

STOICHIOMETRY OF WHEAT GERM AGGLUTININ AS A MORPHOLOGY CONTROLLING AGENT AND AS A MORPHOLOGY PROTECTIVE AGENT FOR THE HUMAN ERYTHROCYTE

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ABSTRACT

The lectin wheat germ agglutinin (WGA) is an unusually effective agent in controlling both the forward and reverse reactions of the reversible morphology conversion discocyte \rightleftharpoons echinocyte for the human erythrocyte. Under conditions severe enough to drive the reactions to completion in either direction without the lectin, WGA is able to stabilize both these morphologies and to fully prevent conversion of either morphology. The lectin can quantitatively block both reactions. The ability of WGA to carry out these functions has no obvious rate limitation. Its effectiveness depends mainly on its binding stoichiometry, particularly toward the transmembrane glycoprotein, glycophorin. The critical binding stoichiometries for both the lectin and the echinocytic agent were determined in relation to the binding isotherms using ^{125}I -labeled WGA and ^{35}S -labeled dodecyl sulfate. There appear to be two principal stoichiometries for WGA binding that are important in its control of erythrocyte morphology. The first stoichiometry marks the threshold of obvious protection of the discocyte against strong echinocytic agents such as detergents and, likely, is simply a 1:1 stoichiometry of WGA: glycophorin, assuming currently recognized values of $3\text{--}5 \times 10^5$ copies of glycophorin per cell. The second important stoichiometry, whereby the cell's morphology is protected against extremely severe stress, involves binding of $\sim 4\text{--}5$ WGA molecules per glycophorin. The controls that WGA exerts can be instantly abolished by added *N*-acetylglucosamine. However, *N*-acetylglucosamine ligands on the erythrocyte are of less importance than membrane neuraminic acid residues in enabling WGA to control the cell's morphology, as is shown by comparing intact cells with completely desialated cells. WGA can also be used to produce elliptocytes in vitro, but it does this at levels approaching monolayer coverage of the cell with WGA.

Lectins have long been recognized as proteins that agglutinate human erythrocytes. Their agglutination properties have been important tools in de-

tecting several of the blood groups (17). Less obvious, however, is the extent of the control lectins exert over the discrete morphologies that may be

obtained from the normal discocyte, if indeed they affect erythrocyte morphology at all.

The echinocyte is a common, well-recognized morphology that can be readily produced from the discocyte by echinocytic ligands such as fatty acid anions and alkyl sulfates. Upon removal of the echinocytic ligands by binding them to a protein such as serum albumin, echinocytes may be quickly returned to discocytes. Therefore, these two morphology conversions may be represented as the reversible reaction discocyte \rightleftharpoons echinocyte. The main emphasis of this paper is the control that wheat germ agglutinin (WGA) can impose on both reactions in this conversion. However, WGA can also produce a discrete morphology from normal erythrocytes, namely, the elliptocyte. That is, WGA can drive the *in vitro* conversion discocyte \rightarrow elliptocyte by itself without the addition of another agent. Ordinarily, elliptocytes are produced only by *in vivo* elliptocytosis.

There are diverse views pertaining to how the human erythrocyte's morphology is controlled and to how the membrane lipids and proteins are involved. One view is that the integral membrane proteins have no control of the cell's morphology, but that the membrane lipids are paramount (37). An alternative view is that the parameters that describe the erythrocyte membrane's mechanical properties are remote from the parameters expected simply from lipid bilayer behavior. There seems to exist a fairly tough matrix, requiring contributions by proteins (10). The majority of authors, considering a number of the biochemical, compositional, and biological aspects of the erythrocyte membrane, believe that there is important involvement of membrane proteins ("mechanoproteins") in both the cytoskeleton and transmembrane proteins that govern cytoskeletal behavior (22).

We subscribe to this second view because of the avenues through which WGA likely exerts its control over erythrocyte morphology. In the search for simple relationships, namely, the stoichiometries, between membrane morphology controlling agents and their receptors, it is necessary to gather detailed data from the regions in which the membrane's morphology changes are brought under control and to determine the binding isotherms for the lectins that can exert such controls. The distinction between amounts bound, denoted ν , and amounts added, denoted y , is important, particularly when the binding constants are intermediate or small. The practical result is that lectin-con-

trolled morphology changes, viewed in their relation to amounts actually bound, ν , may engender a different picture from that evoked simply by the total added levels, y .

The most critical stoichiometries for WGA binding to the erythrocyte, from a correlation of the direct binding studies and their relation to erythrocyte morphology changes, need to be compared with the number of copies of glycophorin per erythrocyte membrane. This is for two reasons: first, glycophorin has been thought to be the principal receptor for WGA (2); second, glycophorin is increasingly thought to be a transmembrane protein, spanning the erythrocyte membrane and linked to the cytoskeleton. Inasmuch as glycophorin is an avenue for control of at least some of the determinants of the cell's morphology (26), WGA might be expected to affect the cell's morphology.

The eleven lectins that we examined are listed with their specificities, which are rather diverse, in Table I. None of the other lectins exhibited nearly the force nor the versatility that WGA can exert as a controlling agent of erythrocyte morphology. Moreover, WGA performs its functions quite rapidly, within 20–100 s in the concentration range of 10^{-7} – 10^{-8} M, unlike the other lectins. Apparently, WGA's interaction with the erythrocyte and the control it has over it depend simply on equilibrium processes, not on rate processes, at 25°C. This is the case for both the forward and reverse reactions in the discocyte \rightleftharpoons echinocyte conversion. Accordingly, our work concentrated on WGA's ability to control erythrocyte morphology.

MATERIALS AND METHODS

Intact Erythrocytes and General Procedures

Erythrocytes were drawn from individuals of various blood types using procedures quite similar to those previously used (21). The cells were washed three times in isotonic Tris-saline (0.015 M Tris, pH 7.4) and were used for experiments within 3 h after the last washing. New stocks were generated for each day's use. All components used to interact with the cells were dissolved in the same Tris-saline buffer and rapidly mixed immediately upon addition. Components were added in amounts that approximately doubled the cell suspension volume in each case, giving a final standard cell concentration of $\sim 1 \times 10^7$ cells/ml, except when agglutinating cell concentrations were used. The sequence of addition of the various reagents is most important and is described below. The standard temperature was $25.0 \pm 0.5^\circ\text{C}$ in all experiments involving discocyte \rightleftharpoons echinocyte equilibria. Both 25° and 37°C temperatures were used in the discocyte \rightarrow elliptocyte conversion. The cells were fixed by the standard procedure for bright-field microscopy with a final concentration of 0.1% of SEM grade glutaraldehyde (Sigma Chemical Co., St. Louis, Mo.), including phenol red to indicate pH 7.4.

TABLE I
Lectins Surveyed

Lectins	Concentration range $\mu\text{g/ml}$	Specificity (14)	
		Monosaccharide	Erythrocyte receptor
Concanavalin A	0-5,000	$\alpha\text{-D-Manp} > \alpha\text{-D-Glcp}$	Band 3 (12)
<i>Ricinus communis</i> (120,000-dalton tetramer)	0-1,000	$\beta\text{-D-Galp} > \alpha\text{-D-Galp}$	Band 3 and others (2)
<i>Lens culinaris</i>	0-200	$\alpha\text{-D-Manp} > \alpha\text{-D-Glcp}$	Glycophorin, band 3 (12)
<i>Phaseolus vulgaris</i>	0-2,000	Oligosaccharides of $\beta\text{-D-Galp}$, $\beta\text{-D-Manp}$, and $\beta\text{-D-GalNacp}$	Glycophorin, band 3 (24)
<i>Arachis hypogaea</i>	0-2,000	$\beta\text{-D-Galp-(1-3)-D-Gal Nacp}$	
<i>Limulus</i>	0-500	NANA	
<i>Solanum tuberosum</i>	0-1,000	$\beta\text{-D-GlcNAcp}$ oligosaccharides	
<i>Bandeiraea simplicifolia</i>	0-1,000	$\alpha\text{-D-Galp} > \alpha\text{-D-GalNacp}$	
Glycine max	0-3,000	$\alpha\text{-D-GalNacp} > \beta\text{-D-GalNacp}$	
<i>Pisum sativum</i>	0-500	$\alpha\text{-D-Manp} > \alpha\text{-D-Glcp}$	
Wheat germ agglutinin (<i>Triticum vulgaris</i>)	0-500	$\beta\text{-D-GlcNAcp}$, oligosaccharides and NANA	Glycophorin (2)

WGA, ¹²⁵I-WGA, and N-acetylglucosamine (NAG)

The lectin was prepared from raw wheat germ using the method of Bouchard et al. (5), followed by affinity chromatography on Ovomucoid-Sepharose according to Marchesi (23). The lectin was crystallized at pH 4.5 in an acetate-NaCl system (0.1-0.2 M concentrations of each component) at 4°C.

For ¹²⁵I labeling, the Fraker and Speck procedure (13) was used. This method has several distinct advantages over lactoperoxidase-based methods for iodination of proteins. The ¹²⁵I-WGA prepared in this way (average specific activity, 5×10^7 dpm/mg) was >95% retained on the affinity column. The molecular weight of WGA is taken as 36,000.

The inhibitor N-acetylglucosamine and sialic acid were purchased from Sigma Chemical Co.

Radiolabeled Sodium Dodecyl Sulfate

³⁵S-labeled dodecyl sulfate (sodium salt) was prepared by use of [³⁵S]chlorosulfonic acid (Amersham-Searle Corp., Arlington Heights, Ill.) in a procedure starting with dodecyl alcohol, using Davidsohn and Milwidsky's general synthetic method (8). After coupling and removal of HCl, the mixture was neutralized to the phenol red endpoint in the cold with NaOH and lyophilized. It was then recrystallized three times from absolute ethanol. The specific activity was 7.5×10^{11} dpm/mol; mol wt, 288.7.

Binding Isotherms and Scintillation Counting

The general procedure used for determining the amount of material bound has been previously described (21). It relies on centrifugation of the cells and calculation of the amount bound as the difference between the amount added and the amount left free after equilibration. Scintillation counting was carried out in Patterson and Greene's fluid (28) in a Beckman LS3133 (Beckman Instruments, Inc., Fullerton, Calif.) at an efficiency of 60-70%.

Electron Microscopy Preparation

Cells were fixed for electron microscopy with a combination of 2 ml of cell suspension and 0.1 ml of 2.1% SEM grade glutaraldehyde in the buffer, with the glutaraldehyde reagent adjusted to the phenol red endpoint (5 mg/liter of phenol red). After 20 min, the concentration of glutaraldehyde was increased to 3% by the addition of 2 ml of 6% glutaraldehyde. The mixture was incubated for 2 h at 25°C. The cells were then washed three times in water. A drop of concentrated cell suspension was placed on a coverslip and immersed in 25% acetone. The acetone concentrations were increased by increments from 25 to nearly 100% acetone. The cells were then dried at the critical point of carbon dioxide. 10-15 Å of gold was condensed onto the mounted cells by evaporation with a Denton DVS02 vacuum evaporator (Denton Vacuum Inc., Cherry Hill, N. J.) at 10^{-7} torr. The cells on their mounts were viewed in a Hitachi SEM Model 450.

Neuraminidase Desialation

Sialic acid residues (N-acetyl neuraminic acid [NANA] residues) were removed by means of *Vibrio cholerae* neuraminidase. The pH range was 7.0-7.4 in Tris-saline isoosmotic buffer, which also contained 10 mM CaCl₂. The cell concentration was 1×10^6 cells/ml (300 Behringwerke U/ml (36)) at 37°C for 1.2 h. The reason for using a Tris buffer in conjunction with this enzyme is that the Ca⁺⁺ stays in solution. Phosphate buffers, which are sometimes used, precipitate calcium (1). The amount of released NANA was quantitated by thiobarbiturate colorimetry (42).

RESULTS

Lectins Surveyed

Two aspects of 11 lectins were studied: their ability to change the morphology of human erythrocytes and their ability to control morphology changes imposed by echinocytic agents. The 11

lectins are listed in Table I, together with their specificities. WGA turned out to be the most reactive lectin in both regards. The controls which the other lectins seem to exert are quite diverse and merit a separate study. A salient difference between WGA and the other lectins is that WGA carries out its effects on the erythrocyte nearly as rapidly as the cell can change shape without the lectin, processes about which detailed knowledge exists (3). The other lectins, especially concanavalin A, are markedly time dependent in whatever controls they exert, at least with respect to the erythrocyte at 25°C. The properties of WGA vis-à-vis the erythrocyte are apparently controlled by whatever thermodynamic limitations exist, whereas the other lectins are rate limited in their overall reactions with the erythrocyte, as far as control of morphology is concerned. Therefore, our main work was confined to the WGA-erythrocyte system.

Erythrocyte Morphologies and Pathways for Conversion

The controls that WGA imposes on erythrocyte morphology depend on cell concentration and on the amount of WGA added (y_{WGA}). Most of the conditions that we used pertain to subagglutinating concentrations of erythrocytes on the order of 10^6 – 5×10^7 cells/ml. However, it is striking how, when agglutinating concentrations of cells are employed with WGA (5×10^8 cells/ml or greater), extreme (and instant) distortion ensues, initiated by WGA's effects on the membrane. Fig. 1*a* illustrates normal (control) discocytes. Fig. 1*b* shows a typical WGA agglutinate of erythrocytes. Under agglutinating conditions, isolated erythrocytes still remain normal discocytes. The extreme distortion to helmet-shaped cells, codocytes, knizocytes, etc., (we use Bessis's terminology [4]) occurs only in the agglutinated clump. Therefore, it appears that such distorted morphologies, facilitated by WGA, are developed in full by cell-cell interaction. Because in other laboratories, proteolytic enzymes are often used to increase agglutinability in conjunction with lectin-erythrocyte interaction (20), we wish to specifically note that proteolysis is not necessary to obtain severe cell distortion from WGA agglutination, as can be seen in Fig. 1*b*.

The other morphologies in Fig. 1 (*c-f*) pertain to subagglutinating cell concentrations. The two principal results that illustrate the control over human erythrocyte morphology changes and the

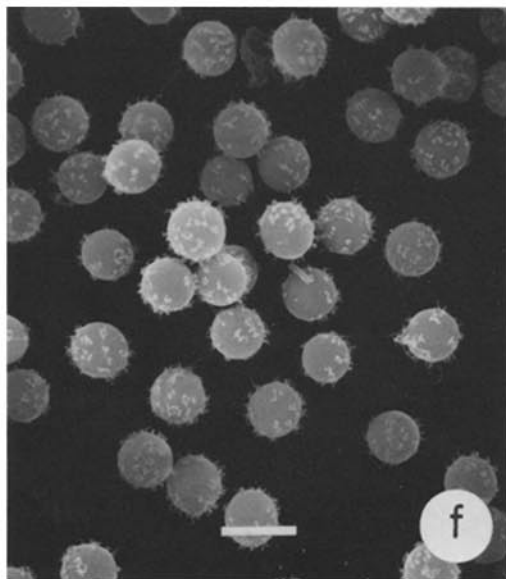
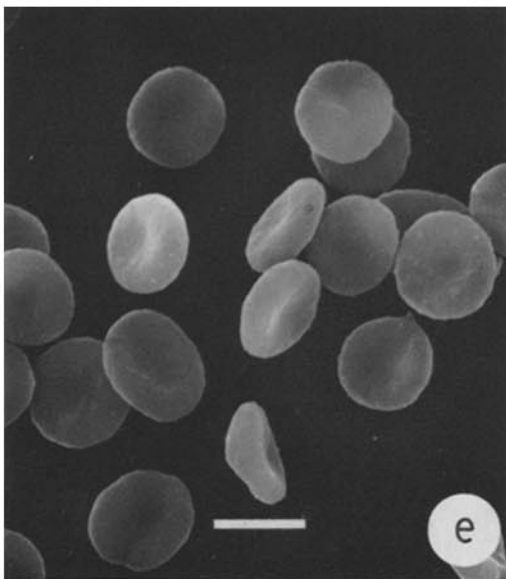
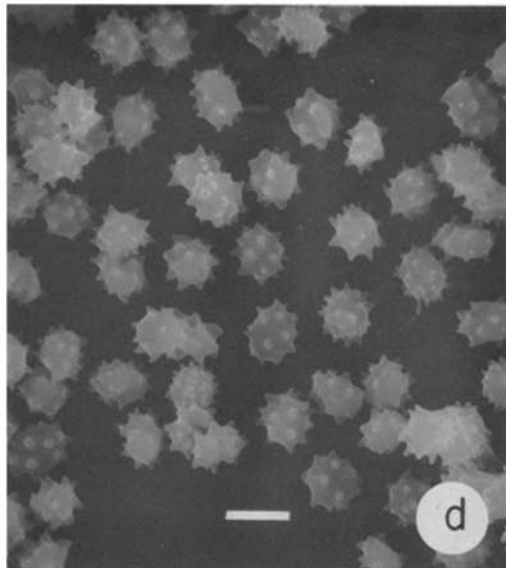
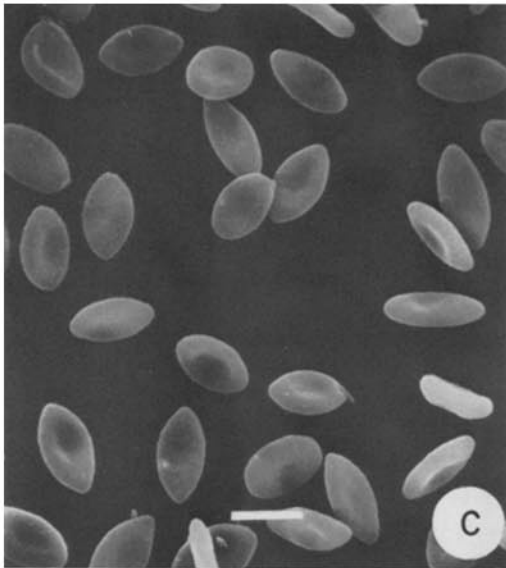
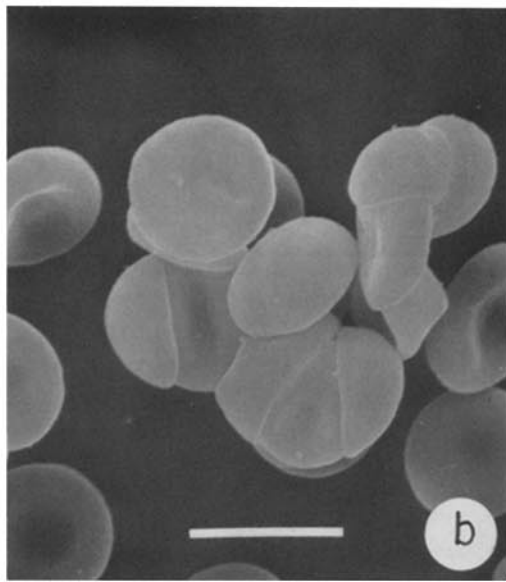
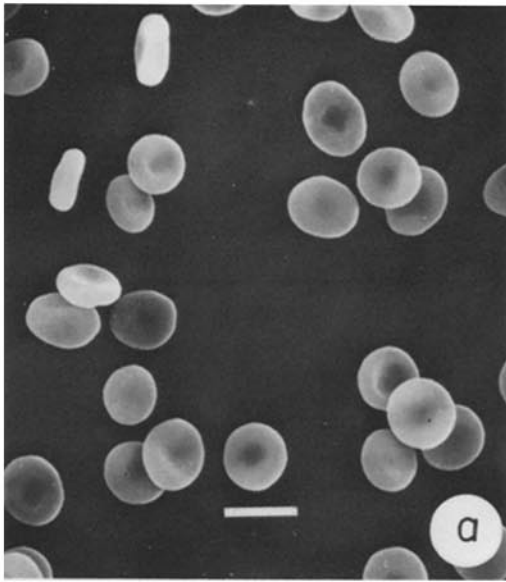
production of a discrete morphology engendered by WGA are displayed in Fig. 2 and are described below.

(*a*) The elliptocyte may be readily produced from normal discocytes *in vitro* by proper adjustment of WGA and cell concentration. At least 80% conversion to elliptocytes can be achieved at 25°C, and the conversion is even more complete at 37°C. Fig. 1*c* shows typical results from the 25°C conversion. Cells from seven different individuals with various blood types were investigated. No major differences were seen within this group in the WGA-driven conversion discocyte → elliptocyte.

(*b*) The lectin WGA is a powerful agent, which can protect or stabilize the normal human erythrocyte in certain of its morphologies, including the discocyte. A vivid illustration of this is provided by our experiments with the strongly echinocytic agent dodecyl sulfate. The sequences of addition, or pathways, are shown in Fig. 2. Echinocytes, which are readily formed by such agents from normal discocytes, are shown in Fig. 1*d*. The lectin WGA can, however, completely block formation of echinocytes if added at the proper point in the sequence. Fig. 1*e* shows cells protected by WGA, essentially retaining the discocyte morphology, in the face of dodecyl sulfate at a concentration 50 to 100 times that ordinarily needed to produce echinocytes such as those shown in Fig. 1*d*. The cells shown in Fig. 1*e* are not perfect discocytes. They retain the overall discocyte morphology but are not smooth or normal like those shown in Fig. 1*a*. An illustration of the extreme distortion that the imperfect discocytes in Fig. 1*e* would have produced if they had not been protected by WGA is provided by Fig. 1*f*. The cells in Fig. 1*f* were subjected to the same concentration of echinocytic agent as the cells in Fig. 1*e*, but the Fig. 1*f* cells were not protected by WGA. Unprotected cells are profoundly distorted and start to lyse. With WGA protection, there is <1% lysis according to hemoglobin measurements.

As will be shown below, the lectin WGA can also protect the echinocyte against forces that ordinarily would completely convert it to the discocyte. In short, WGA can thoroughly block both directions in the reversible morphology conversion discocyte \rightleftharpoons echinocyte.

The concentration tolerances pertaining to Figs. 1 and 2, within which the results are readily reproducible, are given in Table II. These tolerances or ranges are somewhat restricted but are not incon-



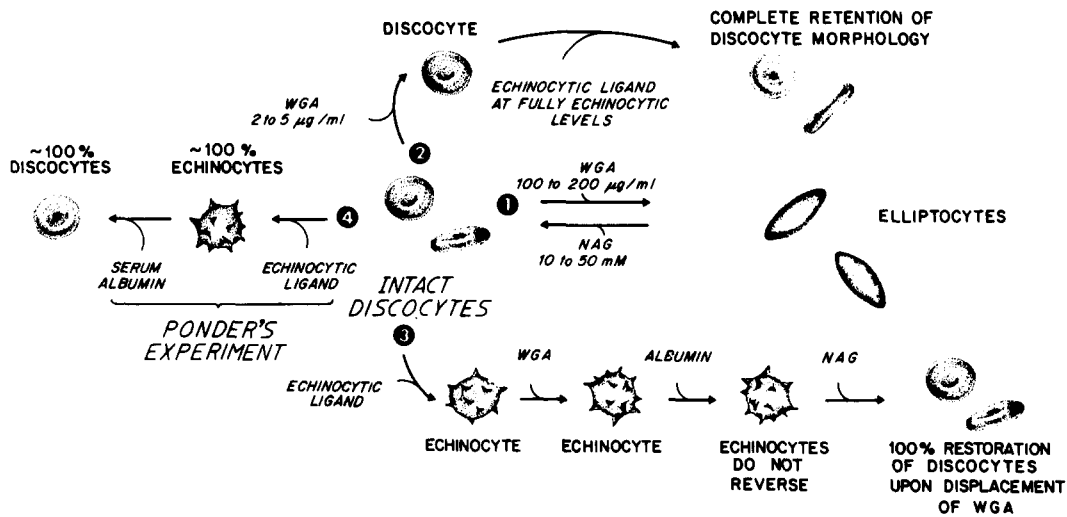


FIGURE 2 The principal controls that can readily be imposed on normal discocyte morphology conversions by WGA in relation to the sequences of addition and concentration ranges given in Table II. "Ponder's experiment" in pathway 4 refers to Ponder's original observations (30).

veniently narrow. The sequence of addition of agents to the cells must be carefully considered and are now described in more detail.

Conditions for Production of the Various Morphologies and Their Control Reactions as Illustrated in Fig. 2

PATHWAY 1: With subagglutinating amounts of cells and relatively high concentrations of WGA, elliptocytes are rapidly formed at a level of $\sim 40 \times 10^{-6} \mu\text{g}$ of WGA added per cell. This corresponds to $\gamma_{\text{WGA}} = 7 \times 10^8$ molecules of WGA added per cell, but fewer than that are bound. The binding data indicate that ν_{WGA} is $\sim 1-2 \times 10^7$ molecules of WGA bound per cell. This is enough, nevertheless, to completely cover the cell surface. If we use $1.5 \times 10^{10} \text{ \AA}^2$ as the available area for the discocyte and a spherical radius for WGA of 25 \AA , a closely packed monolayer of WGA molecules should occur at about 1.5×10^7 molecules of

WGA per cell. There is no proof that such a monolayer actually forms. Clustering of WGA at certain receptors is quite possible.

The discocyte \rightarrow elliptocyte conversion is fully reversible by the WGA-specific ligand, *N*-acetylglucosamine (NAG). No elliptocytes are seen at a 50 mM NAG concentration. Quite discernible reversal is seen at ~ 10 mM NAG. At a 50 mM NAG concentration, there are $\sim 1,300$ moles of NAG per mole of WGA receptor sites, using Nagata and Burger's (27) value of four receptor sites per WGA (mol wt 36,000). The conditions resulting in elliptocyte formation do not give complete conversion. Some discocytes remain. However, the mixture is dominated by elliptocytes, discocytes comprising only 10–20% of the population.

PATHWAY 2: When WGA is added as a first step in the proper amount (Table II), the discocyte remains protected against the echinocytic agent dodecyl sulfate (DOD). It has been shown (21) that DOD at a level of $\nu_{\text{DOD}} = \sim 2 \times 10^7$ ligands

FIGURE 1 Morphologies of the human erythrocyte. (a) Control cells, normal discocytes. (b) Agglutination of erythrocytes by WGA; severe distortion upon cell-cell interaction when agglutinating concentrations of cells are employed. (c) Elliptocytes produced by WGA from normal discocytes, isoosmotic buffered saline, pH 7.4, 25°C. Conditions specified in Table II and pathway 2 of Fig. 2. (d) Echinocytes from normal levels of echinocytic agent, 2×10^7 dodecyl sulfate anions bound per cell. (e) Erythrocytes first protected with WGA in the presence of 100 times the normal echinocytic level of dodecyl sulfate, producing moderate distortion of the discocyte. (f) Spheres and spherocytocytes under the same conditions as in (e) but without WGA. Bar, 5 μm .

TABLE II
Range of Conditions Used in the Pathways for Figure 2

Pathway 1	$\frac{\text{Cell concentration}}{1-5 \times 10^6 \text{ cells/ml}}$	$\frac{\text{WGA}}{100-300 \mu\text{g/ml}}$	$\frac{\text{NAG (GluNAc) reversal}}{10-50 \text{ mM}}$		
Pathway 2	$\frac{\text{Cell concentration}}{10^6-5 \times 10^7 \text{ cells/ml}}$	$\frac{\text{WGA}}{2-50 \mu\text{g/ml}}$	$\frac{\text{Ligand}}{2-10 \times 10^7 \text{ DOD bound/cell}}$	$\frac{\text{GluNAc reversal}}{0.5-5 \text{ mM}}$	
Pathway 3	$\frac{\text{Cell concentration}}{10^6-5 \times 10^7 \text{ cells/ml}}$	$\frac{\text{Ligand}}{\sim 4 \times 10^7 \text{ DOD bound/cell}}$	$\frac{\text{WGA}}{2-5 \mu\text{g/ml}}$	$\frac{\text{Plasma albumin}}{\sim 200 \mu\text{g BSA/ml}}$	$\frac{\text{GluNAc reversal}}{0.5-5 \text{ mM}}$
Pathway 4	$\frac{\text{Cell concentration}}{10^6-5 \times 10^7 \text{ cells/ml}}$	$\frac{\text{Ligand}}{10-20 \times 10^7 \text{ DOD bound/cell}}$	$\frac{\text{Plasma albumin}}{\sim 100 \mu\text{g/ml}}$		

bound per cell is ordinarily sufficient to convert all discocytes to echinocytes. Under conditions under which the WGA is bound first, not only are no echinocytes formed but the cells can readily withstand DOD at considerably greater levels than $\nu_{\text{DOD}} \cong 2 \times 10^7$ without switching morphology. This reaction is referred to as a protection reaction.

The level of WGA needed to protect the cells as discocytes is far lower than that needed to convert them to elliptocytes. About $0.2 \times 10^{-6} \mu\text{g}$ of WGA per cell is needed, 100–200 times less than in pathway 1.

PATHWAY 3: Addition of DOD to discocytes as a first step instantly resulted in the formation of echinocytes (discocyte \rightarrow echinocyte) as expected. Ordinarily, addition of serum albumin removes the ligand DOD from such elliptocytes, and the cells readily revert to discocytes without delay (echinocyte \rightarrow discocyte). This commonly investigated reaction is illustrated in pathway 4. But if the echinocytes are treated with WGA before serum albumin is added, serum albumin, when it is added as a third step, is completely unable to reverse the echinocyte to the discocyte. Hence, WGA performs a function very similar to its function in pathway 2: it quantitatively locks the cells into whatever morphology they have, be they echinocytes or be they discocytes, at the point of addition of WGA. The morphologies are completely stable over long periods (hours).

However, if NAG is then added as a fourth step, the cells instantly and quantitatively revert to normal discocytes. Thus, NAG displaces WGA. Because serum albumin is already present in sufficient quantity at this stage to bind all the echinocytic ligand, the final result is that all the cells immediately return to discocytes.

However, there remains a question: does WGA merely prevent serum albumin's removal of DOD

from the cells? A distinction should be made between echinocytes stabilized by WGA and free of DOD and echinocytes with both WGA and DOD bound in which WGA somehow prevents serum albumin removal of DOD. The distribution of DOD was measured using ^{35}S -labeled DOD. Echinocytes were formed by subjecting discocytes to cold and [^{35}S]DOD, then stabilized by WGA. Serum albumin was then added in various concentrations as part of pathway 3. After equilibration, the system was centrifuged, and the supernate was counted. Serum albumin remaining in the supernate would be expected to gain the ^{35}S -labeled DOD if it removed DOD from the echinocytes. In control experiments, the same dilutions were carried out without protein. Hence, the net amount of DOD removed by serum albumin from the echinocytes was measured as a function of serum albumin concentration. The slope, in units of (molecules DOD bound) (ml) (cell) $^{-1}$ (molecules serum albumin) $^{-1}$ was 3.3×10^{-13} , negative in sign. This corresponds, taking into account the stoichiometry of serum albumin for DOD, which is between 5 and 10 (33), to quantitative transfer of the DOD that originally produced the echinocytes to the serum albumin. Yet the cells remain echinocytes so long as the WGA concentration is maintained. This clearly demonstrates that WGA does not prevent the echinocyte \rightarrow discocyte conversion by simply preventing removal of the DOD. The DOD is exhaustively removed by stoichiometric amounts of serum albumin.

PATHWAY 4: This pathway was established some time ago by Ponder (30). It is illustrated here for completeness and to emphasize again the excellent reversibility of the discocyte \rightleftharpoons echinocyte conversion.

The lectin WGA is indeed effective in controlling erythrocyte morphology and erythrocyte re-

sponse to ligands. At this point, there are two main questions: what is the apparent number of critical sites for WGA binding at which control is exerted and what relationship do they have to the number of membrane components of which the membrane is composed?

Binding Stoichiometries and Binding Isotherms

The number of critical binding sites for WGA control of erythrocyte morphology was sought by direct determination of binding isotherms of WGA in relation to the morphology changes it controls and by quantitating the discocyte → echinocyte titration plots that are governed by WGA. Fig. 3 shows how the fraction of cells that are echinocytes, r , varies with y_{DEC} , the added amount of decyl sulfate. Fig. 3 also shows how WGA shifts such a plot at a concentration of 1.9×10^{-8} M WGA. A major shift in the plot is caused by WGA, as was expected from its behavior in pathway 2. The displacement resulting from WGA's protective effect is measured by the area between the two plots: r vs. y_{DEC} with and without WGA for each prescribed level of WGA. Such an area, hatchmarked in Fig. 3, is denoted L_w . It is equivalent to a circular integration of r dy between the two plots. Area L_w has the dimensions of molecules of ligand added per cell. Area L_w is a measure of the extra amount of echinocytic ligand needed when WGA is present to generate the

discocyte → echinocyte titration plot relative to the amount of ligand needed when $y_{WGA} = 0$. The magnitude of L_w is directly dependent on WGA's powers as a protective agent for the discocyte. The L_w values from families of such plots, dependent on WGA, are shown in Fig. 4.

Fig. 4 indicates two levels of WGA on the abscissa with brackets. The first bracket designates the region of y_{WGA} at which WGA's protection is first made evident ($L_w \geq 0$). It marks the threshold range of y_{WGA} at which WGA obviously starts to protect the discocyte against echinocytic agents. The threshold level is $3-5 \times 10^5$ molecules of WGA per cell. With increasing WGA in native cells, there occurs a second, higher level of WGA (shown by the second bracket of Fig. 4; $\sim 18-20 \times 10^5$ molecules of WGA per cell) where L_w sharply increases. Further addition of WGA beyond this level does not further change the behavior of the system until elliptocytes are formed. In the range $18-20 \times 10^5$ molecules of WGA per cell, the discocytes are maximally protected (in the way indicated by Fig. 1 e), even with huge amounts of echinocytic ligand. With lesser, but still fully echinocytic amounts of ligand, $\sim 10^7-10^8$ molecules of ligand per cell, the protected discocytes are entirely like the control cells (Fig. 1 a). These two levels, $3-5 \times 10^5$ molecules of WGA per cell (threshold level for displacement of the r vs. y_{DEC} plot) and $18-20 \times 10^5$ molecules of WGA per cell (maximum useful WGA level), are consistent with

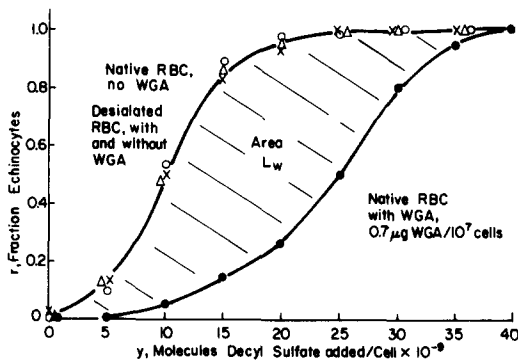


FIGURE 3 Plot of data, r , fraction of cells converted to echinocytes as a function of y , i.e., the amount of echinocytic agent added (decyl sulfate), dependent on WGA. Native cells (1×10^7 cells/ml) are markedly protected by WGA (1.9×10^{-8} M; mol wt, 36,000), producing area L_w , which is a measure of WGA's ability to forestall echinocyte formation. Desialated cells, initially in normal discocyte morphology, fail to be protected by WGA.

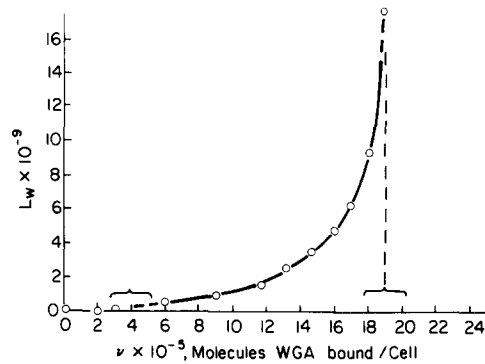


FIGURE 4 Plot of L_w values as a function of amount of WGA bound to the cell, from use of 125 I-WGA. Bracket at the lower level: range of bound WGA, which is the threshold level at which WGA obviously protects the cell against echinocyte formation. Upper level bracket: range of WGA past which extreme (lytic) levels of echinocytic agent must be employed to force the cells out of the discocyte morphology.

other stoichiometries involving morphology conversion reactions that are discussed below.

Isotherms of WGA binding to the erythrocyte membrane were determined in two ways: (a) In relation to the binding isotherms of the ligand DOD and in relation to the boundaries between the discocyte morphology and the echinocyte morphologies. This leads to a "phase diagram," or a morphology behavior diagram, as illustrated in Fig. 5. (b) In a conventional binding isotherm involving only WGA in Scatchard plot, as shown in Fig. 6. This figure clearly shows that the early stages of WGA binding are sharply cooperative, of positive sign, for the native erythrocyte.

Fig. 5 shows how DOD-induced conversion from the normal discocyte proceeds through echinocyte morphology substages I and II (4). Fig. 5 is plotted with respect to amounts of WGA bound, ν_{WGA} , and with respect to amounts of DOD bound, ν_{DOD} , as determined with ^{125}I -WGA and ^{35}S -DOD, respectively. With no WGA, ν_{WGA} , of course, equals zero. The critical level for DOD binding in complete conversion to echinocytes is $\nu_{DOD} = 2.8 \times 10^7$ molecules of DOD ligand per cell, as shown by the intercepts on the abscissa of Fig. 5. This reinforces our previous results (21). The range over which the discocyte is stabilized is increased as ν_{WGA} increases. Thus, the data of Fig.

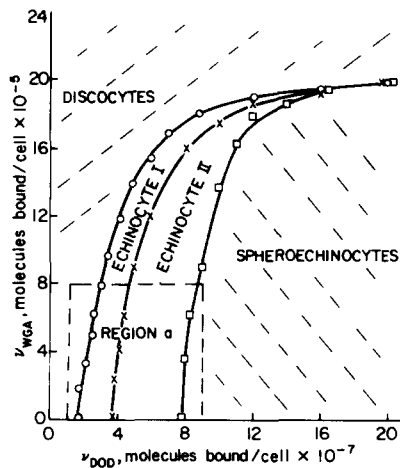


FIGURE 5 Production of principal echinocyte morphologies dependent on ν , i.e., amounts bound, for both echinocytic agent (dodecyl sulfate) and WGA (1×10^7 cells/ml, 25°C). "Region a" marks the areas of the more critical stoichiometries for WGA binding in the protection reaction in relation to binding levels of the echinocytic agent at which substages of morphologies are seen and in relation to intercepts of the plots on the abscissa.

5 quantitates some of the behavior qualitatively outlined by pathway 2 in Fig. 2.

The ranges of the steeper parts of the isoclines in Fig. 5 extend to ν_{WGA} values of $\sim 8-18 \times 10^5$ molecules of WGA bound per cell. In the lower ranges at which protection against echinocyte formation by bound DOD is elicited by bound WGA, the isoclines are sloped and lie roughly (region a) in the range $\nu_{WGA} \cong 0-5 \times 10^5$. This range corresponds to the threshold range for WGA protection, as seen from the L_W plot method in Fig. 4. Another point, which is expressed by the steeply rising nature of the isoclines can clearly be seen in Fig. 5: the binding levels of WGA, ν_{WGA} , are apparently independent of ν_{DOD} . This is seen more directly in Fig. 7, which shows how, in a plot of ν_{WGA} vs. y_{DOD} , spanning the critical (echinocytic) range of $y = 0-10 \times 10^7$ molecules of DOD per cell, ν_{WGA} increases with increasing overall level of WGA, as one might expect. However, the plots are flat, within experimental error, confirming ν_{WGA} 's independence of DOD level. Thus, although the binding levels of WGA and of the echinocytic ligand are surprisingly independent of one another, the consequences of binding on erythrocyte morphology are strongly dependent on: (a) bind-

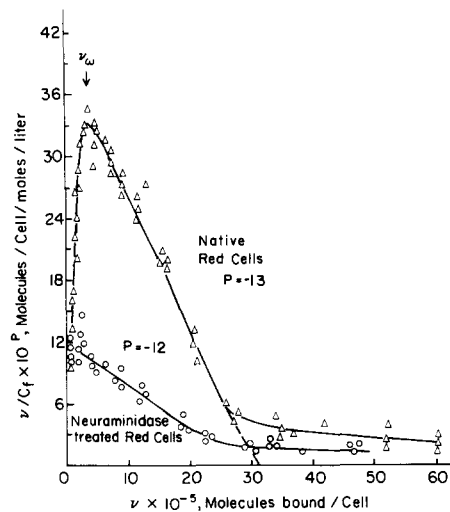


FIGURE 6 Binding isotherms, Scatchard plots, of WGA to native and to desialated cells, showing marked positive cooperativity by the native cells up to the ν_w point and abolishment of positive cooperativity in WGA binding by removal of sialic acid residues. Dashed line and later sectors of native erythrocyte isotherm are in agreement with Adair and Kornfeld's earlier data (2). P, order of magnitude of the ordinate.

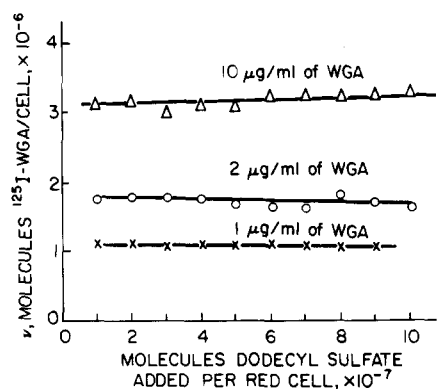


FIGURE 7 Increase of WGA added to native erythrocytes increases the levels of WGA binding (ordinate). However, the ability of WGA to bind at various levels is independent of the amount of echinocytic agent added (dodecyl sulfate, abscissa). This behavior is consistent over the range over which the cells become echinocytes, i.e., between 2 and 8×10^7 DOD added per cell.

ing levels; (b) the sequence of addition of the compounds which produce the behavior illustrated in pathways 2, 3, and 4 of Fig. 2. Nevertheless, there is no discernible time dependence in any of these reactions involving WGA.

Fig. 6 shows the results from direct determination of WGA's binding isotherm to the erythrocyte, plotted according to Scatchard's method. The sharp maximum in the Scatchard plot of Fig. 6, v_{∞} , is similar to plots from some of our earlier work in which erythrocyte binding isotherms exhibited sharp, positive cooperativity (21). A transition is involved near the maximum, which is made visible because of a gross morphology change, in the case of certain ligands. Here, the ability to bind WGA increases rather sharply up to v_{∞} , and a "normal" appearing (negatively sloping) isotherm follows. In the region of $v_{\text{WGA}} \cong 40 \times 10^5$, Adair and Kornfeld (2) also determined a WGA isotherm for binding to erythrocytes, but they gathered no data below the level of $v_{\text{WGA}} \cong 15 \times 10^5$ molecules bound per cell. In the range in which there is overlap between their results and ours, there is reasonable agreement.

The region of binding of WGA in excess of about $v_{\text{WGA}} = 30 \times 10^5$, is marked by a much flatter, only gently sloping portion of the Scatchard plot. This is consistent with an ongoing binding of WGA molecules, of much less affinity, also seen by Adair and Kornfeld (2). This was considered consistent with binding of WGA via sialic acid ligands, because of their large numbers.

Sialic Acid Residues (NANA) as Ligands for WGA Binding. Consequences of Desialation in WGA's Control of Morphology

To determine whether sialic acid residues might be the primary ligands for WGA interaction with its erythrocyte receptor, the sialic acid groups were removed by neuraminidase treatment and quantitated. In four determinations, using neuraminidase in the pH range 7.0–7.4 (25°C), 4.1, 4.4, 5.4, and 3.7×10^7 sialic residues per cell, for an average value of 4.4×10^7 residues per cell, were measured. Seaman et al. (36) obtained an average of 3.5×10^7 residues per cell under somewhat different conditions involving added Ca^{++} .

The morphologies and the morphology interconversion reactions of these desialated human erythrocytes were examined, as illustrated by Fig. 8. First, excellent discocyte morphologies are retained by completely desialated cells. Second, the reversible conversion discocyte \rightleftharpoons echinocyte is retained to a remarkable degree. Echinocytic ligands drive the conversion completely to the right, and serum albumin completely reverses it. The third result is dramatic: WGA's control over both reactions of the desialated cells is completely abolished. WGA exerts no control at all, even at WGA levels of $\sim 60 \mu\text{g/ml}$. This third result is illustrated by Fig. 3, where echinocyte formation behavior for desialated cells in the presence of WGA is plotted. The plot is congruent with that for intact cells without WGA. This third result is strong evidence that, indeed, WGA's control over the intact cell is mediated via sialic acid ligands. The lectin is best displaced from the cells by NAG, not by added sialic acid, however. The reason for this likely lies in the anomeric equilibria that prevail in free sialic acid, as discussed below. In any case, complete desialation does not affect either of the two morphologies nor the interconversion that usually occurs in the absence of the lectin.

The isotherms for ^{125}I -WGA binding were re-determined for completely desialated cells, and the plot is shown in Fig. 6. The entire positively cooperative branch of the isotherm was destroyed as shown. Even so, the "normal," or negative sloping branch of the plot still intercepts on the abscissa at about 30×10^5 molecules bound per cell, as is the case for native erythrocytes and as Adair and Kornfeld also found (2). There is a 32-fold decrease in the apparent association constant of the negative sloping branch, $K_a (-K_{an} = n \cdot (\text{the$

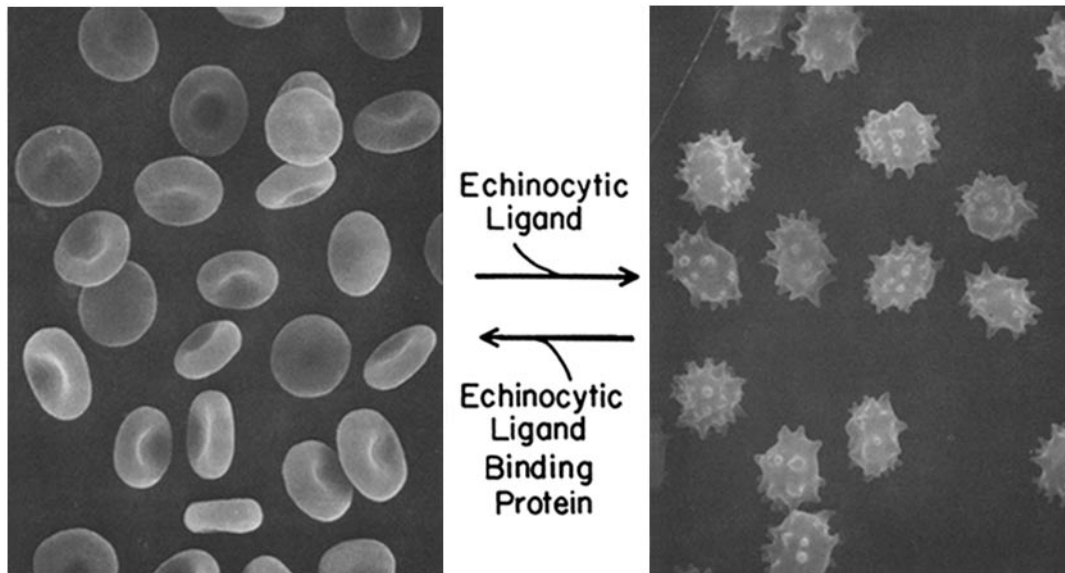


FIGURE 8 Morphology conversion of completely desialated cells: the normal, reversible conversion, discocyte \rightleftharpoons echinocyte is still observed with erythrocytes stripped of surface sialic acids. However, WGA imposes no control over this reaction, in contrast to native cells. 1×10^7 cells/ml, $60 \mu\text{g}$ WGA/ml.

slope); $n \cong 30 \times 10^5$), for desialated cells relative to intact cells. However, neither of these large changes in the binding isotherms, namely, loss of positive cooperativity and decrease in K_a after desialation, occur at the cost of overt alterations in cell morphology, as Fig. 8 shows. This behavior is consistent with good retention of normal discocyte morphology in native cells, up to and beyond the ν_w point as WGA progressively binds.

DISCUSSION

The critical binding level, or the threshold number for WGA binding at which WGA imposes obvious, strong influence over the discocyte \rightleftharpoons echinocyte equilibrium, has the value $\nu_{\text{WGA}} = 3-4 \times 10^5$ molecules of WGA bound per cell. The best earlier estimate of the number of copies of glycophorin is that of Fairbanks et al. (11), who found 2.6×10^5 copies per cell membrane. The latest measurements are by Sadler et al. (35). They lead to close to $6-7 \times 10^5$ chains per membrane. It is likely that glycophorin tends to exist as the dimer in the membrane (25). Therefore, WGA's interaction with the human erythrocyte in the region of the critical stoichiometry at which WGA imposes strong control over the cell's morphology changes might be mediated through a glycophorin dimer rather than simply through a monomer.

The lectin does not produce echinocytes or discocytes at any binding level. Rather, it locks or protects either morphology such that further morphology changes are blocked in the face of conditions which ordinarily would readily force such changes. The control that WGA exerts does not occur by competition, such as prevention of echinocytic agent binding or unbinding, as is shown by the experiments with radiolabeled detergents that were designed to answer the question of competition. It might have been thought that such controls, even if they applied to the discocyte, might not apply to the echinocyte if the distribution of the receptors for WGA in the echinocyte were different from that in the discocyte. However, the membrane cytoplasmic protein spectrin, which is fundamental in regulating cell shape, has been shown by Ziparo et al. (45) to maintain the distribution in the echinocyte that it apparently had in the discocyte.

The discrete morphology that WGA can independently produce is the elliptocyte. It can be estimated that elliptocytes are produced at levels corresponding to a monolayer of WGA molecules bound to the cell surface, using the approximation that the cell surface areas of the elliptocyte and of the discocyte are equal. We know little of the mechanism for production of the elliptocyte, ex-

cept that it is likely very different from production of the echinocyte because the conditions for formation *in vitro* are profoundly unlike. Because the discocyte \rightarrow elliptocyte conversion can take place *in vitro*, there is a possibility that elliptocytes could form from normal discocytes *in vivo*, but this is far from established.

The sharply increasing branch of Fig. 4, the "hyperprotecting" region that marks WGA's more forceful abilities in controlling the cell's morphology, occurs at $\nu_{\text{WGA}} = 18\text{--}20 \times 10^5$. At this level, the discocyte is stabilized, almost regardless of how much echinocytic agent is present (Fig. 1*e* and *f*; pathway 2 of Fig. 2). When ν_{WGA} values are produced that have an apparent stoichiometry of 4:1 or 5:1 with respect to glycophorin, there arises the question of whether some WGA molecules might be distributed elsewhere, even though the 1:1 stoichiometry (threshold protection) indeed helps confirm (2) glycophorin as a principal receptor. There is a connected question. Is WGA likely acting as a cross-link between remote receptors, e.g., different glycophorins or other glycoproteins? These questions need to be gauged with respect to the numbers, affinities, spacing, total valency, and valence directional properties of such groups. Fortunately, Wright (43) has developed a 2.8 Å resolution of WGA's structure complexed with NANA, illustrating how such ligands are oriented in binding.

The molecular weight of glycophorin is 29,000–31,000 (15), and it is 60–64% carbohydrate by weight. It has 8 *N*-acetylglucosamine residues (GlcNAc_p) and 28–33 *N*-acetylneuraminic acid residues (NANA), with some variation between laboratories (19, 35, 40). Until recently, most authors thought that WGA uses GlcNAc groups (in the β -anomeric form) almost exclusively for binding to cell surfaces. However, the monosaccharide of GlcNAc is a ligand of much lower affinity than the oligosaccharides of *N*-acetylglucosamine (32). Although there are enough GlcNAc groups on glycophorin to accommodate a single WGA molecule, there are not nearly enough GlcNAc residues to accommodate four or five WGA molecules, even if the GlcNAc residues were arranged in the most advantageous sequence for generation of large binding affinities, for WGA is tetravalent (27), and it has to exert at least a trivalency, preferably a tetravalency, for best binding (14). Approximately 15–20 GlcNAc residues per glycophorin would have to be present for tight binding of five WGA molecules, and they would cer-

tainly have to be contiguous in groups of three or four, judging by the behavior of WGA with chitooligosaccharides (14). Moreover, in the carbohydrate sequences, the position of GlcNAc groups is almost always "endo," or interior within the type II glycophorin oligosaccharides (35). Yet WGA's interaction with oligomers of GlcNAc is best developed when at least one of the GlcNAc groups is a terminal, or "exo" group. Summarizing what has been discussed so far, the density and arrangement of GlcNAc in glycophorin is not advantageous for binding WGA, even though free GlcNAc or NAG in millimolar concentrations can displace WGA.

In contrast, the density and arrangement of sialic acid (NANA) in glycophorin fills the requirements seen to be emerging (43) for WGA-NANA binding. Quite recently, WGA was found to use NANA as ligands, and WGA often has more affinity for NANA than for GlcNAc (16). The results summarized by Fig. 8 strongly support the view that NANA is particularly important in WGA's control of erythrocyte morphology. This is not to say that NANA is the exclusive ligand for WGA in the erythrocyte surface; GlcNAc possibly becomes involved under some conditions. Nevertheless, viewing the composition of the whole membrane with respect to NANA, both glycophorin and the remainder of the membrane have enough NANA to account for WGA binding, even at the "hyperprotecting" level, $\nu_{\text{WGA}} \cong 18\text{--}20 \times 10^5$ molecules per cell. This requires $\sim 0.8 \times 10^7$ NANA residues if WGA is tetravalent for NANA. Glycophorin alone carries about 1.8×10^7 NANA on the whole cell. The cell's total NANA is $3.0\text{--}4.0 \times 10^7$, and glycophorin contributes 40–60%. Therefore, glycophorin's composition and arrangement with respect to NANA's sequence are favorable for both the 1:1 and the 1:5 ratios for WGA interaction, in contrast to GlcNAc.

In our experiments, free or monomeric NANA did not displace WGA, in contrast to NAG. However, WGA binding is specific for the α -anomer of the saccharide (43), whereas the free, unattached sugar in water mutarotates to a mixture that is 90% β -anomer (43). In this respect, and in certain others involving *O*-glycosidic linkages available in glycophorin but lacking in free NANA, free NANA does not adequately duplicate the sialyl group stereochemistry that prevails in glycophorin.

It is most unlikely that "cross-linking" (à la glutaraldehyde), wherein WGA simultaneously binds two glycophorin receptors, is responsible for

WGA's ability to protect. It has already been noted that WGA needs immediately adjacent, contiguous ligands for best binding. Glycophorin has an average radius of $\sim 25 \text{ \AA}$ (25). Hence, the surface density of NANA in glycophorin is $\sim 160 \times 10^{-4}$ molecules of NANA per square angstrom. The glycophorin receptors are, on the average, $\sim 220 \text{ \AA}$ distant. If WGA could successfully function merely using two intermolecular NANA, the ligand density would be 0.13×10^4 molecules of NANA per square angstrom, 1,200 times lower than for the intramolecular case. The lectin likely has to orient the requisite ligands for proper valence direction in intermolecular binding (43). Moreover, the observed stoichiometry is incorrect for interglycophorin contribution: if WGA does span two glycophorin receptors at the point of the observed morphology control, a 0.5:1.0 stoichiometry would occur. If WGA is tetravalent and operates intermolecularly, the ratio 0.25:1 for WGA:Glycophorin is expected. Glycophorin likely is a dimer or a multimer in the membrane (25). This reinforces the foregoing arguments.

There are four salient aspects of the isotherms for binding WGA to the membrane that we would like to discuss: (a) the positive cooperativity of lectin binding to the discocyte in the early stages ($\nu_{WGA} < \nu_w$) of the association and its erasure upon desialylation; (b) the parallels between what is seen here and binding isotherms of other intracellular proteins using erythrocyte inner membrane receptors; (c) the independence of the binding isotherms of the lectin and of echinocytic ligands with respect to one another; (d) the expression and confirmation of previous work (21) in the results reported in this paper.

Positive cooperativity of the kind seen in Fig. 6 may arise in three general ways (7): a change in the state of association of the binding molecule, a change in state of the receptor molecule, and rearrangement or exposure of "cryptic" sites as binding develops. Both WGA and glycophorin may exist as dimers, so that the first two possibilities, changes in state of association at the molecular level, are good possibilities. Cryptic sites not initially available or an increase in affinity of the first sites which are forced into availability as binding progress fit equally well. (Initially, ν is small but $C_f \gg \nu$, so that ν/C_f is relatively small. Later, ν increases relatively rapidly as ligand is added, so that ν/C_f climbs, and the slope of ν/C_f vs. ν is positive in sign. Eventually, remaining sites become exposed, so that ν/C_f is forced to become

negatively sloped.) The discocyte is not only present but is well stabilized at the ν_w point shown in Fig. 6. This favors assignment of positive cooperativity to molecular association-dissociation equilibria, probably in glycophorin, rather than membrane rearrangement. Positive cooperativity of this kind is increasingly found in lectin binding to membranes other than in the erythrocyte (31, 38) but is also found for drug binding in the human erythrocyte (21).

In the human erythrocyte, the best evidence for positive binding cooperativity involves inner membrane receptors for glyceraldehyde-3-*p*-dehydrogenase (44). The G3PD receptor is the band 3 protein, which also binds aldolase on the cytoplasmic side, perhaps with positive cooperativity in its initial stages (39). Thus, band 3's binding isotherms for these two proteins is kindred to glycophorin's behavior in binding WGA in overall outline. Glycophorin and band 3 are both transmembrane proteins, but they are asymmetrically placed. Edwards et al. (9) clearly showed that glycophorin is exposed to the outside, whereas band 3 is mainly on the cytoplasmic or inner side. The relationship of both proteins to the "Intramembrane Particle Assembly" (IMP) merits careful focus. There are $3-5 \times 10^5$ IMP per human erythrocyte. The IMP may harbor both glycophorin and band 3. Rothstein (34) thinks that band 3 may be "covered" by the sialoglycoprotein, glycophorin. It is likely that there are two copies of band 3 monomer/IMP (one glycophorin per IMP), so that band 3 monomers are possibly dimers in the IMP. As noted above, concanavalin A can control some of the discocyte \rightarrow echinocyte conversions, but much less forcefully than can WGA. Concanavalin A's receptor is band 3 (29).

The binding isotherms for the membrane involving the lectin and the echinocytic agent show that the two binding processes are independent (see Fig. 7). This holds through ranges of binding in which a morphology change occurs. Nevertheless, in control of the cell's morphology, there is an intimate mutual dependence of lectin and echinocytic agent in the consequences of binding, as is vividly seen in Fig. 2. The binding units themselves appear to be independent, but after the complexes form between the lectin and its receptor and the echinocytic agent and its binding unit, the filled units interact strongly. The principal controls appear to be thermodynamic and not rate limited.

It was found earlier (21) that for all echinocytic ligands studied, regardless of their structure, mo-

lecular weight, or total ligand concentration (y_{Ligand}), that discocyte \rightarrow echinocyte conversion always becomes developed when $\nu_{\text{Ligand}} \cong 2 \pm 1 \times 10^7$ ligands bound per erythrocyte. This includes decyl and dodecyl sulfate, ligands used for this work. Fig. 5 confirms the earlier work: the isocline for conversion to the first discernible echinocyte, echinocyte I, intercepts the abscissa at $\nu_{\text{DOD}} \cong 2 \times 10^7$. This points out an avenue for echinocyte formation.

It has commonly been thought that the discocyte \rightarrow echinocyte conversion simply represents an isovolumetric cell change with membrane area expansion. The hydrocarbon chains insert into the membrane lipids, and the increased volume requirement, plus electrostatic repulsion from the anions, presumably are the main driving forces (37). However, in such morphology changes the cell cytoskeleton must at least conform to, if not help produce, the morphology change. Cytoskeleton-controlling assemblies must be reckoned with in many cells (6). For the erythrocyte, several papers (we cite only two, Van Zoelen et al. [41] and Kahane and Gitler [18]) show that glycoporphin is in intimate, extensive contact with membrane lipids. Van Zoelen et al. give a range of from 9 to as many as 80 membrane lipid molecules interacting with each glycoporphin. Taking a rough average of 50 lipids per glycoporphin and $\sim 4 \times 10^5$ glycoporphins per membrane, the product is 2×10^7 lipids per membrane. This may be merely fortuitously close to our critical range, $\nu_{\text{Ligand}} \cong 2 \pm 1 \times 10^7$. However, it appears that when the "lipid bilayer" is invaded, with a number of intruding ligands comparable to the number with which glycoporphin already interacts, not one but two events follow. First, membrane expansion and electrostatic repulsion may indeed occur. But, in addition, glycoporphin becomes perturbed, in turn allowing the cytoskeletal change necessary to form a new morphology. Not only are the forces in the morphology change at stake but the governance of the consequences of dissipating the forces may be of equal importance. Glycoporphin provides such governance.

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