Biochemistry

Volume 16, Number 2 January 25, 1977

Binding of 4-Methylumbelliferyl α-D-Mannopyranoside to Tetrameric and Unmodified or Derivatized Dimeric Concanavalin A: Equilibrium Studies†

Frank G. Loontiens,*,‡ Robert M. Clegg, and Thomas M. Jovin

ABSTRACT: The binding of 4-methylumbelliferyl α-D-mannopyranoside (MUM) and concanavalin A, composed of intact polypeptide chains, was studied by equilibrium dialysis, difference spectroscopy, and fluorescence titration (Dean, B. R., and Homer, R. B. (1973), *Biochim. Biophys. Acta 322*, 141–144), measured either at a fixed wavelength or above 350 nm. Dimeric and tetrameric concanavalin A samples were used under conditions of apparently full metal saturation. The results are consistent with a single carbohydrate-specific site per protomer, without interaction between sites; no indication for additional unspecific binding could be obtained. The values of the association constant are independent of the method or of the saturation range used and 4-methylumbelliferyl α-D-mannopyranoside, bound at a fractional saturation of 0.91, can be totally displaced by methyl α-D-mannopyranoside. The thermodynamic binding parameters for acetylated or succinylated concanavalin A, composed of intact polypeptide chains, were obtained by titration of total MUM fluorescence in the temperature range 9–39 °C. For unmodified dimeric concanavalin A at 25.0 °C, the values are $K = (3.36 \pm 0.04)10^4$ M^{-1} with $\Delta H° = -8.3 \pm 0.1$ kcal mol^{-1} and $\Delta S° = -7.2 \pm 0.3$ eu; for tetrameric concanavalin A, the affinity is increased by 25% and within experimental error the values of $\Delta H°$ and $\Delta S°$ are identical to those for the dimeric protein. Derivatized concanavalin A shows binding characteristics that are entirely comparable to those of the native protein.

Concanavalin A, a protein isolated from the Jack bean (*Canavalia ensiformis*) belongs to the class of lectins (Lis and Sharon, 1973). These proteins mediate a wide range of biological effects as a consequence of their ability to bind to the carbohydrate moiety of glycoprotein receptors on cellular membranes (Tooze, 1973; Nicolson, 1974). The ensuing cell response as well as the interactions of con A with polysaccharides are specifically inhibited by low-molecular-weight carbohydrates (Poretz and Goldstein, 1970). The best inhibitors are α-D-mannopyranosides and, especially, α(1 → 2) linked mannotriose (Goldstein, 1975).

The quaternary structure of concanavalin A is pH dependent: at pH 7.0 or higher the protein is a tetramer of molecular weight 102 000; at pH 5.6 or lower it is a dimer (Kalb and Lustig, 1968; McCubbin and Kay, 1971). Acetylated or succinylated concanavalin A can form only dimers (Gunther et al., 1973). The primary sequence (Wang et al., 1975; Cunningham et al., 1975) and the three-dimensional structure (Becker et al., 1975; Reeke et al., 1975) have been reported. Carbohydrate binding necessitates a transition metal ion and Ca^{2+} (Kalb and Levitzki, 1968; Shoham et al., 1973), but the requirement of Ca^{2+} has been disputed (Brewer et al., 1974).

In solution, concanavalin A possesses one binding site for carbohydrates per protomer (So and Goldstein, 1968; Yariv et al., 1968). The absorption difference spectrum of *p*-nitrophenyl α-D-mannopyranoside (Hassing and Goldstein, 1970) has been used to study the binding kinetics by stopped flow (Gray and Glew, 1973; Lewis et al., 1976). We decided to use 4-methylumbelliferyl α-D-mannopyranoside (MUM[1]), a very sensitive fluorescent probe due to the total quenching of its fluorescence upon binding to concanavalin A (Dean and Homer, 1973). The present paper characterizes the binding equilibria of MUM with dimeric, tetrameric, acetylated, and succinylated con A in terms of thermodynamic parameters. These will be compared in the following paper (Clegg et al., 1977) with the results obtained from temperature-jump relaxation kinetics.

† From the Laboratory of General and Biological Chemistry, State University of Gent, B-9000 Gent, Belgium and from the Abteilung Molekulare Biologie, Max-Planck-Institut für Biophysikalische Chemie, D-34 Göttingen, Postfach 968, West Germany. *Received June 17, 1976.* This work was supported in part by the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek. A preliminary account of this work was delivered at the 10th FEBS meeting in Paris, abstract no. 995, July 20–25, 1975.

‡ Recipient of an EMBO Fellowship.

[1] Abbreviations used are: con A, concanavalin A composed of intact polypeptide chain (the unabbreviated concanavalin A is used for the protein prepared by affinity chromatography on Sephadex G-75); MUM, 4-methylumbelliferyl α-D-mannopyranoside.

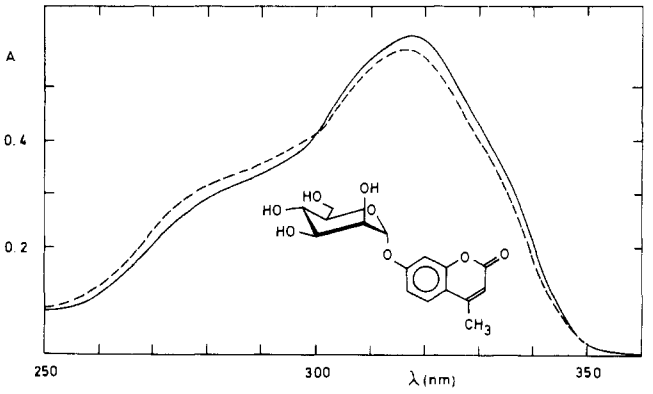

FIGURE 1: Absorption spectrum of 43.9 μM MUM without (full line) and in the presence (dashed line) of 35.1 μM con A, measured with pH 5.5 buffer or 35.1 μM con A in the reference beam, respectively. Optical path length, 1 cm; temperature, 20 °C.

Experimental Procedure

Materials. Concanavalin A was isolated (Agrawal and Goldstein, 1967) from powdered Jack bean meal (Serva, Heidelberg) as a lyophilized powder. Unless stated otherwise, con A (composed of intact polypeptide chains) prepared by mild alkaline treatment (Cunningham et al., 1972) was used throughout. Succinylated con A and acetylated con A, both composed of intact polypeptide chains, were prepared according to Gunther et al. (1973) as lyophilized powders; when examined by thin-layer gel filtration on Sephadex G-200 at pH 7.2, both samples of modified proteins migrated between ovalbumin and serum albumin. Protein samples were dissolved in 1 M NaCl or in an appropriate buffer, always with a 25-fold molar excess of both $NiCl_2$ and $CaCl_2$. These were allowed to stand at room temperature for at least 1 h, prior to exhaustive dialysis at 4 °C against either 0.05 M NaOAc–HOAc, 1 M NaCl, 1 mM $NiCl_2$, 1 mM $CaCl_2$ (pH 5.5) or 0.05 M Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1 M NaCl, 1 mM $NiCl_2$, 1 mM $CaCl_2$ (pH 7.2). These conditions afforded high solubility for dimeric (30 mg/ml) and tetrameric (10 mg/ml) con A, without any precipitation over several months. Protein concentrations were determined at 280 nm in a Zeiss PMQII-M4QIII spectrophotometer, using the experimental $\epsilon_{280} = 1.14$ cm^2 mg^{-1}, in accordance with Agrawal and Goldstein (1967). This value was found to be constant in the pH range 4–8 and was also used for derivatized con A. The molar concentrations of con A are expressed on the basis of a molecular weight of 25 500 (Wang et al., 1971).

MUM (Figure 1) was prepared according to Vervoort and De Bruyne (1970). It was practically free of 7-hydroxy-4-methylcoumarin, as judged by absence of fluorescence on thin-layer chromatography plates corresponding to the latter component. Concentrations of MUM solutions were determined at 318 nm using $\epsilon_M = 1.36 \times 10^4$ M^{-1} cm^{-1}; at 334 nm, this value is 7.94×10^3 M^{-1} cm^{-1}. $\alpha(1 \rightarrow 2)$ linked mannotriose was isolated from the cell wall of *Saccharomyces cerevisiae* (Lee and Ballou, 1965). By thin-layer chromatography, it was identical with authentic samples obtained from C. E. Ballou and P. A. Gorin.

Sedimentation velocity measurements (40 000 rpm) and sedimentation equilibrium measurements (10 000 rpm) were performed at 20 °C in a Spinco, Model E, analytical ultracentrifuge equipped with an automatic photoelectric scanner. Three samples were run simultaneously using 12-mm double-sector cells in a four hole ANF rotor. Samples were scanned in sequence at 4-min intervals, or, after 30 h, at 280 nm (0.61 mg of con A/ml) or 290 nm (3.1 mg/ml). The partial specific volume (0.730) was calculated from the amino acid composition (Cunningham et al., 1975).

Turbidity of con A–glycogen mixtures was determined at pH 7.0 and 25 °C (Loontiens et al., 1973).

Equilibrium dialysis was performed in Perspex cells separated by a semipermeable membrane (A. H. Thomas, Philadelphia, 5/8 in. dialysis tubing). The half-cells were filled with 0.6 ml (reproducibility better than 99.9%) of MUM (20–300 μM) and con A (1, 1.5, and 2 mg/ml). For each concentration couple, six double cells were filled. Equilibrium was established after 24 h (25–26 °C) or 72 h (2–4 °C).

Ultraviolet absorption difference spectra using MUM as a ligand were obtained as reported for *p*-nitrophenyl α-D-mannopyranoside binding to con A (Hassing et al., 1970; Bessler et al., 1974) with a Cary 16 double-beam spectrophotometer, equipped with thermostated cuvette holders (±0.1 °C). Some measurements at 334 nm were made with a Zeiss PMQII-M4QIII spectrophotometer. The baseline was checked with both unmixed and mixed reference and sample cells. Both halves of tandem 2 × 0.437 cm cuvettes were filled with reproducible volumes (0.8 or 0.6 ml) of MUM solution and con A solution. Any nonchromophoric competitive ligands used were contained in the con A filling solution. The association constant was estimated according to Dahlquist et al. (1966).

Fluorescence spectra were recorded with a Zeiss spectrofluorimeter using 0.6-mm slit widths on both M4QIII monochromators, an Osram 450-W Xenon lamp and a thermostated cuvette holder (±0.2 °C). 1 × 1 × 4.5 cm cuvettes were used and the spectra were uncorrected. All solutions used for fluorescence measurements were passed through 0.8-μm membrane filters (Sartorius, Göttingen) prior to use.

Titrations of MUM fluorescence quenching were routinely performed with a Vitatron MPS clinical photometric system equipped with a 45-W Hg lamp. The excitation wavelength (313 nm) was selected with an interference filter with maximal transmission at 316 nm (17% maximal transmittance, half-width 6.5 nm) and a Schott WG 360-2 or WG 360-3 secondary filter (50% cutoff at 348 or 359 nm) was placed at 90° in front of an IP-28 photomultiplier. 3.000 ml of buffered MUM solution (3 μM) was added to the fluorescence cuvette, equipped with a motor-driven stirrer and a capillary inlet. The assembly was mounted in a thermostated (±0.05 °C) copper holder. Buffered protein solution (0.5–1 mM) was added from a calibrated Agla micrometer syringe under continuous stirring. Blank titrations without MUM were performed at each temperature. Three methods of analysis were used to determine the binding parameters: the method of Scatchard (1949), the method of Kurganov et al. (1972), and an iterative nonlinear fitting of the titration data to the binding equation (modified Gauss–Newton method). The starting parameters in the latter method were obtained from the Scatchard plot. With the Kurganov method, a single binding site per 25 500 daltons was assumed and the final amount of quenching was determined graphically (Kurganov et al., 1972) or preferably from the corresponding minimal error of the association constant, as calculated over 15 to 20 equally spread values of relative fluorescence. Dilution of the MUM solution was taken into account in all cases.

Results

Hydrodynamic Properties. At 20 °C and in the pH 5.5 and pH 7.2 buffers containing 1 M NaCl, the $s^0_{20,w}$ values were 3.32 and 5.59, respectively (0.61 mg/ml). The molecular

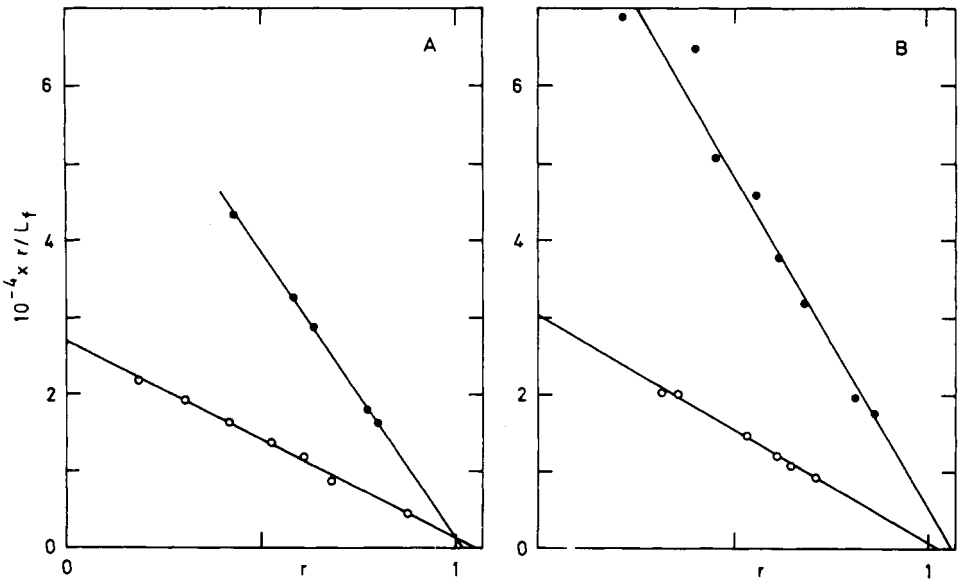

FIGURE 2: Scatchard plots for the binding of MUM to con A from the data obtained by equilibrium dialysis at 2-4 °C (●) and at 25-26 °C (○) at pH 5.5 (A) and 7.2 (B). The binding was monitored at 318 nm for free MUM only. r is the ratio of bound ligand to total protein (expressed as protomer) and L_f is the free MUM concentration at equilibrium.

weights, obtained from sedimentation equilibrium, were 47 600 and 91 060 at pH 5.5 and 7.2. This indicates that at pH 7.2 the con A tetramer shows no appreciable dimerization in the presence of 1 M NaCl. The binding of MUM to con A was studied by four methods: inhibition of turbidity formed between glycogen and con A, equilibrium dialysis, absorption difference spectroscopy, and titration of MUM by fluorescence quenching.

Inhibition of Turbidity. The inhibition of turbidity formed between glycogen and con A shows MUM to be a 4.3 times better inhibitor than methyl α-D-mannopyranoside. The molarity of MUM giving 50% inhibition is 69 μM. This concentration leads to the estimated association constant of 2.5×10^4 M^{-1} (Loontiens et al., 1975), which is in fair agreement with the reported constant of 3.4×10^4 (Dean and Homer, 1973) and with the results given below.

Equilibrium Dialysis. The high absorptivity of MUM (Figure 1) allows a direct determination of the binding parameters by equilibrium dialysis. In order to avoid corrections for the decreased absorptivity (Figure 1) of bound MUM, the extent of binding was calculated from the absorption of MUM in the protein-free half-cell and from its amount initially added. The Scatchard plots for dimeric and tetrameric con A are given in Figure 2. The values for the association constant, K, and the number of binding sites, n, are as follows: at 25–26 °C $K = (2.6 \pm 0.1)10^4$ M^{-1}, $n = 1.04 \pm 0.04$; at 2–4 °C $K = (7.5 \pm 0.1) \cdot 10^4$ M^{-1}, $n = 1.02 \pm 0.02$. For tetrameric con A at pH 7.2: at 25–26 °C $K = (3.0 \pm 0.1)10^4$ M^{-1}, $n = 1.06 \pm 0.03$; at 2–4 °C $K = (8.9 \pm 0.6)10^4$ M^{-1}, $n = 1.06 \pm 0.04$. The affinity of MUM is about 15% higher for tetrameric con A than for dimeric con A. The enthalpy of binding can be estimated as -7.9 ± 0.4 kcal mol^{-1} for dimeric and -8.0 ± 0.8 kcal mol^{-1} for tetrameric con A.

Absorption Difference Spectra. Upon binding to con A, the absorption spectrum of MUM undergoes a blue shift (Figure 1). The resulting difference spectrum shows two extremes at 322 and 334 nm (Figure 3). The occurrence of this difference spectrum is directly related to the binding of carbohydrates, as was evidenced by the inhibition of mannotriose (Figure 3). As shown in Figure 4, the difference spectrum due to MUM

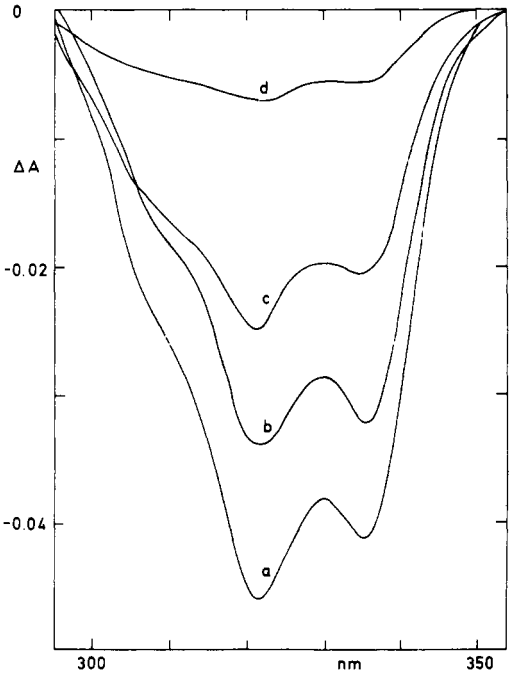

FIGURE 3: Difference absorption spectra formed between 34.5 μM con A and 44.8 μM MUM without (a) and in the presence of increasing concentrations of mannotriose: 10.2 μM (b), 40.8 μM (c), and 123 μM (d). All concentrations are for the cell content after mixing. The pH was 5.5 and the temperature 20.3 °C.

binding at a fractional saturation as high as 0.91 can be completely inhibited by methyl α-D-mannopyranoside. This illustrates that the difference spectrum due to MUM binding only arises from binding at the carbohydrate-specific site. From a series of difference spectra obtained with a constant concentration of MUM at different protein concentrations (Figure 5), the maximal change, $\Delta\epsilon_{max}$, was estimated from a double-reciprocal plot. The values in units of M^{-1} cm^{-1} are -2.37 10^3 at 322 nm ($\epsilon_M = 1.27 \times 10^4$) and -2.21×10^3 at 334 nm ($\epsilon_M = 7.94 \times 10^3$). Measurements at the latter wavelength give

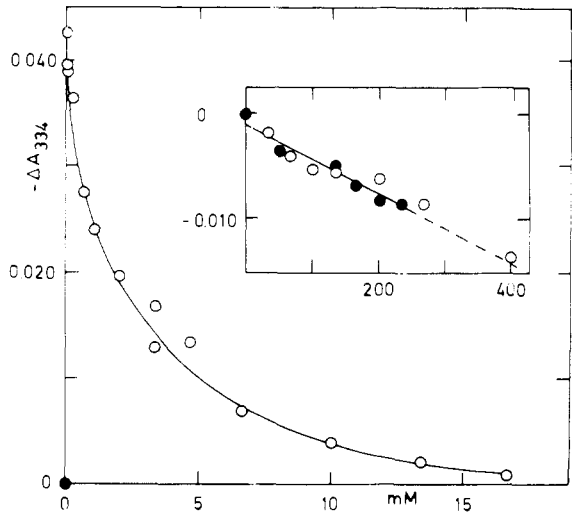

FIGURE 4: Quantitative displacement by methyl α-D-mannopyranoside of MUM bound to con A as measured by absorption difference at 334 nm. The mixed Yankeelov cells contained 25 μM con A and 252 μM MUM in the presence of increasing concentrations of methyl α-D-mannopyranoside (pH 5.5, 20.2 °C, open circles). Absorption differences at concentrations of methyl α-D-mannopyranoside larger than 20 mM (inset) were identical to those observed in the absence of protein (filled circles). In the absence of methyl α-D-mannopyranoside, the fractional saturation is 0.91, as calculated for $K = 4.23 \times 10^4$ M^{-1}.

a maximal absorbance change equal to 29% of the value for the free ligand. Assuming a 1:1 complex, the value of the association constant, K, was estimated (Figure 5) as 4.7×10^4 M^{-1} (pH 5.5; 20.3 °C) according to the equation

$$\log \frac{\Delta A}{\Delta A_{max} - \Delta A} = \log K + \log \left(P_0 - L_0 \left(\frac{\Delta A}{\Delta A_{max}}\right)\right)$$

in which ΔA_{max} is the maximal absorbance change obtained from a plot of $1/\Delta A$ against the reciprocal of the total protein concentration P_0; L_0 is the total ligand concentration, and the log term represents the free protein concentration.

Quenching of MUM fluorescence spectrum by increasing concentrations of con A is represented in Figure 6. No significant blue shift is noticeable. From these results, the value of the association constant, K, can be estimated graphically (Chipman et al., 1967) according to the relation

$$\log \frac{F_0 - F}{F - F_\infty} = \log K + \log \left(P_0 - \frac{L_0(F_0 - F)}{F_0}\right)$$

in which F_0, F, and F_∞ are the values of the fluorescence maximum in the absence of protein, in the presence of protein, and at infinite protein concentration, respectively. All values are corrected for dilution and for blanks. The value of F_∞, as determined from a double-reciprocal plot, was practically equal to zero (Dean and Homer, 1973); experimental values for the percent of quenching were between 98 and 100. The data in Figure 6 yielded $K = 5.8 \times 10^4$ M^{-1} (pH 5.5; 13 ± 0.2 °C). At 20 °C, this value was 4.8×10^4 M^{-1}. When concanavalin A prepared by Sephadex G-75 absorption was used instead of con A composed of intact polypeptide chains, this value was 3.6×10^4 M^{-1} at 20 °C; this is in accordance with the value 3.4×10^4 M^{-1} obtained by Dean and Homer (1973). Their observation that fluorescence quenching of bound MUM is inhibited by carbohydrates was confirmed using mannotriose.

Titration of MUM Fluorescence Quenching. Fluorescence was measured above 350 nm. The experiments were performed in the temperature range 9–39 °C using tetrameric (pH 7.2) and unmodified dimeric, succinylated, or acetylated con A (all at pH 5.5). Blank titrations without MUM gave final readings amounting to 14–20% for the initial value for unquenched MUM, except for succinylated con A which gave a final blank of 30–36%.[2] The values of F_∞/F_0 giving a minimal error in the value of the association constant, calculated over the entire titration curves, were in the range +0.03 to −0.03, except for acetylated con A (0.08 to 0.09). The titrations and blanks were perfectly reproducible. A typical titration curve is given in Figure 7. The calculated values of the association constants were independent of the method of analysis (Table I). In the case of dimeric con A, they agreed with the results obtained by the other methods described above. For dimeric con A, the more reliable series of titrations (Δt °C = ±0.05; see Figure 7) yields $\Delta H° = -8.3 \pm 0.1$ kcal mol^{-1}; within limits of error, this value is identical to that determined for tetrameric con A, $\Delta H° = -8.5 \pm 0.2$ kcal mol^{-1} (Table II). Tetrameric con A, however, has association constants that are about 25% higher than for dimeric con A (Table II). In the temperature range studied, association constants for succinylated con A are comparable with those for native dimeric con A, while the constants for acetylated con A lie between those for the two oligomeric forms of underivatized con A.

TABLE I: Binding Parameters Determined by Titration of MUM Fluorescence Quenching as a Function of Temperature at pH 5.5.

t (°C) (±0.15 °C)	$10^{-4} \times K$ (M^{-1}) (Kurganov)[a]	Nonlinear Fitting $10^{-4} \times K$ (M^{-1})	n
14.0	5.54 ± 0.07	5.89 ± 0.33	1.00
18.6	4.51 ± 0.07	4.47 ± 0.57	1.06
23.0	3.71 ± 0.04	3.98 ± 0.21	1.00
26.7	3.03 ± 0.03	3.09 ± 0.26	1.01
35.3	2.13 ± 0.02	2.16 ± 0.33	1.05

[a] The number of binding sites was assumed to be one. Graphical estimation yielded 95% quenching. A minimal standard deviation for the association constant, K, calculated from 15 fluorescence readings, was also found for 94–95% quenching, using a 348-nm cutoff filter. [b] The maximum error in the number of binding sites, n, was 10% and the quenching found was in the range 95–96%. These values changed to 97.5–99.5% when a 359-nm cutoff filter was substituted for the 348-nm filter used in the above experiments. The value of K determined by equilibrium dialysis at 25.5 °C was 3.37×10^4 M^{-1} using the same protein preparation.

Discussion

4-Methylumbelliferyl glycosides are interesting ligands for the study of carbohydrates interacting with proteins. The fluorescence of 4-methylumbelliferyl chitooligosaccharides is enhanced upon binding to lysozyme (Delmotte et al., 1975) or is totally quenched upon binding to the lectin from wheat germ (Privat et al., 1974). A similar effect was also noted for MUM binding to concanavalin A (Dean and Homer, 1973) but was small for the lectin from *Pisum sativum* (Loontiens et al., unpublished). In addition to the total quenching of MUM fluorescence upon binding to con A (Dean and Homer, 1973), we have observed a blue shift of its absorption spectrum, similar to the one found with *p*-nitrophenyl α-D-mannopyranoside (Hassing and Goldstein, 1970). It allows sensitive measurements at the two extremes at 322 and 334 nm. The high absorption coefficient of MUM also allows photometric monitoring of equilibrium dialysis experiments.

[2] This larger background for succinylated con A was attributed to scattered light impinging upon the WG 360-2 cutoff filter which shows a considerable autofluorescence.

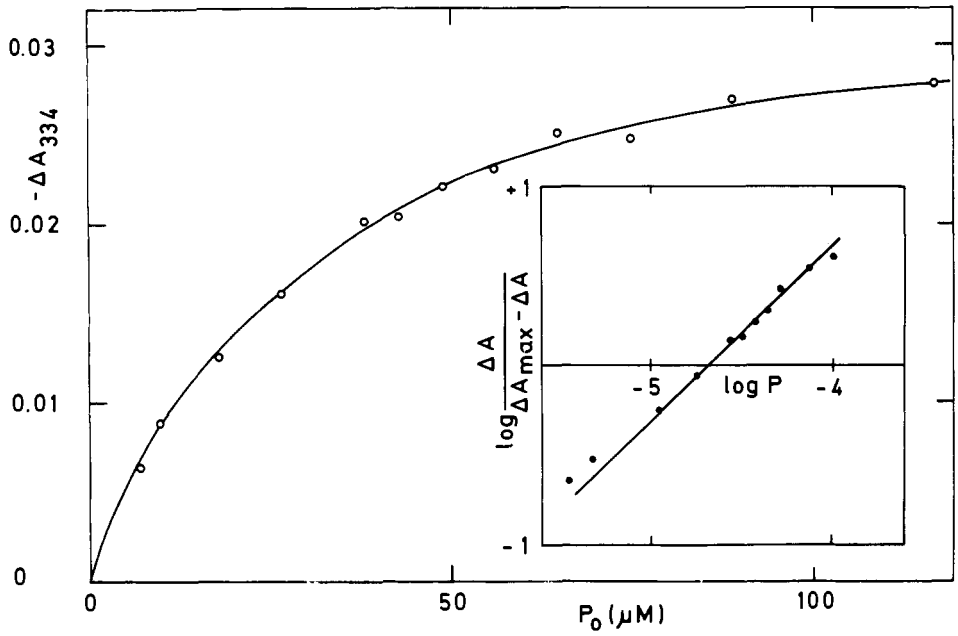

FIGURE 5: Estimation of the association constant for MUM from absorbance difference spectra. The final concentration of MUM was constant (17.9 μM) over the range of con A concentrations used. ΔA_{max} is the maximal absorbance change (334 nm) at infinite protein concentration; P_0 is the total protein concentration, and P the free protein concentration. Graphical estimation of the association constant (inset) gives 4.7×10^4 M^{-1} (pH 5.5, 20.3 °C).

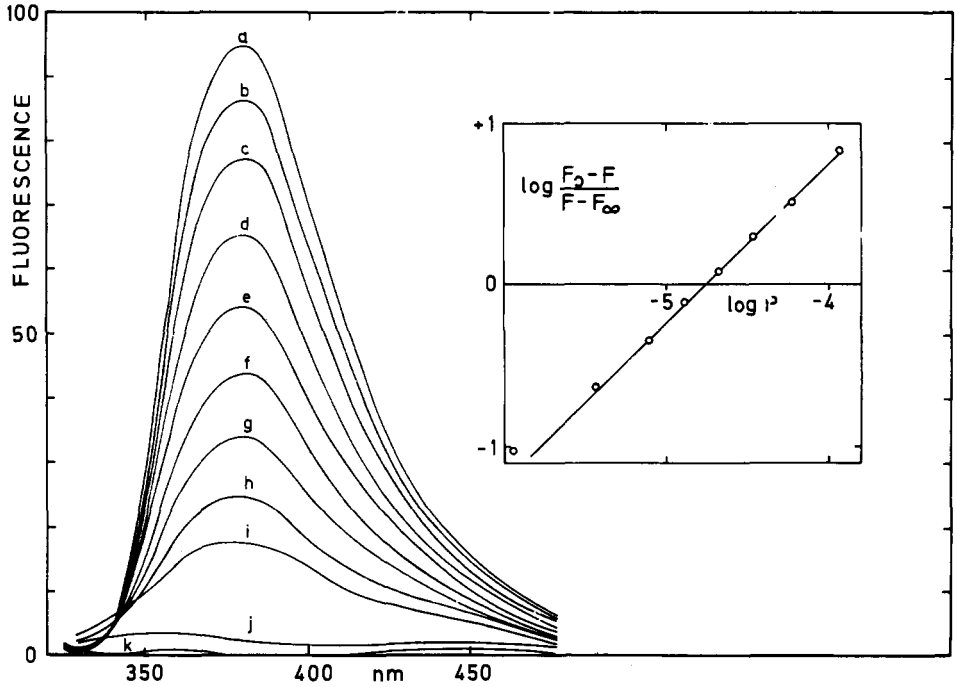

FIGURE 6: Quenching of the MUM fluorescence spectrum upon addition of con A. To 3.007 ml of a 3.29 μM solution of MUM (pH 5.5, 13.0 °C) samples of a 877 μM solution of con A were added successively in total amounts as indicated. The spectra were recorded exciting at 318 nm: (a) without con A; (b) 5 μl; (c) 15 μl; (d) 30 μl; (e) 50 μl; (f) 80 μl; (g) 130 μl; (h) 230 μl; (i) 480 μl. Curve j is for con A alone, at a concentration corresponding to curve i; curve k is for buffer. After correction of the fluorescence maxima for these blanks and for dilution, the value of the association constant was determined graphically (inset) as 5.8×10^4 M^{-1}.

Most of the binding experiments, however, involved quenching of MUM fluorescence. In agreement with Miller and Nwokedi (1975), no appreciable quenching of protein fluorescence was observed upon binding nonchromophoric mannose derivatives to con A saturated with Ni^{2+} and Ca^{2+}. Any such apparent decrease of protein fluorescence upon addition of MUM was an artifact, caused by a filter effect of MUM and could not be restored upon addition of methyl α-D-mannopyranoside in large excess. The filter effect also occurred with 4-methylumbelliferyl β-D-galactopyranoside. Dean and Homer (1973) concluded that MUM binds to an apolar site and is therefore quenched. They find that solvents of less polarity than water reduce the quantum yield of MUM considerably. Experimental values for the amount of MUM quenching by con A slightly increased with temperature and were smaller (91–92%) for acetylated con A than the almost

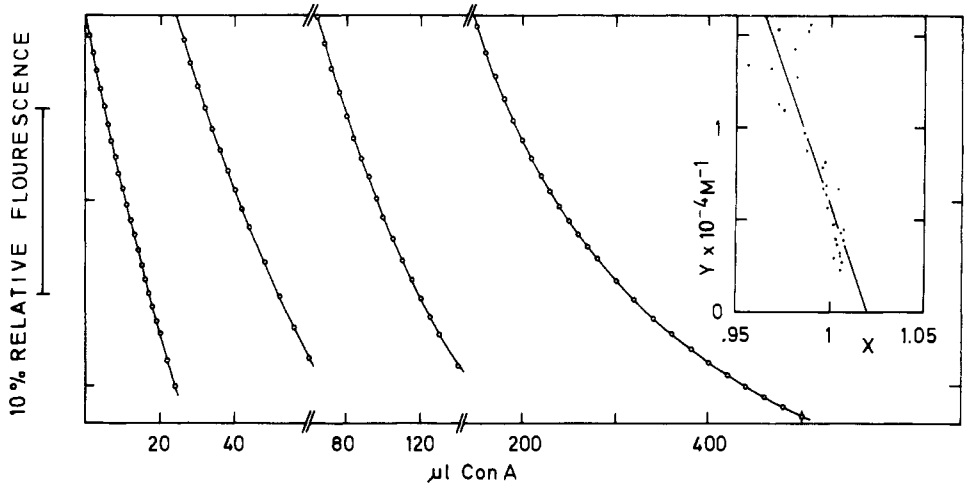

FIGURE 7: Titration of MUM fluorescence quenching upon continuous addition of 1.00 mM con A to 3.017 ml of 3.54 μM MUM. Fluorescence was measured at wavelengths larger than 359 nm. The values (O) are corrected for a blank titration without MUM. The graph is calculated for F_∞/F_0 = 0.02 and $K = 3.58 \times 10^4$ M^{-1}, assuming one binding site per 25 500 daltons; the variance of experimental and calculated relative fluorescence is 4×10^{-6} over the entire titration. Experimental conditions are pH 5.5 and 23.70 ± 0.05 °C. The value of F_∞/F_0 is identical when calculated from the minimal error for K over the entire titration curve or when determined graphically according to Kurganov (inset). For construction of the latter graph, values of F/F_0 larger than 0.8 were not used; $X = [\kappa(1 - F'/F_0) - (1 - F/F_0)]/[(\kappa - 1)(1 - F/F_0)(1 - F'/F_0)]$ and $Y = [\kappa(F'/F_0) - (F/F_0)]/[(\kappa - 1)P_0]$. The intercept on the X axis equals $(1 - F_\infty/F_0)^{-1}$; the values of κ used are 2, 3, and 4. The experimental values of F/F_0 showed a slight but systematic decrease as the temperature was raised from 9 to 39 °C. For native dimeric con A: F/F_0 = 0.03 to 0.01; for native tetrameric con A: 0.025 to −0.03; for succinylated con A: 0.025 to −0.03; for acetylated con A 0.09 to 0.08.

TABLE II: Thermodynamic Functions for the Binding of MUM and Related Mannosides to Different con A Samples.

α-D-Mannopyranoside	Lectin	$10^{-4} \times K$ (M^{-1}) at 25.0 °C	$-\Delta H°$ (kcal mol^{-1})	$-\Delta G°$ (kcal mol^{-1})	$-\Delta S°$ (eu)
4-Methylumbelliferyl[a]	Unmodified dimer	3.36 ± 0.04	8.3 ± 0.1	6.18 ± 0.01	7.2 ± 0.3
	Unmodified tetramer	4.26 ± 0.07	8.5 ± 0.2	6.32 ± 0.01	7.4 ± 0.6
	Succinylated (dimer)	3.14 ± 0.06	8.8 ± 0.3	6.14 ± 0.01	9.0 ± 0.9
	Acetylated (dimer)	3.80 ± 0.06	7.4 ± 0.1	6.25 ± 0.01	3.7 ± 0.5
Methyl[b]	Unmodified tetramer	0.9	6.1	5.4	2.3
p-Nitrophenyl[c]	Unmodified dimer	1.49	6.8	5.7	3.6

[a] All con A samples used for MUM binding were composed of intact polypeptide chains and the data were obtained by continuous titration of MUM fluorescence quenching in the temperature range 9–39 °C. The van't Hoff plots were all linear. In the regression equations, N is the number of temperatures (error given as δt °C) used, R is the regression coefficient, and S is the standard error of the estimated ln K. For unmodified dimeric con A, a series with δt °C = 0.05 gave data that were consistent with those in Table I (corresponding to $\Delta H° = -7.9 \pm 0.1$ kcal mol^{-1}) according to: $\ln K = -3.618 + (4.189 \pm 0.041) \times 10^3/T$ ($N = 7$, $R = 0.9997$, $S = 0.010$). The values of K obtained by equilibrium dialysis (Table I), absorption difference spectroscopy (Figure 4), and titrations at a discrete wavelength (Figure 5) were fully consistent. For unmodified tetrameric con A with δt °C = 0.05: $\ln K = 3.740 + (4.299 \pm 0.084) \times 10^3/T$ ($N = 6$, $R = 0.9991$, $S = 0.016$). For succinylated con A with δt °C = 0.2: $\ln K = 4.546 + (4.445 \pm 0.131) \times 10^3/T$ ($N = 5$, $R = 0.9984$, $S = 0.019$). For acetylated con A with δt °C = 0.05: $\ln K = -1.851 + (3.698 \pm 0.072) \times 10^3/T$ ($N = 7$, $R = 0.9989$, $S = 0.015$). [b] Calculated from Bessler et al. (1974) at pH 7. [c] From Loontiens et al. (1973) at pH 5.4.

total quenching observed for all other types of con A investigated. The values were always identical when calculated by either of the two fitting procedures or when extrapolated according to Kurganov et al. (1972). The differences in small residual fluorescence, observed at infinite protein concentrations in individual titration series, are probably due, in part, to minor variations in the amount (less than about 0.2%) of 7-hydroxy-4-methylcoumarin in the MUM solutions. The fluorescence of this free aglycon of MUM is much larger—even at low pH—than for MUM itself (Delmotte et al., 1975).

There is a single binding site for MUM per protomer of 25 500 daltons in dimeric and tetrameric con A without noticeable interaction between sites in the oligomers. This is shown unequivocally by equilibrium dialysis (Figure 2) and is confirmed by nonlinear fitting of the MUM fluorescence titration curves (Table I). The Scatchard plots for equilibrium dialysis are linear in the range of fractional saturation from 0.2 to 0.8 and show no deviation at high saturation that would indicate an additional low-affinity site. This is in agreement with earlier studies obtained with simple carbohydrates (So and Goldstein, 1968; Yariv et al., 1968), with p-nitrophenyl α-D-mannopyranoside (Hassing and Goldstein, 1970; Loontiens et al., 1973; Bessler et al., 1974), with p-nitrophenyl α-D-glucopyranoside (Alter and Magnuson, 1974), and o-iodophenyl β-D-glucopyranoside (Becker et al., 1975). The ab-

sence of interaction between identical sites is further substantiated by the consistency of values for K found by equilibrium dialysis and by the methods involving a low degree of saturation (Figure 7). Similar evidence was obtained from titration and dilution type of temperature-jump experiments (Clegg et al., 1977).

The MUM binding site is carbohydrate specific: its association constant can be predicted from inhibition of con A–glycogen interaction and both the observed difference spectrum and the MUM fluorescence quenching can be inhibited by methyl α-D-mannopyranoside and mannotriose. Furthermore, MUM bound at a fractional saturation of 0.91 can be totally displaced from the protein by methyl α-D-mannopyranoside (Figure 4). By any of the methods used, no indication has been found for binding at an additional unspecific site. Such an additional site might have been the binding site for 2-p-toluidinylnaphthalenesulfonate probably at the center of 222 symmetry in tetrameric con A and also present in dimeric con A (Yang et al., 1974); it is known to be independent of carbohydrate binding. An alternative unspecific site might have been the cleft at 20 Å from Mn^{2+}, known to bind apolar molecules (Hardman and Ainsworth, 1973) and o-iodophenyl β-D-glucopyranoside (Becker et al., 1975) probably via its aglycon. In view of NMR data (Brewer et al., 1973; Villafranca and Viola, 1974; Alter and Magnuson, 1974) and recent crystallographic data (Becker et al., 1976; Hardman and Ainsworth, 1976), carbohydrates bind at a shallow pocket, about 10 Å from the transition metal ion. Part of this pocket most removed from the transition metal ion is lined by Tyr-12, Leu-99, and Tyr-100, and these could provide the region of less polarity invoked to explain the fluorescence quenching of MUM, bound in an orientation corresponding to ^{13}C NMR data for methyl α-D-glucopyranoside.

The binding of glycosides, such as p-nitrophenyl α-D-mannopyranoside to con A (Hassing and Goldstein, 1970) is known to be exothermic. In order to determine the enthalpy and entropy for binding of MUM to con A, the fluorescence titrations were done in the temperature range 9 to 35 °C (Table II). For dimeric con A, $\Delta H° = -8.3 \pm 0.1$ kcal mol^{-1}. This value is in the same range as found for other mannosides (Table II), as would be expected for carbohydrate-specific binding. As compared with methyl α-D-mannopyranoside, the effect of the large aglycon of MUM causes only a minor perturbation: binding is more exothermic by 2.3 kcal mol^{-1} and is partly counterbalanced by a decrease of $\Delta S°$ by 5 eu. This would be consistent with an interaction of the aglycon, possibly involving Tyr-12, Leu-99, and Tyr-100, with concomitant restriction of rotational freedom among the other entropy factors which might be involved.

The van't Hoff plots for the association constants of MUM, obtained with dimeric, tetrameric, acetylated, and succinylated con A, are all linear in the temperature range 9–39 °C. Only minor differences in the thermodynamic parameters were observed for all four types of con A studied (Table II). The consistency of binding equilibrium data for native and derivatized con A is relevant in deducing the binding mechanism (Clegg et al., 1977).

The affinity of MUM is about 25% higher for tetrameric than for dimeric con A. Although the tetramer is known to dissociate into dimers below 25 °C (Huet et al., 1974; Huet, 1975), no downward deviation could be observed in the van't Hoff plot for tetrameric con A. At the lowest temperature (11.5 °C), there should be about 50% dissociation, causing a decrease in the value of K expected to be five to ten times larger than the average error of its calculated value (Table II). This may indicate that the difference in the binding affinity observed for dimeric and tetrameric con A is due primarily to a pH difference (i.e., the affinity of the two forms of con A may be identical). This is further indicated by the identity of the values of $\Delta H°$ and $\Delta S°$ for the dimer and tetramer of con A (see Table II).

The exothermic binding of simple carbohydrates to con A is in marked contrast with the "endothermic" interaction of this lectin with cells, as determined by agglutination or by binding studies using ^{125}I- or $^{63}Ni^{2+}$-labeled con A (Betel et al., 1972; Huet et al., 1974; Huet, 1975). This phenomenon has been shown (Huet et al., 1974) to arise from the dissociation of con A below 25 °C. The dimers show a reduced potency for producing certain biological effects, probably due to the decreased valence compared to the tetramer (Gunther et al., 1973) with concomitant decreased accessibility for the receptors. This difference is much larger than for simple carbohydrates like MUM. An additional observation is the sharp increase in the binding of ^{3}H-acetylated con A to Py3T3 cells at 15 °C (Kaneko et al., 1973; Noonan and Burger, 1973). This effect may be caused by a phase transition in the lipid bilayer, which increases the mobility of the lectin receptors, leading to unspecific binding and/or pinocytosis among the different factors involved (Nicolson, 1974).

The observed temperature dependence of MUM binding to con A, as characterized in this paper, can be followed either by absorbance changes or preferably by the total quenching of MUM fluorescence and provides an excellent probe for the application of fluorescence temperature-jump relaxation kinetics (Clegg et al., 1977).

Acknowledgments

The help of W. Bähr, M. Cambier, and A. Van Landschoot in different parts of this work was essential. The authors thank Professor C. K. De Bruyne for reading the manuscript.

References

Agrawal, B. B. L., and Goldstein, I. J. (1967), *Biochim. Biophys. Acta 147,* 262.

Alter, G. M., and Magnuson, J. A. (1974), *Biochemistry 13,* 4038.

Becker, J. W., Reeke, G. N., Jr., Cunningham, B. A., and Edelman, G. M. (1976), *Nature (London) 259,* 406.

Becker, J. W., Reeke, G. N., Jr., Wang, J. L., Cunningham, B. A., and Edelman, G. M. (1975), *J. Biol. Chem. 250,* 1513.

Bessler, W., Shafer, J. A., and Goldstein, I. J. (1974), *J. Biol. Chem. 249,* 2819.

Betel, I., and Van den Berg, K. J. (1972), *Eur. J. Biochem. 30,* 571.

Brewer, C. F., Marcus, D. M., and Grollman, A. P. (1974), *J. Biol. Chem. 249,* 4614.

Brewer, C. F., Sternlicht, H., Marcus, D. M., and Grollman, A. P. (1973), *Biochemistry 12,* 4448.

Chipman, D. M., Grisaro, V., and Sharon, N. (1967), *J. Biol. Chem. 242,* 4388.

Clegg, R. M., Loontiens, F. G., and Jovin, T. M. (1977), *Biochemistry 16* (following paper in this issue).

Cunningham, B. A., Wang, J. L., Pflumm, M. N., and Edelman, G. M. (1972), *Biochemistry 11,* 3233.

Cunningham, B. A., Wang, J. L., Waxdal, M. J., and Edelman, G. M. (1975), *J. Biol. Chem. 250,* 1503.

Dahlquist, F. W., Jao, L., and Raftery, M. A. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 26.

Dean, B. R., and Homer, R. B. (1973), *Biochim. Biophys. Acta*

322, 141.
Delmotte, F. M., Privat, J.-P. D. J., and Monsigny, M. L. P. (1975), *Carbohydr. Res. 40,* 353.
Goldstein, I. J. (1975), *Adv. Exp. Med. Biol. 55,* 35.
Gray, R. D., and Glew, R. H. (1973), *J. Biol. Chem. 248,* 7547.
Gunther, G. R., Wang, J. L., Yahara, I., Cunningham, B. A., and Edelman, G. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1012.
Hardman, K. D., and Ainsworth, C. F. (1973), *Biochemistry 12,* 4442.
Hardman, K. D., and Ainsworth, C. F. (1976), *Biochemistry 15,* 1120.
Hassing, G. S., and Goldstein, I. J. (1970), *Eur. J. Biochem. 16,* 549.
Huet, C., Longchampt, M., Huet, M., and Bernadac, A. (1974), *Biochim. Biophys. Acta 365,* 28.
Huet, M. (1975), *Eur. J. Biochem. 59,* 627.
Kalb, A. J., and Levitzki, A. (1968), *Biochem. J. 169,* 669.
Kalb, A. J., and Lustig, A. (1968), *Biochim. Biophys. Acta 168,* 366.
Kaneko, I., Satoh, H., and Ukita, T. (1973), *Biochem. Biophys. Res. Commun. 50,* 1087.
Kurganov, B. I., Sugrobova, N. P., and Yakovlev, V. A. (1972), *FEBS Lett. 19,* 308.
Lee, Y. C., and Ballou, C. E. (1965), *Biochemistry 4,* 257.
Lewis, S. D., Shafer, J. A., and Goldstein, I. J. (1976), *Arch. Biochem. Biophys. 172,* 689.
Lis, H., and Sharon, N. (1973), *Annu. Rev. Biochem. 42,* 541.
Loontiens, F. G, Van Wauwe, J. P., and De Bruyne, C. K. (1975), *Carbohydr. Res. 44,* 150.
Loontiens, F. G., Van Wauwe, J. P., De Gussem, R., and De Bruyne, C. K. (1973), *Carbohydr. Res. 30,* 51.
McCubbin, W. D., and Kay, C. M. (1971), *Biochem. Biophys. Res. Commun. 44,* 101.
Miller, J. N., and Nwokedi, G. I. C. (1975), *Biochim. Biophys. Acta 393,* 426.
Nicolson, G. L. (1974), *Int. Rev. Cytol. 39,* 89.
Noonan, K. D., and Burger, M. M. (1973), *J. Biol. Chem. 248,* 4286.
Poretz, R. D., and Goldstein, I. J. (1970), *Biochemistry 9,* 2890.
Privat, J. P., Delmotte, F., and Monsigny, M. (1974), *FEBS Lett. 46,* 229.
Reeke, G. N., Jr., Becker, J. W., and Edelman, G. M. (1975), *J. Biol. Chem. 250,* 1525.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51,* 660.
Shoham, M., Kalb, A. J., and Pecht, I. (1973), *Biochemistry 12,* 1914.
So, L. L., and Goldstein, I. J. (1968), *Biochim. Biophys. Acta 165,* 398.
Tooze, J. (1973), The Molecular Biology of Tumour Viruses, Cold Spring Harbor Laboratories, Chapter 3.
Vervoort, A., and De Bruyne, C. K. (1970), *Carbohydr. Res. 12,* 277.
Villafranca, J. J., and Viola, R. D. (1974), *Arch. Biochem. Biophys. 160,* 465.
Wang, J. L., Cunningham, B. A., and Edelman, G. M. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1130.
Wang, J. L., Cunningham, B. A., Waxdal, M. J., and Edelman, G. M. (1975), *J. Biol. Chem. 250,* 1490.
Yang, D. C. H., Gall, W. E., and Edelman, G. M. (1974), *J. Biol. Chem. 249,* 7018.
Yariv, J., Kalb, A. J., and Levitzki, A. (1968), *Biochim. Biophys. Acta 165,* 303.