gels. The most useful differences in R_{Bac} between compounds of high and low MW were developed using Sephadex G-50 or G-100 superfine.

In TLGC studies of more than 120 known compounds of MW less than 1200 on Sephadex G-50 superfine, none have shown R_{Bac} values greater than 100, but the sharpness of the molecular sieving effect is blurred particularly in the case of G-10 and G-200 (Fig. 4).

The addition of β-mercaptoethanol, ethylenediamine tetraacetate (EDTA), concentrated urea or guanidine hydrochloride did not significantly affect the relative migration rates of BSA, ribonuclease, or other solutes on thin-layer plates of Sephadex G-50.

Performic acid oxidation of BSA caused molecular degradation resulting in two major fragments with R_{Bac} 95 and 180, respectively. The former component showed strong fluorescence when exposed to UV at 366 nm. Neither dimethyl sulfoxide nor dimethylformamide caused a significant change of R_{Bac} for BSA.

Binding experiments

For the determination of molar binding ratios of ligands to proteins, solutions of BSA were treated with varying concentrations of the required ligand. The resultant mixtures were applied to thin-layer plates of Sephadex G-50 superfine, for chromatographic separation as described above.

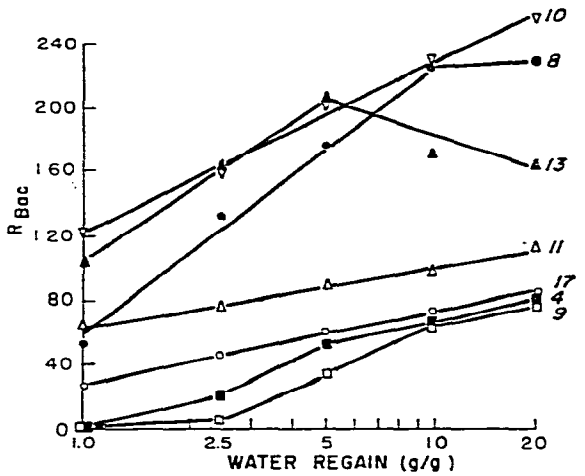

Fig. 4. Semi-log plot of R_{Bac} *vs.* $\log_{10}$ of Sephadex water regain values (grams water per gram of dry Sephadex.) Numbers refer to compounds listed in Table I.

When the unbound ligand, ANS, is present in excess, it migrates at a rate which is about 0.2 that of the protein bound ligand. Using appropriate concentrations of A and P the molar binding ratio, r, is determined where P–A migrates as a single component leaving a minimal residue of A.

Fluorimetry demonstrates that ANS binds to BSA forming a highly fluorescent product whose quantum yield is about 200-fold greater than that of free ANS[21].

Using the thin-layer gel technique here described, the molar binding ratio for ANS to BSA is 2. Mixtures of ANS and BSA were prepared over a range of concentrations. Samples of each mixture were treated chromatographically as described above. When the molar ratio of ANS:BSA was in excess of 2, a residue of unbound ANS appeared as a slow moving spot ($R_{Bac} = 60$) of weak fluorescence on the thin-layer plate. The ANS/BSA bound complex formed a highly fluorescent spot which moved at the same rate as native BSA ($R_{Bac} = 208$) (Fig. 1B). Sephadex gel containing ANS–BSA spots as well as ANS were scraped off, eluted with Tris buffer and assayed by UV absorbance and by fluorimetry.

To confirm the chromatographic observations, UV absorption and fluorimetric characteristics were determined. ANS binds to BSA to produce an adduct which differs markedly in UV absorption and in fluorimetric characteristics from either component alone[21]. ANS was titrated with BSA to maximum fluorescence at the wavelength of maximum excitation (380 nm) and maximum fluorescent emission (470 nm). The results of this titration confirmed those obtained by TLGC. Maximum fluorescence was achieved at a mole ratio ANS:BSA of 2. The same mole ratio was obtained by fluorimetry using BSA concentrations in the range 10^{-7}–10^{-4} M. The concentrations of bound and unbound ligands were determined by UV absorbance of samples eluted from the Sephadex gels. Semiquantitative stoichiometric estimations of binding ratios may be made in the case of BPB and of ANS simply by inspection of developed TLGC plates in UV and white light. Using BPB the protein bound ligand is visible as a blue spot. The excess unbound BPB is also visible as a blue spot. However, when the molar ratio (BPB:BSA) = 1, the bound ligand moves as a single

component, leaving only a minimal residue in the position occuried by BPB. This observation was used in further model experiments on the binding of BPB to BSA. Mixtures were prepared parallel to those with ANS. Location of bound and unbound ligands by virtue of the blue dye was convenient. Elution and quantification of appropriate samples by UV absorbance resulted in recovery of 98% of the total BPB in the protein bound ligand with the optimum molar ratio BPB:BSA = 1. These results were obtained at pH 7.5 as well as 3.5. BPB shows only slight fluorescence in 0.05 *M* Tris buffer or in sodium acetate pH 7.50 but after drying on a thin-layer plate of Sephadex G-50 or G-25 it shows red fluorescence ($\lambda_{max.}$ of fluorescent emission of BPB in Tris buffer, pH 7.5, is 430 nm upon excitation at 366 nm). This fluorescence has been found unavailable for fluorimetric estimation of *r* in solution for upon binding of BPB to BSA quenching of fluorescence occurs. Bac, whether in solution or allowed to dry on thin-layer plates of Sephadex, also shows strong fluorescence when exposed to UV (366 nm). When BSA is denatuted with performic acid or with 8 *M* urea TLGC demonstrates that binding of BPB does not occur. Treatment of BSA with dimethyl sulfoxide or dimethylformamide does not interfere with the binding of BPB.

Mixtures of coherin peptides were prepared as described above. TLGC of those solutions on Sephadex G-50 demonstrated the formation of a highly fluorescent adduct as a result of ANS binding to Coh C (Fig. 2 and Table II).

The molar binding ratio (ANS:Coh C) determined by TLGC was in agreement with that by fluorimetry. Neither Coh A, nor Coh B reacts in a similar way with ANS or with BPB to yield a bound ligand in the range of concentrations used in this study.

Coh A and B, respectively are bound to C in a molar binding ratio of 2. Coh A, B and C, when treated individually with fluorescamine react to form covalently bound fluorescent products which have the same R_{Bac} values as the unbound peptides (Table II).

DISCUSSION

Molecular weights

Previous determinations of molecular weights by TLGC have been applied mainly to proteins[10,12,16] and to a smaller number of peptides[13]. Present results demonstrate the applicability of this principle within limitations to the determination of binding characteristics of polypeptides and other small molecules of varied structures.

On the basis of the hypothesis that chromatography on cross-linked dextran gels may operate by a mechanism of osmotic pressure it is reasonable to assume that this mechanism should apply to the movement of small molecules as well as large into and out of the gel micelles.

Proteins of MW > 10,000 are considered excluded by the highly cross-linked dextran gels, Sephadex G-10, G-25 and G-50. Nevertheless, the method here described demonstarates fractionation in these categories of Sephadex and shows that the migration rates of a substantial number of compounds are approximately within the range predicted by their MWs.

In order to obtain consistent values in the determination of MWs of proteins by gel chromatography on columns, it has been found useful [13,17,18,22–24] to dissolve the

proteins in solutions containing one of the denaturants, guanidine hydrochloride, LiCl, β-mercaptoethanol, urea or EDTA. The denatured molecules are excluded from gel media which normally admit the native protein. Such denaturants should be unnecessary in the case of peptides and other compounds of low MW.

Using the thin-layer procedure, the above reagents did not affect the migration ratios of either the proteins or of small ligand molecules. Urea (5 or 8 *M*) or guanidine HCl (8 *M*) caused a decreased migration rate of all solutes but the rates relative to Bac remained unchanged.

Our observations relative to the effects of urea on BSA are in accord with those of previous workers who concluded that serum albumin in concentrated urea undergoes virtually no change in MW [25].

Blue dextran 2000, because of its high MW ($2 \cdot 10^6$), is considered excluded from the gel granules and is therefore used as a marker for void volume in columns of Sephadex gels[26].

Present results are in accord with earlier studies[23,24] which demonstrate that the migration characteristics of blue dextran on thin layers of Sephadex differ from those on columns. For in addition to excessive zone spreading in TLC on Sephadex G-10, G-25 and G-50, the R_{Bac} values of blue dextran are lower than that of BSA, and in G-100 and 200 they are lower than that of γ-globulin (Table I and Fig. 4).

To avoid these difficulties, alternative markers for void volume have been introduced[23].

Whether migration rates of proteins in Sephadex gels are best correlated with Stokes radii or with molecular weights has been the subject of extensive discussion[16,24,27]. However it is assumed that in the range of low MWs where the frictional ratios and partial specific volumes are closely similar, the linear correlation between migration rates and MWs are valid[16].

In determinations of MWs of proteins by gel chromatography those compounds which do not fall on the logarithmic curve are said to exhibit "anomalous MWs"[12]. A number of "anomalous" values were noted in the present study, especially in the category of low MW ligands. The differences of migration ratios are reproducible and significant. Whether they can all be attributed to differences in MW is debatable. However, these differences might reflect properties such as hydration, solvation, dimer or polymer formation[28]. Despite the "anolmaous" values observed in TLGC of numerous reference compounds, the MWs determined by this method may be considered a useful first approximation.

Although departures from linearity in the relationship between R_{Bac} and log MW are numerous, the applicability of TLGC to the study of binding small ligands to proteins is not impaired, for the differences in R_{Bac} between the ligands and protein are sufficient to demonstrate binding irrespective of the exact adherence to the linear equation.

Binding characteristics of proteins and peptides

In the range of concentrations used here, a large excess of *A* does not affect the molar binding ratio *A*:*P*. Thus it appears that the high binding constant of ANS to BSA ($3.2 \cdot 10^6\ M^{-1}$, ref. 26) or BPB to BSA results in the complete saturation of the binding sites of BSA and that having been filled no further binding occurs even in the presence of a multimolar excess of free ligand.

Although protein bound ligands migrate on thin layers of Sephadex G-50 at about the same rate as that of unbound *P*, the migration rate of pure (*A*) is somewhat lower than that in the presence of *P*. For example R_{Bac} for pure unbound ANS is 58, compared with R_{Bac} of ANS isolated from the mixture with BSA, R_{Bac} 65. It is unlikely that this increase in R_{Bac} represents the formation of a new adduct, for its low R_{Bac} value would preclude a protein bound product. The increase in R_{Bac} under these conditions may be the result of continuous dissociation of the bound complex as discussed by Cann and Hinman[7].

Migration ratios of bound ANS–BSA are in accord with the fluorescent titration data (Fig. 3). The addition of 2 moles of ANS would be expected to alter the molecular weight of BSA by only about 0.5%. The fact that the ANS–BSA adduct in which $r = 2$ moves as a single spot with R_{Bac} the same as unbound BSA is further indication of the stability of this protein-bound ligand .

The present TLGC procedure, although most useful in reactions with high binding constant (K_b) values, provides a useful adjunct to fluorimetric titration as a guide to binding for it allows the convenient identification and quantification of the bound ligand in isolation from either excess protein or excess ligand. Either of the latter excesses may result in quenching of fluorescence.

Among the R_{Bac} values for free and bound ligands, some specific features are noted. Whereas the ionically bound ligand ANS–BSA migrates on TLGC (G-50) to the position of unbound BSA ($R_{Bac} = 213$), the major fluorescent product of bound ANS–Coh C migrates to a position nearly identical with that of unbound ANS ($R_{Bac} = 50$, Fig. 1). On the other hand the covalently[30] linked fluorescamine product, Flu–Coh C, migrates to the position of unbound Coh C ($R_{Bac} = 121$). Similarly Flu–bound Coh A and Coh B, respectively migrate to the position of the unbound peptides (Table II).

The interpretation of these differences in migration rates between ionic and covalent bound coherin peptides is the subject of further study.

Since Coh A_1 and B under present conditions are bound to Coh C ($r = 2$), we conclude that Coh C may indeed function as a carrier for these highly enteroactive peptide molecules.

A comparison of dextran gels of various degrees of cross-linking has demonstrated that Sephadex superfine G-50 and G-100 are the most suitable for use in TLGC binding studies (Fig. 4). R_{Bac} values are the most consistent and the technical difficulties encountered such as dehydration or adjustment of solvent flow, across the plate are the least troublesome. However, in principle, any one of the gels listed here could be adapted for specific binding experiments. Our earlier studies[1] using Sephadex G-10 superfine for example, together with the present report, have demonstrated a substantial fractionation of peptides and other molecules having MWs between 200 and 4000. Sephadex G-10 in TLGC appears to function by a mechanism combining adsorption and molecular sieving.

It is noteworthy that, with some exceptions, the migration ratios determined by TLGC bear a logarithmic relationship to the water regain values of Sephadex gels (Fig. 4). Water regain is a function not only of pore diameter[31] but of the hydrophilicity and polarity of the gel. The more highly cross-linked dextrans (G-10, G-25) are at the same time less polar and more hydrophobic due to the obliteration of glycosidic hydroxyl groups.

CONCLUSIONS

Because of its simplicity and its successful application to the characterization of proteins and peptides in the microgram range, the literature on TLGC continues to expand at an accelerating rate. Technical limitations which have been a deterrent to more extensive use of this approach[16] have been significantly reduced by the present procedures including improved visualization and recording of migration rates. Although quantification may still be somewhat cumbersome for precise determinations of ligand concentrations, semiquantitative, binding data may be developed rapidly with short exposure time and with minimal dissociation of bound ligand. K_b values derived by TLGC are in reasonable agreement with those derived by direct fluorimetry. This method when applied to ligand binding has the advantages of and resembles that previously referred to as "frontal analysis"[5]. TLGC has been a useful technique in the present study of binding interactions between coherin peptides. Since these are all compounds of low MW, available in extremely small quantity, the classical methods involving equilibrium dialysis would be hazardous.

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