Equilibrium and Kinetic Studies of the Binding of *Lens culinaris* Lectin to Rabbit Erythrocytes by a Quantitative Fluorometric Method†

Johan Hoebeke, André Foriers, Alain B. Schreiber, and A. Donny Strosberg*

ABSTRACT: The binding of *Lens culinaris* lectin to the receptors of rabbit erythrocytes was studied by a quantitative fluorometric method using the fluoresceinated conjugate. Equilibrium data showed the presence of 1.2×10^6 receptors/cell with an association constant varying from 8 to 3×10^6 M^{-1} over a temperature range of 5 to 37 °C. The binding reaction is exothermic with a $\Delta H = -4.6$ kcal/mol and a $\Delta S = +15$ eu. It appears that most of the bound lectin molecules had only a single site occupied, the agglutination being due to a very low fraction (0.0003–0.0056) of lectin molecules with two sites occupied. The specificity of the reaction was demonstrated through its inhibition by nonfluoresceinated LL and by the specific sugars methyl α-D-glucoside and methyl α-D-mannoside. From the inhibition curves an association constant of 4×10^2 M^{-1} was calculated for methyl α-D-glucoside and 9×10^2 for methyl α-D-mannoside. The association and dissociation rate constants of the binding reaction are, respectively, in the range of $3–10 \times 10^3$ and $3–33 \times 10^{-4}$ M^{-1} s^{-1} in the temperature range of 5 to 37 °C. The activation energies of the forward and reverse reactions are 7 and 13 kcal/mol, respectively. The association constants and the binding enthalpy calculated from the activation energies were fully consistent with those obtained by equilibrium measurements.

Lectins are sugar-binding and cell-agglutinating proteins which, in recent years, have been used as a major tool in the study of the nature of cell-surface changes (Lis and Sharon, 1973). The importance of these changes in relation to cell function is reflected, for example, by the fact that the binding of many lectins to the cell-surface carbohydrate structures of lymphocytes results in lymphocyte transformation or blastogenesis (Sharon, 1976).

Although, in this respect, one of the most used lectins is Con A,[1] little is known about the physicochemical binding characteristics of this protein to cell-surface receptors. Indeed, the thermodynamic and kinetic approach to the reaction is hampered for two main reasons: Con A displays a dimer–tetramer transition with increasing temperature which renders the study of the lectin–receptor interaction virtually impossible (Huet, 1975), and the rearrangement of lectin binding sites induced by the microtubular and microfilamentous network underlying the membranes of nucleated cells (Edelman et al., 1973; Bourguignon and Singer, 1977) introduces another variable, making an unequivocal physicochemical interpretation very difficult.

To avoid these problems, we have chosen to study the binding of *Lens culinaris* lectin (LL) to erythrocytes. LL has a sugar-binding specificity similar to Con A but exists only in a dimeric state (Howard and Sage, 1969). The use of erythrocytes lacking the patching and capping properties of nucleated cells renders a straightforward thermodynamic approach possible. Finally, the use of fluoresceinated lectin was preferred over ^{131}I-labeled protein used in a previous study (Kornfeld et al., 1971) because manipulation of fluoresceinated material requires less precautions or facilities than radiolabeled protein and has proved to be very accurate and sensitive for the study of membrane receptors (Schreiber et al., 1978).

This paper presents a complete quantitative equilibrium and kinetic analysis of the binding of LL to the erythrocyte receptors. The internal consistency of the thermodynamic parameters obtained by both analyses and the comparison of the results obtained here with those from the literature indicate the validity of the approach and its potential for tackling more complex problems.

Experimental Procedure

Materials

Fluorescein isothiocyanate was purchased from British Drug Houses (Great-Britain); NP 40 (a nonionic detergent) and methyl α-D-glucoside were from Fluka (Switzerland); methyl α-D-mannoside was from Aldrich (Beerse, Belgium); and bovine serum albumin was from Sigma Chemical Co. All other reagents were of analytical grade.

LL was purified from commercial lectins by 80% $(NH_4)_2SO_4$ precipitation, followed by adsorption on Sephadex G-75 and desorption with 0.1 M methyl α-D-glucoside (Howard et al., 1971). The lectin preparation was homogeneous as shown by sequence studies (Foriers et al., 1978). The lectin was stored as a precipitate in $(NH_4)_2SO_4$. To fluoresceinate the lectin, the precipitate was dialyzed against 1 M NaCl and the pH brought to 8.5 with Na_2CO_3 in the presence of 0.1 M methyl α-D-glucoside to protect the binding sites. To 5 mL of the resulting solution, which contained approximately 1 mg/mL lectin, was added 0.5 mg of fluorescein isothiocyanate dissolved in 0.05 mL of acetone, and the reaction was allowed to proceed for 16 h at 4 °C. Unbound fluorescein was separated from the labeled lectin by molecular sieving on Bio-Gel P10 and extensive dialysis against 1 M NaCl. The dye/protein ratio was calculated as 1.46 from absorbance measurements at 280 and 493 nm ($E_{280}^{1\%} = 12.5$, $\epsilon_{493} =$

† From the Afdeling Pathologische Biochemie en Chemie der Proteinen, Vrije Universiteit te Brussel, Instituut voor Molekulaire Biologie, Paardenstraat, 65, B-1640 Sint Genesius Rode, Belgium. *Received March 14, 1978.* This work was supported in part by the Belgian Fonds voor Kollektief Fundamenteel Onderzoek, the Fonds voor Geneeskundig Wetenschappelijk Onderzoek, and the Kankerfonds van de Algemene SpaarenLijfrentekas.

[1] Abbreviations used are: LL, *Lens culinaris* lectin; FITC-LL, fluoresceinated *Lens* lectin; PBS–BSA 0.1% phosphate-buffered saline (pH 7.4) containing 1 mg/mL bovine serum albumin; NP-40, the detergent Nonidet P 40; Con A, concanavalin A.

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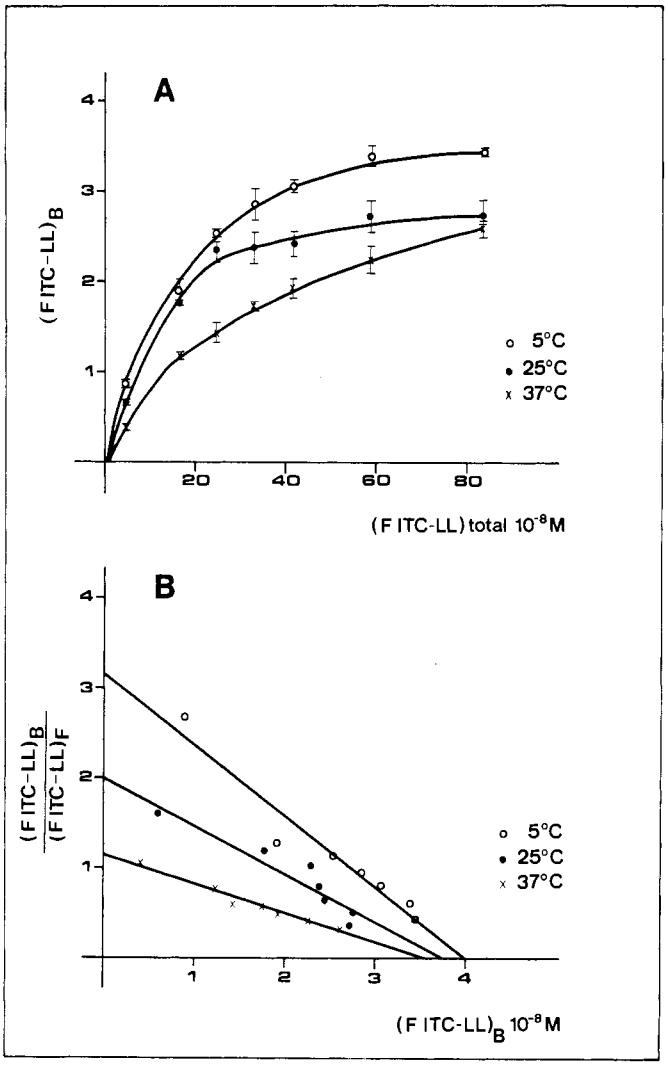

FIGURE 1: (a) Actual binding curves of FITC-LL to rabbit erythrocytes (2×10^7/mL) at three different temperatures. Nonspecific binding is substracted. (B) Scatchard plots calculated from the curves in A. Correlation coefficients (R) were 0.965, 0.928, and 0.992 for, respectively, 5, 25, and 37 °C. The straight lines represent linear fits through the data.

73 000). Assuming that the heterogeneity in labeling follows a Poisson distribution (Green, 1964), 23% of the lectin is unlabeled, 34% mono-, 25% bi-, 12% tri- and 4% tetralabeled. The stock solution containing 1.7×10^{-4} M labeled lectin was divided into 0.05-mL aliquots and stored at −18 °C until use. Working solutions were made up by diluting 0.05 mL of stock solution to 1 mL in 0.1% PBS-BSA. Agglutination titers of LL and FITC-LL were the same.

Rabbit erythrocytes were prepared from blood obtained from the ear vein with an equal volume of Alsever's solution. Working suspensions were made up by washing 0.8 mL of the citrated blood twice in 10 mL of 0.1% PBS-BSA and resuspending the erythrocytes in 10 mL of 0.1% PBS-BSA. The citrated blood was never stored longer than 15 days and was discarded if any hemolysis was visible. The number of cells in the working suspension was controlled by a haemocytometer.

Methods

Equilibrium and inhibition studies were performed in an end volume of 1 mL containing 0.1 mL of the erythrocyte suspension and different amounts of FITC-LL and inhibitors. After 30 min of incubation, the erythrocytes were spun down at 200g for 5 min in a Sorvall RC3 centrifuge, and the pellet was washed once with 1 mL of 0.1% PBS-BSA and dissolved in detergent solution containing 0.5% NP-40 and 0.1 M methyl α-D-glucoside in PBS. For every FITC-LL concentration, a blank was set up by incubating the erythrocytes in the presence of 0.1 M methyl α-D-glucoside. Blanks amounted to 15 ± 4% of the specific binding. Every series was accompanied by a standard consisting of a known amount of FITC-LL dissolved in 1 mL of detergent solution to which 0.1 mL of erythrocyte suspension was added to compensate for the hemoglobin absorption quenching.

Association kinetics were followed by adding 0.1 mL of erythrocyte suspension at different time intervals before centrifugation to 0.9 mL of 0.1% PBS-BSA in which the FITC-LL was already dissolved. The cells were centrifuged in an Eppendorf table centrifuge for 1 min at 8000g, the walls of the centrifuge tubes were wiped off with a paper towel, and the cell pellet was dissolved directly in the detergent solution. Blanks were made up as described previously but at the same time intervals before centrifugation as the samples.

Dissociation was initiated by adding 0.1 mL of 1 M methyl α-D-glucoside 30 min after preincubation of a mixture containing 0.1 mL of erythrocyte working suspension and FITC-LL in 0.9 mL of 0.1% PBS-BSA. At different time intervals after the addition of the sugar, the cells were spun down at 8000g for 1 min, the centrifuge tubes were cleaned with a paper towel, and the cell pellet was dissolved in the detergent solution. A blank was made up in the presence of 0.1 M methyl α-D-glucoside during preincubation.

Fluorescence measurements were carried out in a Jobin-Yvon spectrofluorometer JY3 at room temperature in a glass microcuvette. The excitation wavelength was 493 nm and maximal emission was at 515 nm. A standard curve was constructed by dissolving different amounts of FITC-LL in 1 mL of detergent solution containing 0.1 mL of the erythrocyte working suspension. The fluorescence intensity at 515 nm was a linear function of the concentration over the whole range of FITC-LL concentrations used.

TABLE I: Thermodynamic Parameters of the Receptor-LL Interaction Obtained from the Equilibrium Data.[a]

t (°C)	$10^{-6} \times n$	$10^{-6} \times K_A$ (M^{-1})	$-\Delta G$ (kcal/mol)	$-\Delta H$ (kcal/mol)	$+\Delta S$ (eu)
5	1.3 ± 0.2	7.8 ± 0.9	8.8	4.6 ± 0.8	15.1
25	1.1 ± 0.2	5.3 ± 0.9	9.2		15.4
37	1.1 ± 0.2	3.2 ± 0.2	9.2		14.8

[a] The number of receptors per cell (n) and the association constants were calculated from the Scatchard plots (Figure 2); ΔH was calculated from the van't Hoff equation (R = 0.969); ΔS was calculated from $\Delta G = \Delta H - T\Delta S$. Standard deviations were calculated from the least-square analysis.

Results

Equilibrium Studies. As can be seen from Figure 1, the specific binding of FITC-LL to the rabbit red blood cells, i.e., total FITC-LL bound minus FITC-LL bound in the presence of 0.1 M methyl α-D-glucoside, was saturable and could be analyzed by a Scatchard plot (Scatchard, 1949). The number of sites per cell was calculated as 1.2×10^6 and showed no significant change for different temperatures. The thermodynamic parameters obtained from the Scatchard plots are summarized in Table I. The association constant decreases with increasing temperature, corresponding to a negative en-

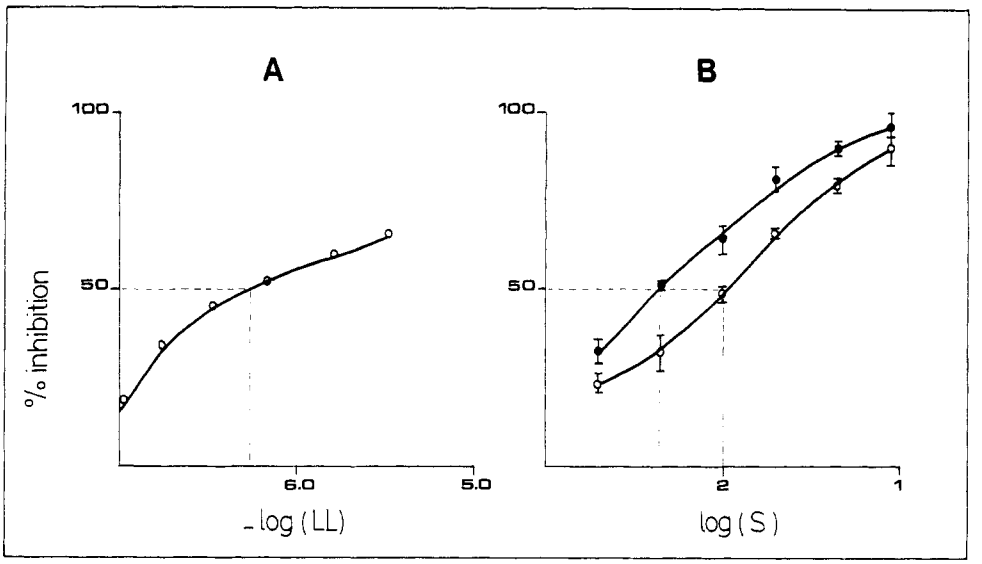

FIGURE 2: (A) Inhibition of the specific binding of FITC-LL to rabbit erythrocytes at 5 °C by nonfluoresceinated LL. The determination of the IC_{50} is indicated (see "Results"). (B) Inhibition of the specific binding of FITC-LL to rabbit erythrocytes at 5 °C by the specific monosaccharides methyl α-D-glucoside (○) and methyl α-D-mannoside (●). The determination of the IC_{50} is indicated.

TABLE II: Calculation of the Fraction of Receptors Linked to a Single or Doubly Occupied LL Molecule.[a]

$10^8 \times$ [FITC-LL]$_0$ (M)	$10^8 \times \bar{X}$ (M)	θ_R	θ_L	θ_L^2	$\theta_L^2\theta_R$	no. of linked receptors/ cell
4	0.6	0.15	0.075	0.00563	0.00084	1012
85	3	0.75	0.018	0.00031	0.00023	280

[a] $\bar{X}$, the quantity of receptors saturated was calculated from eq 1, taking 4×10^{-8} M as value for $[R]_0$ and 5×10^6 M^{-1} for K_A. θ_R is the fraction of receptors occupied ($\theta_R = \bar{X}/[R]_0$); θ_L is the fraction of lectin sites occupied ($\theta_L = \bar{X}/2[\text{FITC-LL}]_0$); θ_L^2 is the fraction of lectin molecules doubly occupied and $\theta_L^2\theta_R$ is the fraction of receptors saturated with a doubly occupied lectin. This last figure multiplied by the number of receptors/cell gives the number of lectin linked receptors.

thalpy of 4.6 kcal/mol and an entropy change of 15 eu.

From the association constant and the number of receptors/cell, the number of receptors and lectin sites occupied ($\bar{X}$) can be calculated by resolving eq 1:

$$K_A = \frac{\bar{X}}{([R]_0 - \bar{X})([LL]_0 - \bar{X})} \quad (1)$$

for a known value of receptor sites ($[R]_0$) and lectin ($[LL]_0$). This has been done for the lowest and the highest concentration of FITC-LL used, taking for K_A an approximate value of 5×10^6 M^{-1}. Table II summarizes the results. It appears that, even at the lowest concentration of FITC-LL, only 0.56% of the lectin molecules have both sites occupied. This means that, over the concentration range used, the lectin molecule can be considered monovalent and suggests that only a very limited number of lectin molecules is involved in the agglutination process. At the highest concentration of FITC-LL, only 280 receptors bound per cell are necessary to obtain agglutination.

Inhibition Studies. The specificity of the FITC-LL binding to the erythrocytes could be demonstrated by inhibition by nonfluoresceinated LL and its specific sugars, methyl α-D-glucoside and methyl α-D-mannoside, respectively (Stein et al., 1971). From the plot of the logarithm of the inhibitor concentration vs. percent inhibition, the IC_{50} (the concentration necessary to inhibit 50% of the binding) could be calculated (Figure 2). Using a modified version of the formula of Cheng and Prusoff (1973):

$$K_{A,inh} = \frac{1 + [\text{FITC-LL}]K_{A,\text{FITC-LL}}}{IC_{50}} \quad (2)$$

the association constants for the two inhibitors were calculated. The validity of the formula was verified by studying the sugar inhibition at two lectin concentrations (16.6 and 41.5×10^{-8} M). The association constants were not significantly different in the temperature range of 5 to 37 °C and were $3.5 \pm 0.6 \times 10^2$ and $9.7 \pm 1.6 \times 10^2$ M^{-1}, respectively, for methyl α-D-glucoside and methyl α-D-mannoside. Since the enthalpy change is small, the reaction is mainly driven by an increase in entropy. The association constant of nonfluoresceinated LL is 8.1×10^6 M^{-1} at 5 °C.

Association Kinetics. The association reaction can be written as follows:

$$\text{R} + \text{FITC-LL} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{R-FITC-LL}$$

$$+ \text{R} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{R-FITC-LL-R} \quad (3)$$

in which k_1 is the rate-limiting constant. From the equilibrium data, it could be concluded that, under the conditions used, most of the lectin molecules can be considered monovalent. Since, on the other hand, an excess of FITC-LL has been used for the kinetic measurements, eq 3 can be simplified to a pseudomonomolecular one:

$$\text{R} \underset{k_{-1}}{\overset{k_1'}{\rightleftharpoons}} \text{R-FITC-LL} \quad (4)$$

$$k_1' = k_1[\text{FITC-LL}] \quad (5)$$

The solution of the kinetic equation is as follows:

$$\frac{d[\text{FITC-LL}]_{\text{bound}}}{dt} = k_1'[R] - k_{-1}[\text{R-FITC-LL}] \quad (6)$$

Since at equilibrium:

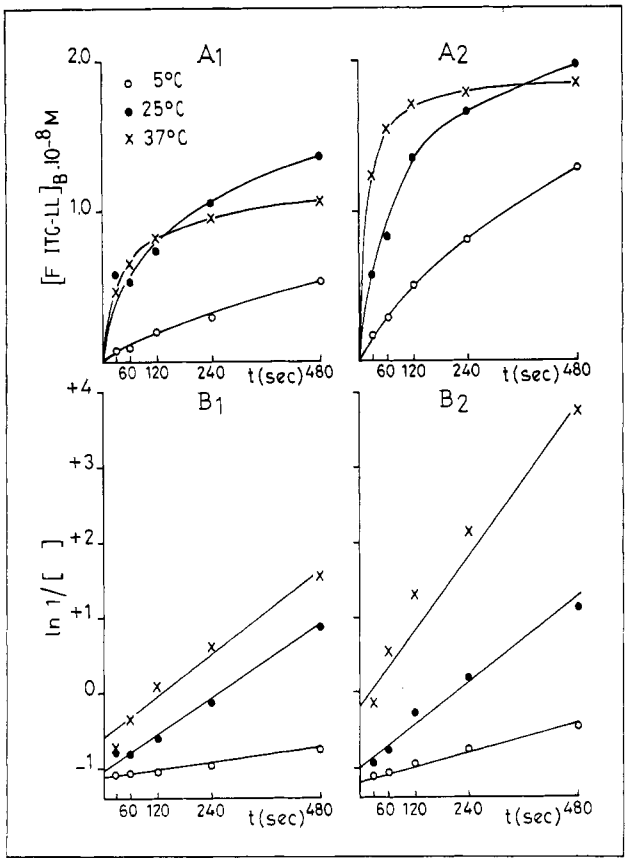

FIGURE 3: (A) Association curves of FITC-LL to rabbit erythrocytes in function of time. Two FITC-LL concentrations were used: 16.6×10^{-8} M represented in A_1 and 41.5×10^{-8} M represented in A_2. Erythrocyte number was held constant (2×10^7/mL). Measurements were performed at three temperatures. (B) Linearization of the curves from A by means of eq 10 (see Results). The linear fit through the data is presented. Correlation coefficients are given in Table IV.

$$k_{-1} = \frac{k_1'[R]_{eq}}{[R\text{-FITC-LL}]_{eq}} \quad (7)$$

and

$$[R] = [R]_{total} - [FITC\text{-LL}]_{bound} \quad (8)$$

$$\frac{d[FITC\text{-LL}]_{bound}}{dt} = k_1' \left\{ [R]_{total} - [FITC\text{-LL}]_{bound} \times \left(1 + \frac{[R]_{eq}}{[FITC\text{-LL}]_{eq}}\right)\right\} \quad (9)$$

Integration of eq 9 gives:

$$\ln \left\{ \frac{1}{[R]_{total} - (1 + [R]_{eq}/[FITC\text{-LL}]_{eq})[FITC\text{-LL}]} \right\} = \left(1 + \frac{[R]_{eq}}{[FITC\text{-LL}]_{eq}}\right) k_1' t \quad (10)$$

Figure 3 shows the experimental association curves and their linearization by means of eq 10. Two concentrations of FITC-LL were used, respectively, in 5- and 15-fold excess over the receptor concentration. Table III summarizes the association rate constants calculated from eq 10. As can be seen from the correlation coefficients ($R > 0.95$), the agreement between the data and eq 10 are satisfactory. Moreover, the association rate constants, obtained for two FITC-LL concentrations, were fully consistent at a given temperature. The small variations observed at the two concentrations are probably due to the experimental determination of the equilibrium parameters in eq 10.

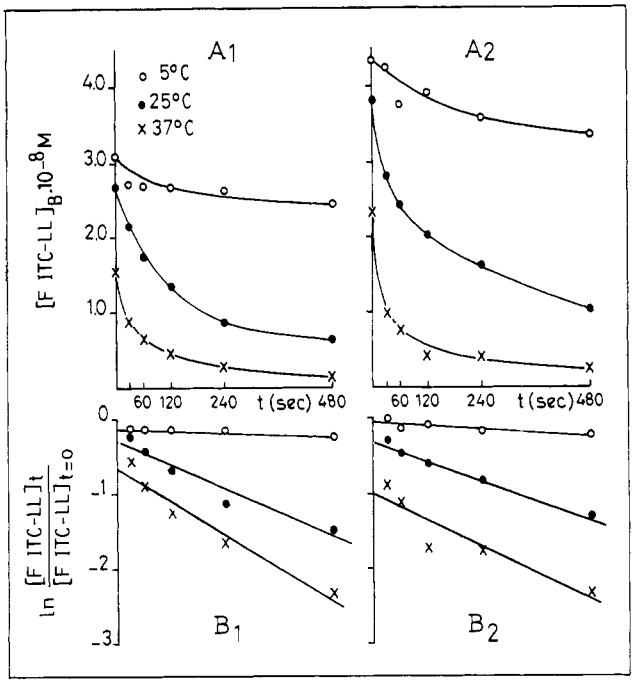

FIGURE 4: (A) Dissociation curves of FITC-LL from rabbit erythrocytes obtained by adding 0.1 M methyl α-D-glucoside after a 30-min preincubation. Initial concentrations of FITC-LL were 16.6×10^{-8} and 41.5×10^{-8} M for, respectively, A_1 and A_2. Erythrocyte number was held constant (2×10^7/mL). The dissociation was studied at three temperatures. (b) Linearization of the curves from A by means of eq 12 (see Results). The linear fit through the data is presented. Correlation coefficients are given in Table IV.

TABLE III: Kinetic Parameters of the Association and Dissociation of the LL-Receptor Interaction.[a]

t (°C)	[FITC-LL]tot $10^8 \times 16.6$ M	[FITC-LL]tot $10^8 \times 41.5$ M
	assoc rate constant: $10^{-3} \times K_1$ (M^{-1} s^{-1})	
5	2.3 ± 0.1 (R = 0.999)	3.4 ± 0.1 (R = 0.992)
25	10.0 ± 0.6 (R = 0.995)	6.7 ± 0.5 (R = 0.991)
37	10.2 ± 0.7 (R = 0.962)	10.4 ± 1.2 (R = 0.982)
	dissoc rate constant: $10^4 \times K_{-1}$ (s^{-1})	
5	2.4 ± 0.3 (R = 0.971)	4.4 ± 1.3 (R = 0.887)
25	27.5 ± 4.4 (R = 0.964)	21.5 ± 1.6 (R = 0.991)
37	36.9 ± 4.3 (R = 0.981)	28.9 ± 6.9 (R = 0.923)

[a] The rate constants were calculated from the plots shown in Figures 3 and 5; the correlation coefficients for the straight lines are indicated between brackets after each constant. Standard deviations were calculated from the least-square analysis.

Dissociation Kinetics. The dissociation has been studied as a monomolecular reaction brought about by displacing the ligand by means of an excess of methyl α-D-glucoside (Boeynaems, 1976).

$$\frac{-d[FITC\text{-LL}]_{bound}}{dt} = k_{-1}[R\text{-FITC-LL}] \quad (11)$$

and after integration:

$$\ln [FITC\text{-LL}]_{bound} = -k_{-1}t \quad (12)$$

Figure 4 shows the actual dissociation curves and their transformation by means of eq 12. The curves were obtained using the same FITC-LL concentrations and the same temperatures

TABLE IV: Thermodynamic Constants Calculated from the Rate Constants.[a]

t (°C)	$10^{-6} \times K_A$ (M^{-1})	E_A (assoc) (kcal/mol)	E_A (dissoc) (kcal/mol)	$-\Delta H$ (kcal/mol)
5	8.7 ± 0.9	7.2	12.8	5.6
25	3.4 ± 0.3			
37	3.2 ± 0.4			

[a] The association constant was calculated as k_1/k_{-1}, taking the mean of the values obtained at the two concentrations (Table III); the activation energy (E_A) was calculated by the Arrhenius plot: the plot for the association having $R = 0.98$, the plot for the dissociation $R = 0.97$; ΔH was calculated as E_A(assoc) − E_A(dissoc).

as for the associations kinetics. Table IV summarizes the calculated dissociation rate constants.

The experimental data, especially for longer time intervals, do not exactly fit the straight lines (Figure 4), suggesting the existence of another equilibrium with a very low affinity. Nevertheless, the calculation of the initial dissociation rate constants gives approximately the same values for the two FITC-LL concentrations tested at the same temperature.

Discussion

Methodology. The use of fluorescent-labeled lectins has been almost restricted to the study of the localization of receptors on different cell structures. To our knowledge, quantitative fluorometry has not yet been applied to the physicochemical study of lectin–receptor interactions.

The advantages of the approach are multiple: besides avoiding the use of radioisotopes, the binding of the reagents can be tested directly by fluorescence microscopy and, if the presence of cells with different binding characteristics can be demonstrated, they can be separated by flow microfluorometry in a fluorescence-activated cell sorter.

Linearity of fluorescence vs. concentration can be obtained in a very wide range (10^{-9} to 10^{-6} M). The only limit is set by the sensitivity of the fluorometer. In this study it was 10^{-9} M and is to be compared with 10^{-11} M for radioiodinated lectins (Cuatrecasas, 1973; Kornfeld et al., 1971). However, it must be mentioned that the lower sensitivity in our studies is due to the absorption quenching of the fluorescence by the liberated hemoglobin. Studies with nucleated cells (Schreiber et al., 1978) showed a 10^{-10} M sensitivity.

From the inhibition curve with nonfluorescent LL, it appears that the fluoresceination does not alter the binding characteristics of the molecule (Figure 2). The quantitative fluorometric method seems, therefore, to be a useful tool, which could be extended to other physicochemical studies of ligand–receptor interactions.

Equilibrium Data. Human erythrocytes have been reported to possess 5.5×10^5 receptors per cell for LL (Kornfeld et al., 1971) and 5.2×10^5 for *Phaseolus vulgaris* lectin (Kornfeld, 1969). Con A receptors on human erythrocytes are less numerous (1.8×10^5) (Schnebli et al., 1977). Mouse erythrocytes, on the other hand, have 6.8×10^6 receptors per cell for Con A (Edelman and Millette, 1971). The value obtained for rabbit erythrocytes (1.2×10^6) in the present work is intermediate between the values obtained for human and mouse erythrocytes.

In addition to the limited variation of the number of receptors on cells of different origins, the affinity constant found in our experiments was similar to that obtained for human erythrocytes (4.4×10^6 M^{-1}) by Kornfeld et al. (1971) and comparable to that found for the Con A receptors on the same cells (3.0×10^6 M^{-1}) by Schnebli et al. (1976). The linearity of the Scatchard plot, indicating lack of cooperativity of the receptors, was further confirmed by the angular coefficient of the Hill plot which, for the three temperatures, approaches unity. This lack of cooperativity, which contrasts with reports on Con A binding on nucleated cells (Chang and Cuatrecasas, 1976; Wright and Ceri, 1977), could be due to the absence of the reversible dimer-tetramer transition in LL or the absence of a microtubular–microfilamentar system in the erythrocytes.

From the saturation binding curve, it appears that, even at low saturation, less than 1% of the lectin molecules have their two binding sites occupied. From calculation of the surface of the erythrocyte, the mean average distance between the receptors was estimated at 10 nm. Since the diameter of the lectin molecule is 3.5 nm, doubly occupied lectin molecules would only be found in high-density receptor regions. The interpretation of our thermodynamic results suggests that, as far as the lentil lectin is concerned, high-density receptor regions are rare. Agglutination is thus achieved through a low number of intercellular lectin bridges.

Thermodynamic Parameters of the Lectin–Ligand Interactions. Due to the very low affinity of LL for the specific hexoses, the direct determination of the association constants for D-mannose and D-glucose varies widely, yielding values between 10^2 and 10^3 M^{-1} (Stein et al., 1971). More recently, the association constant for methyl α-D-glucoside and methyl α-D-mannoside were calculated, respectively, as 2×10^2 and 4×10^2 by an indirect method (Horejsi et al., 1977). The values obtained in this work are higher and confirm the higher affinity of the lectin for the mannose residue over the glucose residue. The reaction occurs practically without enthalpy change, the binding reaction being driven by an entropy change. When compared to the thermodynamic parameters calculated from data of the literature for Con A (Loontiens et al., 1977), the differences are striking, since this reaction is exothermic ($\Delta H = -6.1$ kcal/mol) with a low decrease in entropy ($\Delta S = -2.3$ eu).

The reaction of LL with the erythrocyte receptors is exothermic, while ΔH is negligible for the binding to monosaccharides. It is tempting to suggest that the enthalpy change is due to the structural complexity of the ligand on the erythrocyte membrane (Kornfeld et al., 1971), resulting in a tighter interaction. The negative enthalpy change of the reaction contrasts with the observations on Con A receptor interactions (Betel and Van den Berg, 1972; Huet et al., 1974) which show a positive enthalpy change. This could be explained by the endothermic dimer-tetramer transition of Con A, which is absent in LL. Therefore, LL showing no such transition behaves as other lectins, in which a decrease in the association constant with temperatures was observed (Steck and Wallach, 1965; Rigas and Johnson, 1964).

Kinetic Parameters of the Lectin–Receptor Interactions. Few reports have been published about the association kinetics of lectins to their receptors. From the dissociation rate and the association constant, Steck and Wallach (1965) calculated an association rate constant k_1 for 2.0×10^3 M^{-1} s^{-1} (1 °C) for the binding of *Phaseolus vulgaris* lectin to Ehrlich carcinoma cells; Podder et al. (1974) obtained similar values for the association of Con A to the carbohydrate moiety of *Ricinus communis* lectin ($k_1 = 3.0 \times 10^3$ M^{-1} s^{-1} at 29 °C). Somewhat lower values were reported for the binding of Con A to neuroblastoma cells (0.6×10^3 M^{-1} s^{-1} at 1 °C) (Rosenberg and Charalampous, 1977). The k_1 calculated from our experiments correspond to these values, and are only ten times slower than those calculated for the binding of the monosac-

charides to Con A (Gray and Glew, 1973; Clegg et al., 1977).

The dissociation rate constant k_{-1}, however, was found to be 40 times slower than that of the Con A–*Ricinus communis* lectin (Podder et al., 1974) and 1000 to 2000 times slower than that for the binding of the monosaccharides to Con A (Gray and Glew, 1973; Clegg et al., 1977). These results confirm other reports of the very slow dissociation of lectin–receptor complexes (Cuatrecasas, 1973).

The activation energy calculated from the Arrhenius plot shows that the dissociation requires almost twice the amount of energy necessary for the association. Activation energies and rate constants thus show that the high affinity of the lectin to the receptor is mainly due to the slow and energetically unfavorable dissociation reaction.

Finally, the association constants calculated from the rate constants and the enthalpy change calculated from the activation energies (Table IV) are fully consistent with those parameters obtained from the equilibrium data, giving an internal control of the experimental approach.

In summary, this work demonstrates the validity of the fluorometric method in the thermodynamic and kinetic interpretation of the lectin–receptor interactions. It shows that, under conditions when even a low number of receptors are saturated, most lectin molecules are monovalently bound to the receptor, the agglutination phenomenon being accounted for by a very low fraction of linked receptors ($\leq$0.02%). The high affinity to the membrane receptor, contrasting with the low affinity for the specific monosaccharides, can be explained by the very slow dissociation reaction, which requires twice the activation energy of the association reaction.

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References

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

Boeynaems, J. H. (1976), *Anal. Biochem. 70,* 366.

Bourguignon, Y. W., and Singer, S. J. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 5031.

Chang, K. J., and Cuatrecasas, P. (1976), in Concanavalin as a Tool, Bittiger, C. H., and Schnebli, H. P., Ed., London, Wiley, p 201.

Cheng, Y., and Prusoff, W. H. (1973), *Biochem. Pharmacol. 22,* 3099.

Clegg, R. M., Loontiens, F. G., and Jovin, T. M. (1977), *Biochemistry 16,* 167.

Cuatrecasas, P. (1973), *Biochemistry 12,* 1312.

Edelman, G. M., and Milette, C. F. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2436.

Edelman, G. M., Yahara, I., and Wang, J. L. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1442.

Foriers, A., De Neve, R., Kanarek, L., and Strosberg, A. D. (1978), *Proc. Natl. Acad. Sci. U.S.A. 75,* 1136.

Gray, R. D., and Glew, R. H. (1973), *J. Biol. Chem. 248,* 7547.

Green, N. M. (1964), *Biochem. J. 90,* 564.

Horejsi, V., Ticha, M., and Kocourek, J. (1977), *Biochim. Biophys. Acta 499,* 290.

Howard, I. K., and Sage, H. J. (1969), *Biochemistry 8,* 2436.

Howard, I. K., Sage, H. J., and Stein, M. D. (1971), *J. Biol. Chem. 246,* 1590.

Huet, C., Longchampt, M., Huet, M., and Bernadac, A. (1974), *Biochim. Biophys. Acta 365,* 28.

Huet, M. (1975), *Eur. J. Biochem. 59,* 627.

Kornfeld, S. (1969), *Biochim. Biophys. Acta 192,* 542.

Kornfeld, S., Rogers, J., and Gregory, W. (1971), *J. Biol. Chem. 246,* 6581.

Lis, H. L., and Sharon, N. (1973), *Annu. Rev. Biochem. 42,* 541.

Loontiens, F. G., Clegg, R. M., and Jovin, T. M. (1977), *Biochemistry 16,* 159.

Podder, S., Suralia, A., and Bachhawat, B. K. (1974), *Eur. J. Biochem. 44,* 151.

Rigas, A. A., and Johnson, E. A. (1964), *Ann. N.Y. Acad. Sci. 113,* 800.

Rosenberg, S. B., and Charalampous, F. C. (1977), *Arch. Biochem. Biophys. 181,* 117.

Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.

Schnebli, H. P., Roeder, C., and Tarcsay, L. (1976), *Exp. Cell Res. 98,* 273.

Schnebli, H. P., Lustig, A., Zulauf, M., Winterhalter, K. H., and Joss, U. (1977), *Exp. Cell Res. 105,* 151.

Schreiber, A. B., Hoebeke, J., Bergman, Y., Haimovich, J., and Strosberg, A. D. (1978), *J. Immunol. 121,* 19.

Sharon, N. (1976), in Mitogens in Immunobiology, New York, N.Y., Academic Press, p 31.

Steck, T. L., and Wallach, D. F. H. (1965), *Biochim. Biophys. Acta 97,* 510.

Stein, M. D., Howard, I. K., and Sage, J. H. (1971), *Arch. Biochem. Biophys. 146,* 353.

Wright, J. A., and Ceri, H. (1977), *Biochim. Biophys. Acta 469,* 123.