Interaction of Wheat Germ Agglutinin and Concanavalin A with Isolated Fat Cells†

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ABSTRACT: The binding to isolated fat cells of high specific activity (2–4 μCi/μg) ^{125}I derivatives of wheat germ agglutinin and concanavalin A has been studied in detail. The binding of wheat germ agglutinin to fat cells is quite complex and suggests the existence of multiple and interacting classes of binding sites. A single fat cell can bind a maximum of about 3×10^8 molecules of wheat germ agglutinin. The rate of wheat germ agglutinin–cell complex formation is very rapid whereas the spontaneous rate of dissociation is very slow; only 10–15% of the cell–lectin complex dissociates during 90 min at 24°. Upon addition of *N*-acetyl-D-glucosamine, ovomucoid, or ovomucoid–agarose, rapid and profound dissociation of the cell–lectin complex occurs. Fat cells also possess heterogenous classes of binding sites for concanavalin A, and there is evidence which suggests the existence of interactions between these binding sites. A maximum of about 4×10^8 molecules of concanavalin A can bind to a single fat cell. Equilibrium binding of this plant lectin occurs rapidly, but the complex formed dissociates negligibly during a 100-min period of incubation at 37°. The simple sugar, α-methyl-D-mannopyranoside, causes the lectin–cell complex to dissociate rapidly. Very high concentrations of concanavalin A and insulin cause a slight fall in the binding of ^{125}I-labeled wheat germ agglutinin to fat cells. Similarly, the binding of [^{125}I]concanavalin is altered only by very high concentrations of insulin or of wheat germ agglutinin. Digestion of fat cells with high concentrations of trypsin and Pronase results in the loss of about half of the binding sites for the plant lectins, and neuraminidase digestion of the cells decreases the binding of wheat germ agglutinin but not of concanavalin A. Intracellular membrane structures also possess specific lectin-binding sites; the binding sites for wheat germ agglutinin are susceptible to neuraminidase digestion. Under the conditions used here intracellular translocation of the plant lectins appears to be negligible. The ability of simple sugars to rapidly dissociate the highly stable cell–lectin complexes suggests that lectin molecules bound to cells possess unoccupied saccharide-binding sites. Binding of sugars to these sites results in changes in the lectin molecule which drastically alter the existing cell–lectin interaction. The maximal number of binding sites in a given cell for wheat germ agglutinin, concanavalin A, or insulin appears to be relatively independent of cell size since the number is nearly constant in cell populations whose average surface area varies more than sevenfold. Rapid reduction of fat cell size by starvation does not appreciably change the total number of binding sites per cell of these surface receptors.

The plant lectins, concanavalin A and wheat germ agglutinin, have been very useful as markers for exploring the surface topography of a variety of normal and neoplastic mammalian cells (reviewed by Sharon and Lis, 1972). In few cases, however, have detailed binding studies been performed with these plant lectins, and little information is available concerning the nature of lectin–cell interactions.

It has recently been demonstrated that the plant lectins, concanavalin A and wheat germ agglutinin, have very marked insulin-like properties on isolated fat cells (Cuatrecasas and Tell, 1972). Very low concentrations of these proteins are as effective as insulin in enhancing the rate of glucose oxidation and in inhibiting epinephrine-stimulated lipolysis in fat cells, and in inhibiting the adenylate cyclase activity of fat cell membranes. These plant lectins in addition appear to be capable of interacting directly with the insulin receptor of fat cells in a way which markedly perturbs the insulin–receptor interaction (Cuatrecasas, 1972). Low concentrations of wheat germ agglutinin enhance the apparent affinity of the insulin–cell complex while higher concentrations of this protein can prevent the binding of insulin to its receptor. Concanavalin A blocks the specific binding of iodoinsulin to fat cells.

In the present studies iodinated plant lectin derivatives of very high specific activity have been used to examine the nature of the interaction of wheat germ agglutinin and concanavalin A with isolated fat cells.

Materials and Methods

Insulin, concanavalin A, wheat germ agglutinin, Na^{125}I, α-methyl-D-mannopyranoside, and *N*-acetyl-D-glucosamine were the same as described previously (Cuatrecasas, 1972). Glucagon was purchased from Eli Lilly, trypsin and neuraminidase from *Clostridium perfringens* (1.5 units/ml) from Worthington, Pronase from Calbiochem, antiserum against concanavalin A from Miles, ovomucoid from Sigma, and phospholipase C (*C. perfringens*) from Nutritional Biochemicals. For some experiments neuraminidase was purified extensively by affinity chromatography on oxamic acid–agarose columns (Cuatrecasas and Illiano, 1971). Isolated fat cells were prepared by the method of Rodbell (1966). Ovomucoid was coupled to agarose by the cyanogen bromide procedure (Cuatrecasas, 1970) using 200 mg of cyanogen bromide and 12 mg of ovomucoid per milliliter of packed Sepharose 4B; the final derivative contained 8 mg of ovomucoid/ml of agarose.

For the preparation of ^{125}I-labeled wheat germ agglutinin,

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10 μl of 0.1 M sodium phosphate buffer, pH 7.4, containing 200 μg of the protein, was added to 100 μl of 0.25 M sodium phosphate, pH 7.4, containing 1.5–2 MCi of carrier-free $Na^{125}I$ (Union Carbide). Twenty microliters (100 μg) of chloramine-T (Hunter and Greenwood, 1962) was added and, after 50 sec at 24°, 20 μl (200 μg) of sodium metabisulfite was added. After 10 sec 0.3 ml of 0.1 M sodium phosphate buffer, pH 7.4, was added. An aliquot of this reaction mixture was diluted in the same buffer, containing 0.5% (w/v) albumin, to determine the per cent of total radioactivity which had been incorporated into protein; this value, determined according to the precipitability of radioactivity by 7% trichloroacetic acid, was generally between 40 and 60%. The iodinated wheat germ agglutinin was purified by affinity chromatography by immediately adding the remainder of the reaction mixture to a 1-ml ovomucoid–agarose column (Pasteur pipet) which had been equilibrated with 0.1 M sodium phosphate buffer, pH 7.4. The column was washed with about 40 ml of the same buffer. Ninety per cent of the trichloroacetic acid precipitable radioactivity adsorbed to the column. Nearly quantitative elution was achieved with 0.2 M acetic acid. The eluted material was neutralized by addition of an equal volume of 0.25 M sodium phosphate buffer, pH 7.4, followed by titration with 0.1 M NaOH; the solution was immediately adjusted with albumin to achieve a concentration of 0.1% (w/v). The specific activity of the purified ^{125}I-labeled wheat germ agglutinin varied between 2 and 4 μCi/μg; 90% of this radioactive material was precipitated by 7% trichloroacetic acid in 0.1 M sodium phosphate (pH 7.4) containing 0.5% albumin, 96% adsorbed to an ovomucoid–agarose column, and 90% could be bound to fat cells or liver membranes. The iodinated wheat germ agglutinin was stable (−20°) for at least 1 month, as judged by its ability to bind to fat cells.

Concanavalin A (50–200 μg) was iodinated by essentially the same procedure using 1–2 MCi of carrier-free $Na^{125}I$. The iodinated concanavalin A was purified from the reaction mixture by affinity chromatography on a 1-ml column (Pasteur pipet) of Sephadex G-100 equilibrated with 50 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The column was washed with 40 ml of the same buffer, and the concanavalin A was selectively eluted with 0.3 M α-methyl-D-mannopyranoside in 0.1 M Tris-HCl, pH 8.5, containing 0.3% (w/v) albumin.[1] The eluted material was dialyzed for 2 days at 4° against large volumes of 50 mM Tris-HCl, pH 7.5. After dialysis the sample was adjusted to 1 mM with $MgCl_2$ and $CaCl_2$. The specific activity of the [^{125}I]concanavalin A varied between 2 and 16 μCi/μg, depending on the ratio of concanavalin A and ^{125}I used; nearly all of the studies described in the present report were performed with material having a specific activity of 3 μCi/μg. About 85% of the radioactivity is precipitable by 7% trichloroacetic acid (using 0.1 M sodium phosphate buffer, pH 7.4, 0.5% albumin), and 90% of the radioactivity can adsorb to a small column of Sephadex G-100 using the conditions described above. Virtually no loss in the ability of [^{125}I]concanavalin A to bind to fat cells could be detected during storage for 1 month at −20°. The ability of wheat germ agglutinin and concanavalin A to stimulate glucose oxidation in fat cells (Cuatrecasas and Tell, 1972) was not appreciably altered by the iodination and purification procedures described above.

The assay procedures used to measure the specific binding of ^{125}I-labeled wheat germ agglutinin and [^{125}I]concanavalin A to fat cells were similar to those described earlier for measuring the binding of [^{125}I]insulin (Cuatrecasas, 1971a,b). Isolated fat cells (about 10^4 cells/ml) were incubated in disposable, polystyrene tubes (12 × 75 mm) for 30–40 min at 24° in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 1 mM $CaCl_2$, 0.5% (w/v) albumin, and the iodinated lectin (2×10^4–5×10^5 cpm, as indicated in legends). Three milliliters of the same buffer (ice cold) are added to the tubes, the contents are poured on appropriate Millipore filters (25 mm) positioned with vacuum, and the filters are washed under vacuum with 10 ml of ice-cold Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin. The steps of dilution, filtration, and washing consume 10–15 seconds. The filters are counted in a well-type γ counter using disposable plastic tubes.

A variety of filters of different chemical composition were tested to select those which adsorb the least amount of radioactivity in the absence of cells. The ones found to be most suitable, which were used in the present studies, were of nylon (NRWP, Millipore Corporation) for wheat germ agglutinin and of Teflon (LSWP, Millipore Corporation) for concanavalin A. Less than 1% of the total ^{125}I-labeled wheat germ agglutinin adsorbs to the nylon filters and about 1.5% of the [^{125}I]concanavalin A adsorbs to the Teflon filters under the conditions described above in the absence of cells. The adsorption of radioactivity to the filters is unaffected by the presence of high concentrations of the specific simple sugars (*N*-acetyl-D-glucosamine or α-methyl-D-mannopyranoside) or by the presence of very high concentrations (50 μg/ml) of the native plant lectins. Virtually all of the filters composed of cellulose or of cellulose esters which were examined bound very large amounts of the plant lectins, and this binding was modified by the presence in the buffer of the simple sugars or of the native plant lectin.

All of the binding data are expressed in terms of "specific" binding, as described in studies of the binding of [^{125}I]insulin to cells (Cuatrecasas, 1971a,b). Specific binding refers to the amount of ^{125}I-labeled protein bound to the cells which can be specifically displaced by adding to the cells high concentrations of the native protein before addition of the iodoprotein. Alternatively, this can be determined by measuring the difference in the amount of radioactivity bound to the cells in the absence and presence of high concentrations of a specific simple sugar which binds to the active site of the plant lectin. In this case the sugar is also added to the cells before addition of the iodoprotein. In the present studies the native protein produced more profound and more reproducible displacement of binding than was observed with the simple sugars. Thus, in the presence of the sugars some nonspecific binding is still being measured. The use of the native plant lectin was therefore adopted in the standard assay procedures. All binding determinations were performed in two sets, each consisting of duplicate or triplicate samples; the two sets differed only in the presence or absence of native wheat germ agglutinin (200 μg/ml) or native concanavalin A (500 μg/ml). When the concentration of the iodinated wheat germ agglutinin was less than 5 μg/ml more than 95% of the bound radioactivity was displaced by the native protein, indicating negligible nonspecific binding. The two sets of samples described above were nonetheless performed for all determinations presented in the present studies since it was not known beforehand if under certain circumstances nonspecific binding would be a significant factor. Since the nonspecific binding of iodoconcanavalin A was usually 10–15% of the total radio-

[1] The iodinated concanavalin A can also be eluted effectively with 0.2 M acetic acid; the material used in all of the present studies, however, was obtained by the elution procedure described in the text.

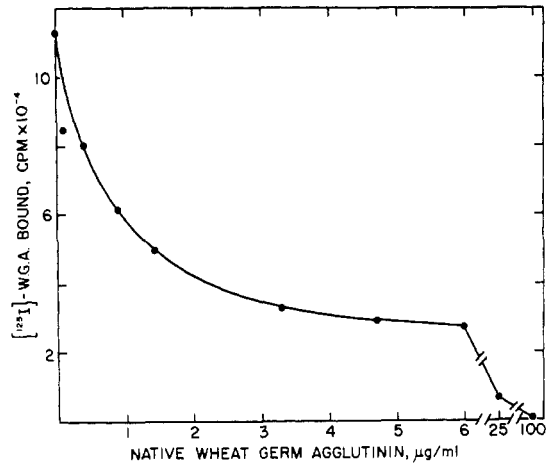

FIGURE 1: Displacement by native wheat germ agglutinin of specific binding of ^{125}I-labeled wheat germ agglutinin (W.G.A.) to isolated fat cells. Cells (2.4×10^4 cells) were incubated for 10 min at 24° in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and various concentrations of the native protein. The iodinated lectin (6×10^4 cpm) was then added and the samples were incubated for 50 min before determining specific binding, as described in the text.

activity bound it was essential in all cases to perform careful controls and to make appropriate corrections.

With the assay procedures described above it was possible to detect specific binding of the ^{125}I-labeled plant lectins to fat cells with concentrations of iodoprotein as low as 10^{-11} M. For calculations of molar concentrations of the plant lectins the molecular weight of wheat germ agglutinin was assumed to be 25,000 (Ozanne and Sambrook, 1971; Nagata and Burger, 1972) and that of concanavalin A, 100,000 (Sumner *et al.*, 1938; Kalb and Lustig, 1968; Hardman *et al.*, 1971; Edmundson *et al.*, 1971; and Edelman and Millette, 1971).

Results

General Features of the Binding of Wheat Germ Agglutinin to Fat Cells. ^{125}I-labeled wheat germ agglutinin binds to isolated fat cells very effectively. With a relatively low fat cell concentration nearly 20% of the iodinated plant lectin is bound to the cells when the concentration of the lectin is about 4×10^{-9} M (Figure 1). The bound radioactivity is readily displaced by low concentrations of native wheat germ agglutinin. Complete displacement is observed with 100 μg/ml of the native protein.

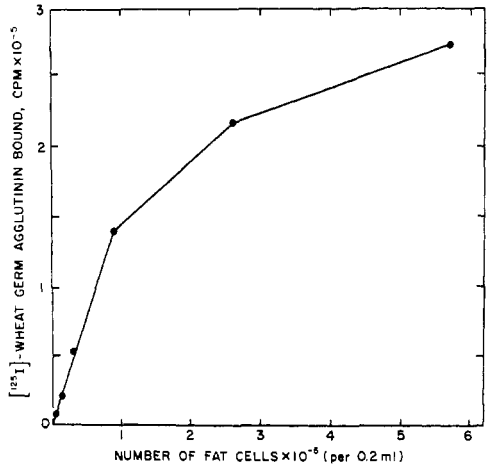

FIGURE 2: Effect of fat cell concentration on the specific binding of ^{125}I-labeled wheat germ agglutinin. The various cell samples were incubated for 50 min at 24° in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and ^{125}I-labeled wheat germ agglutinin (6.2×10^5 cpm). Specific binding was determined as described in the text. In this experiment nearly half of the radioactive plant lectin is bound with the highest cell concentration used.

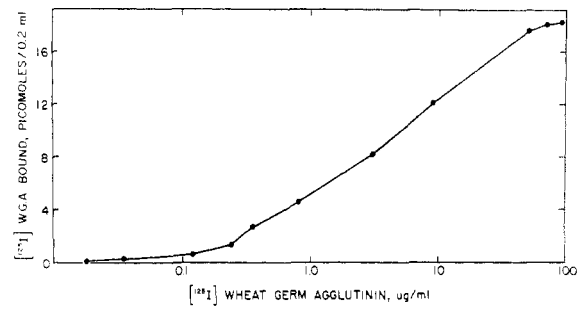

FIGURE 3: Specific binding of ^{125}I-labeled wheat germ agglutinin (W.G.A.) to isolated fat cells as a function of the concentration of the agglutinin. The cells (3.4×10^4 cells) were incubated for 40 min at 24° in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and the indicated concentration of ^{125}I-labeled wheat germ agglutinin (depicted in log scale). Total binding was determined by filtration over nylon NRWP Millipore membranes, as described in the text. These values were corrected for nonspecific binding, which was determined from identical samples which were preincubated for 10 min with native wheat germ agglutinin before addition of the iodinated lectin. The concentration of native wheat germ agglutinin was 200 μg/ml (for the samples containing 1 μg or less per milliliter of labeled lectin) or 1 mg/ml. The ^{125}I-labeled wheat germ agglutinin (3 μCi/μg) was used as such and after mixing with the native protein to obtain preparations of varying specific activity. Samples of different specific activity were used in overlapping concentrations to ascertain if the iodinated material behaves essentially the same as the native protein. No significant differences were detected in such regions of overlap, suggesting that the molecules bind in similar fashion and that modifying the specific activity in this way is valid. The detailed binding data of this experiment showing the results with concentrations of wheat germ agglutinin less than 0.1 μg/ml are described in Figure 4.

The binding of ^{125}I-labeled wheat germ agglutinin to fat cells is proportional to the concentration of fat cells in the incubation mixture provided sufficiently high concentrations of the iodoprotein are used relative to the fat cell concentration (Figure 2). In this experiment binding is linear over a tenfold range of fat cell concentration; the linear relationship is not maintained when more than 25% of the total radioactive lectin originally present in the incubation medium is bound to the cells. When lower concentrations of ^{125}I-labeled wheat germ agglutinin are used it is possible to demonstrate that more than 90% of the radioactive protein can be bound specifically to the fat cells. This suggests that nearly all of the iodinated plant lectin, which is purified by affinity chromatography, is undamaged and is capable of specifically interacting with the fat cell. These results are also in agreement with data obtained independently which demonstrate that under the conditions used in the present experiments there is no significant inactivation or degradation of the wheat germ agglutinin after incubation with isolated fat cells.

The above results suggest that the fat cell must possess some binding sites with very high affinity for wheat germ agglutinin. Although it can be demonstrated that the binding of wheat germ agglutinin to isolated fat cells is a saturable process with respect to the plant lectin (Figure 3), the detailed saturation curve is clearly peculiar and suggests the presence on the fat cell of heterogenous interactions. The apparent affinity con-

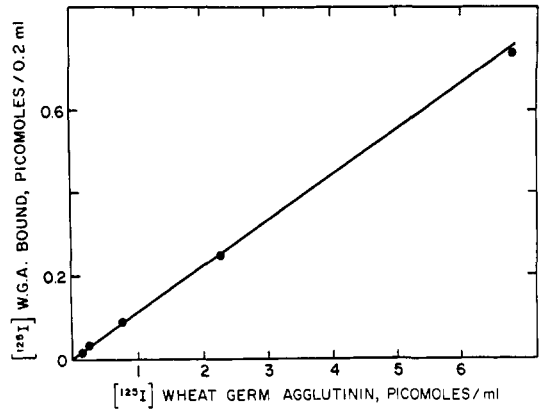

FIGURE 4: Specific binding of low concentrations of ^{125}I-labeled wheat germ agglutinin to isolated fat cells. These data were obtained from the same experiment described in Figure 3.

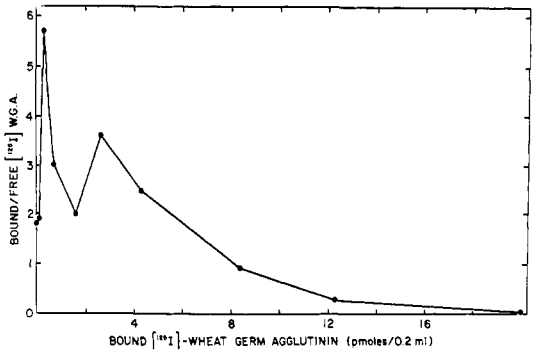

FIGURE 5: Scatchard (1949) plot of the specific binding of ^{125}I-labeled wheat germ agglutinin to isolated fat cells. The data were obtained from experiments performed as described in Figures 3 and 4.

stant for the overall binding process, estimated on the basis of the concentration required to achieve half-maximal saturation, is about 8×10^{-8} M. This constant, however, is not very meaningful since, as will be demonstrated shortly, there probably exist on the fat cells numerous classes of binding sites of grossly differing properties.

From the data described in Figure 3 it can be determined that 10^4 cells can bind to a maximum of about 5 pmol of wheat germ agglutinin, or that one fat cell can bind about 3×10^8 molecules of wheat germ agglutinin. More detailed examination of the low range of lectin concentration in the saturation curve depicted in Figure 3 reveals that under the conditions of this experiment the binding of wheat germ agglutinin to fat cells is directly proportional to the concentration of the lectin over the range 10^{-10}–5×10^{-9} M (Figure 4). It is notable that under these conditions (Figure 4) nearly half of the wheat germ agglutinin in the medium is bound to the cells. If the data described in Figure 3 are plotted according to the scheme of Scatchard (1949), a bizarre and uninterpretable binding curve is obtained (Figure 5). Although the general pattern of such curves is reproducible, the precise pattern over the low range of wheat germ agglutinin concentration varies considerably depending on the concentration of fat cells utilized. The data suggest that fat cells possess multiple binding sites of differing affinity, and that important interactions probably exist between binding sites. It is unfortunately not possible to clearly identify from such data meaningful specific groups of binding sites or to assign discrete binding constants. More detailed kinetic expressions of the binding data are at present unwarranted because of the obvious complexities of the binding process.

Since the insulin-like effects of wheat germ agglutinin on isolated fat cells are observed in the range of 10^{-9}–5×10^{-8} M (Cuatrecasas and Tell, 1972) most of the present binding studies were performed using this range of ^{125}I-labeled wheat germ agglutinin concentration. It is apparent that the ability of this plant lectin to initiate insulin-like metabolic effects, as well as to perturb the binding of insulin to cells (Cuatrecasas, 1972), results from the interaction of this plant lectin with only a small fraction of the total potential binding sites for this protein.

Rates of Association and Dissociation of the Wheat Germ Agglutinin–Fat Cell Complex. ^{125}I-Labeled wheat germ agglutinin binds very rapidly to fat cells, even with relatively low concentrations of the plant lectin (Figure 6). The rate of spontaneous dissociation of the lectin–cell complex is extremely slow (Figure 6). Only 14% of the bound lectin dissociates during a 90-min period of incubation at 24°. Similarly slow dissociation of the cell–lectin complex is observed if dissociation is measured in cells which have been washed to remove the free lectin in the medium. Such a slow rate of dissociation is consistent with the very strong apparent affinity of the lectin–cell complex described above for the lower range of lectin concentrations. This slow dissociation, however, could also be a reflection of phagocytosis or another type of internalization of the protein by the cell, although the very rapid rate of binding and the stability of the binding plateau (Figure 6) suggesting achievement of an equilibrium state argue against such a possibility. It is not possible to calculate meaningful kinetic expressions (rate constants) for the processes of dissociation or association since it is not known whether under these conditions binding is occurring with a discrete population of binding sites and since the number of free binding sites in a given class cannot be determined.

Dissociation of the Wheat Germ Agglutinin–Cell Complex by N-Acetyl-D-glucosamine and Ovomucoid. The lectin–cell complex dissociates very rapidly and profoundly (Figure 7) upon

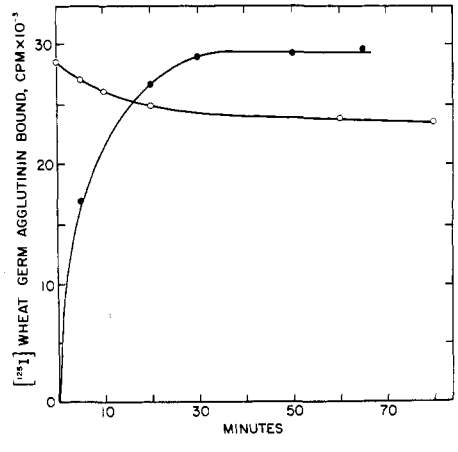

FIGURE 6: Rates of formation (●) and of dissociation (○) of fat cell–wheat germ agglutinin complex. Isolated fat cells were incubated at 24° for various times in Krebs–Ringer–bicarbonate buffer containing 0.1% albumin and 50 ng/ml of ^{125}I-labeled wheat germ agglutinin. To measure dissociation (○), native wheat germ agglutinin (250 μg/ml) was added after the cells had been incubated for 50 min with the iodoagglutinin (zero time), and specific binding was determined after various time periods of continued incubation at 24°.

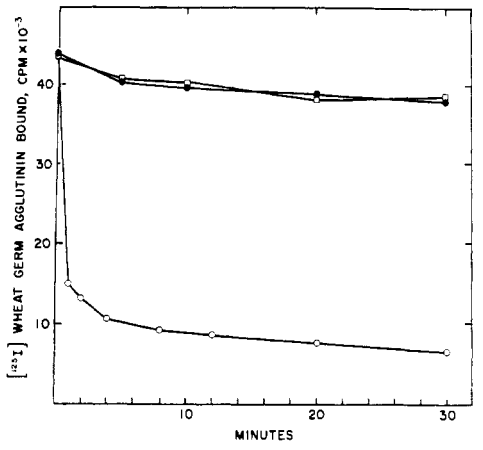

FIGURE 7: Dissociation of the fat cell–wheat germ agglutinin complex by native wheat germ agglutinin (●), α-methyl-D-mannopyranoside (□), and *N*-acetyl-D-glucosamine (○). After incubating fat cells for 50 min at 24° with 50 ng/ml of ^{125}I-labeled wheat germ agglutinin as described in Figure 6, 250 μg/ml of the native protein (●) and 50 mM of the sugar (□, ○) were added and specific binding was determined after varying periods of continuing incubation at 24°.

addition of a specific simple sugar which can bind to this protein, *N*-acetyl-D-glucosamine (Burger and Goldberg, 1967). This is in contrast to the very slow rate of spontaneous dissociation which can be observed after addition of the native protein or after washing the cells to remove the free lectin in the medium. α-Methyl-D-mannopyranoside, a simple sugar which is not bound to wheat germ agglutinin but which binds to concanavalin A (Yariv *et al.*, 1968; So and Goldstein, 1967), has no effect by itself and it does not enhance the spontaneous rate of dissociation of the wheat germ agglutinin–cell complex (Figure 7).

The wheat germ agglutinin–fat complex also dissociates very rapidly if ovomucoid, a glycoprotein having specific carbohydrate determinants for the plant lectin, is added to the medium (Table I). A concentration of 5 mg/ml of this glycoprotein is nearly as effective as 50 mM *N*-acetyl-D-glucosamine. The combination of ovomucoid and *N*-acetyl-D-glucosamine is slightly more effective than either agent alone. Ovomucoid–agarose beads are also capable of dissociating the fat cell–wheat germ agglutinin complex. These results, together with the observations described earlier, indicate that nearly all of the specific binding of ^{125}I-labeled wheat germ agglutinin occurs on the surface of the fat cells and that negligible amounts of the plant lectin are sequestered inside the cells.

These results are of special interest since the ability of *N*-acetyl-D-glucosamine and ovomucoid to cause dissociation of the lectin–cell complex must result from unusual interactions of these compounds with the cell-bound lectin rather than from interactions with the lectin which is free in solution or which spontaneously dissociates from the cell. The ability of *N*-acetyl-D-glucosamine to cause lectin–cell dissociation is dependent on the concentration of the sugar in the medium. With conditions (8-min incubation) similar to those described in Table I, 1 mM *N*-acetyl-D-glucosamine dissociates only 13% of the complex, whereas concentrations of 5, 20, 60, and 100 mM dissociate the complex to an extent of 40, 58, 69, and 78%, respectively. Relatively high concentrations of the sugar are required, which is consistent with the low affinity of wheat germ agglutinin for this simple sugar. It was important to determine if the effect of *N*-acetyl-D-glucosamine was confined only to the specific binding sites which are being studied with the relatively low concentrations (50 ng/ml) of ^{125}I-labeled wheat germ agglutinin utilized in the previous experiments. This is apparently not the case since the effect of *N*-acetyl-D-glucosamine on dissociating the lectin–cell complex is nearly identical on samples of the complex which are formed under conditions where the ^{125}I-labeled wheat germ agglutinin concentration is varied over an 80-fold range. It appears that although different concentrations of the plant lectin bind to different types of cell surface determinants, the simple sugar is

TABLE I: Dissociation of the Fat Cell–Wheat Germ Agglutinin Complex with *N*-Acetyl-D-glucosamine and Ovomucoid.[a]

Addition	Time of Dissoc (min)	Sp Binding of ^{125}I-Labeled Wheat Germ Agglutinin (cpm)
None	0	44,700 ± 1,400
None	6	41,400 ± 750
N-Acetyl-D-glucosamine	6	6,300 ± 300
Ovomucoid	6	8,100 ± 200
N-Acetyl-D-glucosamine + ovomucoid	6	4,200 ± 600
None	12	39,800 ± 1,000
N-Acetyl-D-glucosamine	12	5,500 ± 450
Ovomucoid	12	5,800 ± 500
N-Acetyl-D-glucosamine + ovomucoid	12	3,800 ± 300

[a] *N*-Acetyl-D-glucosamine (50 mM) or ovomucoid (5 mg/ml) was added to isolated fat cells which had been incubated at 24° for 40 min with ^{125}I-labeled wheat germ agglutinin (50 ng/ml). Specific binding to cells was then determined after incubation periods of 6 and 12 min.

TABLE II: Effect of Concanavalin A and Insulin on the Binding of ^{125}I-Labeled Wheat Germ Agglutinin to Isolated Fat Cells.[a]

Addition	Sp Binding of ^{125}I-Labeled Wheat Germ Agglutinin (pmol × 10^2)	
	2.5 × 10^5 cells	0.5 × 10^5 cells
None	18.4	3.31 ± 0.03
Concanavalin A, 20 μg/ml	18.3	2.70 ± 0.02
100 μg/ml	17.1	2.35 ± 0.04
500 μg/ml	13.4	
1 mg/ml	10.2	1.98 ± 0.04
2.5 mg/ml		1.77 ± 0.03
7.5 mg/ml		1.43 ± 0.03
Insulin, 0.1 μg/ml	18.1	3.33 ± 0.02
5 μg/ml	15.4	3.11 ± 0.03

[a] Fat cells at a concentration of 2.5 or 0.5 × 10^5 cells per 0.2 ml were preincubated with insulin or concanavalin A before determining the specific binding of ^{125}I-labeled wheat germ agglutinin (30 ng/ml) as described in the text.

TABLE III: Displacement of Binding of ^{125}I-Labeled Wheat Germ Agglutinin to Isolated Fat Cells by Concanavalin A and Insulin.[a]

Addition	^{125}I-Labeled Wheat Germ Agglutinin Concn	Sp Binding (pmol × 10^2)	% Fall in Binding
None	6 ng/ml	1.30 ± 0.05	
Concanavalin A	6 ng/ml	0.75 ± 0.03	42
Insulin	6 ng/ml	1.01 ± 0.04	22
None	30 ng/ml	6.50 ± 0.10	
Concanavalin A	30 ng/ml	3.70 ± 0.08	43
Insulin	30 ng/ml	5.00 ± 0.05	23
None	15 μg/ml	560 ± 39	
Concanavalin A	15 μg/ml	140 ± 21	75
Insulin	15 μg/ml	410 ± 18	26
None	50 μg/ml	790 ± 21	
Concanavalin A	50 μg/ml	180 ± 12	77
Insulin	50 μg/ml	608 ± 24	23

[a] Isolated fat cells (8×10^4 cells) were incubated for 50 min at 24° in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and, where indicated, insulin (50 μg/ml) or concanavalin A (1 mg/ml). ^{125}I-Labeled wheat germ agglutinin at the indicated concentration was added, and after an additional period of incubation (40 min), the specific binding was determined as described in the text.

TABLE IV: Effect of Digesting Fat Cells with Neuraminidase, Trypsin, and Phospholipase C from *Clostridium perfringens* on the Specific Binding of Wheat Germ Agglutinin.[a]

Enzymic Digestion, Concn (μg/ml)	Sp Binding of ^{125}I-Labeled Wheat Germ Agglutinin (cpm)
No digestion	14,900 ± 400
Neuraminidase, 2	13,800 ± 350
5	11,300 ± 600
20	3,800 ± 300
50	800 ± 150
Trypsin, 0.2	12,700 ± 650
0.7	10,400 ± 500
50	7,600 ± 450
300	7,300 ± 400
2	7,100 ± 400
Phospholipase C, 20	15,100 ± 350
100	15,200 ± 600

[a] The fat cells obtained from the epididymal fat pads of three rats (120 g) were suspended in 15 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin. Samples (0.2 ml) were digested with the indicated enzyme for 30 min at 37°. Soybean trypsin inhibitor was then added to the samples digested with trypsin; the concentration was 400 μg/ml, 800 μg/ml, or 3 mg/ml for the samples containing 50 μg/ml or less, 300 μg/ml, and 2 mg/ml of trypsin, respectively. ^{125}I-Labeled wheat germ agglutinin (1.1×10^5 cpm) was added to all the samples, and after incubating for 40 min at 24° the specific binding was determined as described in the text. Essentially the same results are obtained if the digested cells are washed before examining their capacity to bind ^{125}I-labeled wheat germ agglutinin, indicating that the effects of these enzymes do not result from their possible modification of the plant lectin.

capable of binding to all of these different types of lectin–cell complexes to initiate similar if not identical dissociative events. In all cases free binding sites for the simple sugar must exist on the cell-bound plant lectin.

Effect of Insulin and Concanavalin A on the Binding of Wheat Germ Agglutinin to Cells. In view of the ability of concanavalin A and of wheat germ agglutinin to perturb the insulin–receptor interaction in fat cells (Cuatrecasas, 1972), it was of interest to determine if insulin or concanavalin A could modify the binding of wheat germ agglutinin to fat cells. The specific binding of ^{125}I-labeled wheat germ agglutinin can be slightly decreased by these compounds, but this is observed only with extremely high concentrations of insulin or concanavalin A (Table II). Significant displacement of binding of low concentrations (50 ng/ml) of ^{125}I-labeled wheat germ agglutinin requires 600–3000 times greater concentrations (on a weight basis) of concanavalin A and more than 600 times greater molar concentrations of insulin. No effect is observed with concentrations of insulin which are less than 1 μg/ml. The displacement effects are somewhat more apparent when the cell concentration, and therefore the amount of bound wheat germ agglutinin, is low (Table II). High concentrations of insulin alter to an equal extent the binding of low and of high concentrations of ^{125}I-labeled wheat germ agglutinin to fat cells (Table III). In contrast, concanavalin A is much more effective in decreasing the binding of ^{125}I-labeled wheat germ agglutinin when the concentration of the iodinated plant lectin is high (Table III). It thus appears that the binding sites for wheat germ agglutinin which are measured with high concentrations of the lectin are not only of lower apparent affinity but are also less specific for this plant lectin.

Enzymic Digestion of Fat Cells and the Binding of Wheat Germ Agglutinin. Digestion of isolated fat cells with neuraminidase from *C. perfringens* results in a marked loss of binding of ^{125}I-labeled wheat germ agglutinin (Table IV). Similar results were obtained with enzyme preparations purified by affinity chromatography (see Methods). No effect on wheat germ agglutinin binding is observed if the cells are digested with phospholipase C. Tryptic digestion of cells results in loss of wheat germ agglutinin binding. However, less than half of the binding is lost even with very drastic conditions of digestion. Pronase digestion of cells similarly results in loss of only half of the binding sites for wheat germ agglutinin (Table V). No recovery of binding activity could be detected after incubating Pronase-, trypsin-, or neuraminidase-treated cells in Krebs–Ringer–bicarbonate buffer containing 1% (w/v) albumin for 3 hr at 37°. These results are consistent with the requirement for wheat germ agglutinin binding of sialic acid residues on carbohydrate determinants of the cell surface. The results also point to heterogeneous chemical composition of surface binding sites for this plant lectin.

Intracellular Binding Sites for Wheat Germ Agglutinin. The data presented earlier indicate that no significant quantities of wheat germ agglutinin enter the cell during the usual condition of incubation. The binding of ^{125}I-labeled wheat germ agglutinin to total particulate preparations of homogenates of

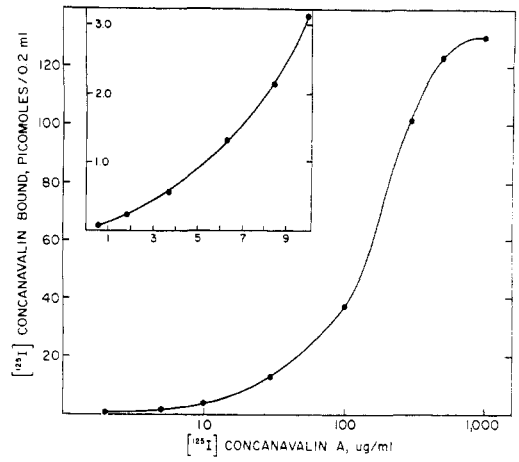

FIGURE 8: Specific binding of [^{125}I]concanavalin A to isolated fat cells as a function of the concentration of the plant lectin. Fat cells (2.2×10^5 cells) were incubated for 80 min at 24° in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and varying concentrations of [^{125}I]concanavalin A (log scale in main figure, not in insert). Specific binding was determined as described in the text. The specific activity of the iodoprotein was varied as described for wheat germ agglutinin (Figure 3). The binding observed with low concentrations of concanavalin A is depicted in the insert.

isolated fat cells is apparently greater than the binding observed to the intact cells (Table VI). The appearance of new, intracellular binding sites upon cell breakage is even more apparent if the surface or exposed binding sites of the intact cells are largely destroyed by neuraminidase digestion before cell homogenization. Under these conditions a very large increase in wheat germ agglutinin binding is readily detected. The apparently intracellular binding sites for wheat germ agglutinin are as readily destroyed by neuraminidase digestion as are the cell surface binding sites. The binding of wheat germ agglutinin and concanavalin A to nuclei and mitochondria has previously been described (Nicholson *et al.*, 1972; Hirano *et al.*, 1972). The subcellular localization of the intracellular, membrane-bound wheat germ agglutinin binding structures has not been determined.

TABLE V: Effect of Digesting Fat Cells with Pronase on the Specific Binding of Wheat Germ Agglutinin.[a]

Pronase Concn	Sp Binding of ^{125}I-Labeled Wheat Germ Agglutinin (cpm)
None	23,300 ± 600
0.2 μg/ml	14,600 ± 400
0.7 μg/ml	13,200 ± 400
0.4 μg/ml	12,800 ± 350
2 mg/ml	12,100 ± 300

[a] Fat cells (4×10^6 cells) were digested for 50 min at 37° in 2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and the indicated concentration of Pronase. The cells were then washed two times with 8 ml of the same buffer and suspended in a volume of 2 ml. Samples (0.2 ml) were incubated for 50 min at 24° with ^{125}I-labeled wheat germ agglutinin (1×10^5 cpm) to determine specific binding to cells.

TABLE VI: Homogenization and the Appearance of Intracellular Binding Sites for Wheat Germ Agglutinin.[a]

Cells	Sp Binding of ^{125}I-Labeled Wheat Germ Agglutinin (cpm)
Undigested, intact	31,200 ± 1,200
Undigested, homogenized	39,100 ± 1,400
Undigested, homogenized and digested with neuraminidase	10,300 ± 1,100
Digested, intact	12,500 ± 2,200
Digested, homogenized	27,500 ± 1,900
Digested, homogenized and redigested with neuraminidase	8,200 ± 800

[a] The isolated cells of fat pads from two rats were suspended in 8 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin. Portions (3 ml) of this suspension were incubated at 37° for 30 min in the absence and presence of neuraminidase (60 μg/ml) from *C. perfringens*. The cells were washed two times with 8 ml of the buffer described above, and the cells were suspended in 3 ml of this buffer. Portions of these cell suspensions were homogenized for 25 sec with a Polytron PT-10 (Brinkman), and portions of these homogenates were digested with neuraminidase as described above. Samples (0.2 ml) of the cells and homogenates were examined for specific binding of ^{125}I-labeled wheat germ agglutinin (2.1×10^5 cpm) after incubating at 24° for 40 min (see text). Control samples (not shown in the table) demonstrated that incubation of the homogenate (37°, 30 min) in the absence of neuraminidase did not alter binding.

Binding of Concanavalin A to Fat Cells. The binding of [^{125}I]concanavalin A to fat cells is a saturable process with respect to the plant lectin (Figure 8). Although the saturation curve has a more normally sigmoidal shape (log scale) than the curve obtained with wheat germ agglutinin (Figure 3), it is apparent that heterogenous binding sites also exist for concanavalin A. For example, careful examination of the binding process using very low concentrations of concanavalin A reveals the existence of binding sites of much greater affinity than are observed with the higher concentrations of the plant lectin (Figure 8, insert). Furthermore, the concanavalin A–cell interaction at these low lectin concentrations suggests the existence of interactions between binding sites.

As in the case of wheat germ agglutinin binding to fat cells, it is not possible to designate meaningful kinetic expressions to the fat cell–concanavalin A interaction because of the complex and heterogeneous nature of these interactions. The data shown in Figure 8, however, indicate that a fat cell can bind a maximum of about 4×10^8 molecules of concanavalin A.

The possibly cooperative behavior of concanavalin A binding sites is again suggested by studies of the effect of varying the concentrations of native concanavalin A on the binding of low concentrations of [^{125}I]concanavalin A to fat cells (Figure 9). At low concentrations the native protein clearly enhances the binding of the iodinated protein to the cells. The binding of [^{125}I]concanavalin A is readily displaced

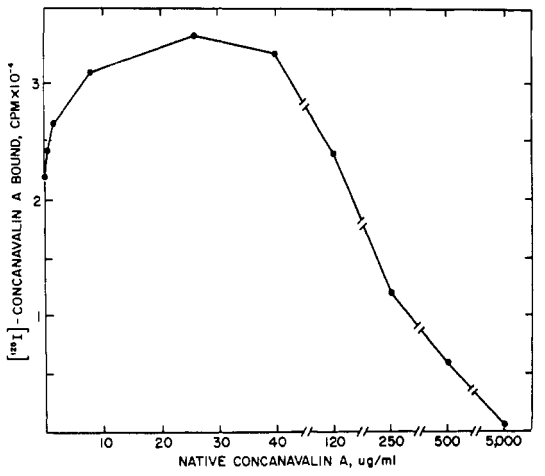

FIGURE 9: Effect of native concanavalin A on the specific binding of low concentrations of [^{125}I]concanavalin A to fat cells. Fat cells were incubated at 24° for 10 min in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and the indicated concentration of concanavalin A. [^{125}I]Concanavalin A (0.2 μg/ml) was added and specific binding was determined after incubating the samples for 70 min at 24°.

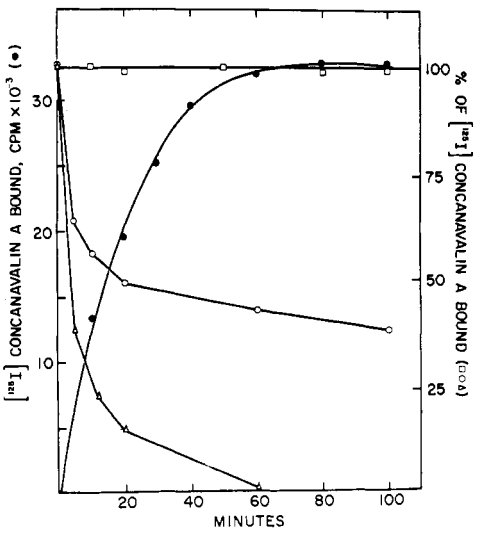

FIGURE 10: Rates of association and of dissociation of fat cell–concanavalin A complex. Fat cells were incubated at 24° in Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and [^{125}I]concanavalin A (0.3 μg/ml), and specific binding to cells was determined at various times (●) by filtration on LSWP Millipore membranes as described in the text. The dissociation was studied by incubating the cells with iodoconcanavalin A for 60 min as described above, followed by the addition of 5 mg/ml of native concanavalin A (□) or 50 mM α-methyl-D-mannopyranoside (○, △). The suspensions were then incubated for various times at 24° (○) or 37° (□, △). No discernible dissociation occurs in samples incubated at 24° with native concanavalin or in cells which are washed to remove the free concanavalin A from the medium (not shown in figure).

with high concentrations of the native protein. It is possible to achieve virtually complete displacement of [^{125}I]concanavalin, indicating that under most circumstances the nonspecific binding of this plant lectin to the cells and to the filters is very low.

Concanavalin A binds very rapidly to fat cells at 24° (Figure 10). The spontaneous rate of dissociation of the concanavalin A–cell complex is extremely slow. Even at 37° there is negligible dissociation during a period of 100 min. As demonstrated in the studies of wheat germ agglutinin, the concanavalin A–cell complex also dissociates very rapidly after addition of a simple sugar which has specificity for this plant lectin, α-methyl-D-mannopyranoside (Figure 10). The rate of dissociation in the presence of this sugar is temperature dependent. Virtually complete dissociation of the cell–lectin complex occurs with this sugar, and the rate of dissociation is dependent on the concentration of the simple sugar.

Partial displacement of binding of concanavalin A to fat cells occurs with very high concentrations of wheat germ agglutinin (Table VII). A very slight decrease in concanavalin A binding is observed in the presence of very high concentrations of insulin (Table VII).

Vigorous digestion of fat cells with trypsin results in a modest loss of the binding capacity for concanavalin A (Table VIII). Pronase digestion of cells is more effective than trypsin in causing a fall in the binding of the plant lectin to cells. Even the most extreme digestions with Pronase, however, result only in the loss of about half of the binding sites under the conditions tested. Unlike the findings described in the studies with wheat germ agglutinin, neuraminidase digestion of cells does not alter the binding of concanavalin A to these cells (Table VIII).

Changes in the Density of Cell Surface Receptors during Rapid Changes in Cell Size. The availability of sensitive procedures for quantitative measurements of cell surface structures which presumably represent glycoprotein components of the cell membrane provides opportunities for exploring the properties of cell surfaces under a variety of conditions. An example presented here deals with the comparative density of different membrane glycoproteins, and with the relative changes in the densities of these membrane structures which may accompany relatively rapid changes in cell size. The studies presented earlier in this report indicate that fat cells from small (about 140 g) rats can bind a maximum of about 3×10^8 molecules of wheat germ agglutinin and 4×10^8 molecules of concanavalin A per fat cell. Since these cells have an average diameter of about 35 μm, it can be calculated

TABLE VII: Effect of Wheat Germ Agglutinin and Insulin on the Binding of [^{125}I]Concanavalin A to Isolated Fat Cells.[a]

Addition	Binding of [^{125}I]-Concanavalin A (cpm)
No additions	13,300 ± 250
Concanavalin A, 5 mg/ml	930 ± 70
Wheat germ agglutinin, 7 μg/ml	11,800 ± 310
35 μg/ml	11,300 ± 540
140 μg/ml	9,200 ± 300
1 mg/ml	8,700 ± 190
Insulin, 0.25 μg/ml	13,000 ± 470
1 μg/ml	12,900 ± 250
5 μg/ml	10,800 ± 510
50 μg/ml	7,500 ± 480

[a] Fat cells were incubated at 24° for 10 min with the added compound and the specific binding of [^{125}I]concanavalin A (0.2 μg/ml) was determined after a 40-min period of incubation.

TABLE VIII: Effect of Digesting Fat Cells with Various Enzymes on the Specific Binding of Concanavalin A.[a]

Enzymic Digestion, Concn (mg/ml)	Sp Binding of [^{125}I]Concanavalin A (cpm)
None	38,200 ± 1,100
Trypsin, 0.1	33,200 ± 1,000
1.0	32,600 ± 600
Pronase, 0.1	26,800 ± 900
0.5	19,100 ± 1,200
1.0	18,400 ± 900
Neuraminidase, 0.1	37,100 ± 1,400

[a] The isolated fat cells obtained from six rats were incubated in 18 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin. Samples (2 ml) of the cell suspensions were incubated at 37° for 50 min with the indicated enzyme. The cells were washed two times with 10 ml of the buffer described above and suspended in 4 ml of the buffer. Samples (0.2 ml) of these cells were then incubated at 24° for 50 min in the presence of [^{125}I]concanavalin A to determine specific binding, as described in the text.

TABLE IX: Effect of Reduction of Fat Cell Size by Starvation on the Density of Various Cell Surface Receptors.[a]

^{125}I-Labeled Protein	Sp Binding of ^{125}I-Labeled Protein (cpm/10^5 cells)	
	Big Cells	Small Cells
Wheat germ agglutinin	40,400 ± 1,400	37,200 ± 1,900
Concanavalin A	12,100 ± 300	11,700 ± 500
Insulin	8,700 ± 400	9,300 ± 300

[a] A very large (580 g) Sprague-Dawley rat was starved for 10 days, at which time it weighed 410 g. The fat pads of the control rat weighed 5 g and of the starved rat 0.5 g. The average diameter (300 cells were measured) of the isolated control cells was 90 μm and of the cells from the starved animal 34 μm. Fat cells (5×10^4 cells for wheat germ agglutinin and concanavalin A, and 2×10^5 for insulin) were suspended in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% albumin and the specific binding of saturating concentrations of ^{125}I-labeled wheat germ agglutinin (130 μg/ml, 25 μCi/mg) and [^{125}I]insulin (1.2×10^{-9} M, 2000 MCi/μmol) was determined as described in the text after incubating for 40 min at 24°. The existence of virtual saturation with the concentrations indicated above was confirmed in these studies by the inclusion of samples in which the concentration of the proteins was varied (not shown in the table). Essentially the same results were obtained in four separate experiments performed as described here. In a similar experiment, cells (diameter, 100 μm) from control rats were found to bind the same amount of concanavalin A, wheat germ agglutinin, and insulin as the cells (diameter, 65 μm) from 3-day starved rats.

that the cell surface density of binding sites for wheat germ agglutinin is about 8.5×10^4 sites per μm^2 and for concanavalin A about 11×10^4 sites per μm^2.[2]

Fat cells obtained from very large (about 550 g) rats can maximally bind very nearly the same number of wheat germ agglutinin and concanavalin A molecules per cell (Table IX) as the small cells obtained from starved animals, despite a nearly threefold difference in the diameter of these cells. Starvation of the large rats for 3 and for 10 days leads to very substantial reductions in the size of the fat cells obtained from the epididymal fat pads (Table IX). However, the maximum number of molecules of wheat germ agglutinin, concanavalin A, or insulin which can bind per cell does not change appreciably despite these rapid and large changes in cell size. The cell surface densities of the receptors for these three proteins change in unison and the magnitude of the change can be more than two- (3-day starvation) and seven- (10-day starvation) fold.

Discussion

The binding of various radioactively labeled plant phytohemagglutinins to lymphocytes (Kornfeld and Kornfeld, 1971; Hauemann and Rubin, 1968), erythrocytes (Kornfeld *et al.*, 1971), and platelets (Majerus and Brodie, 1972) has been studied in some detail. Cline and Livingston (1971) described the binding of [^{3}H]concanavalin A (specific activity, 0.01 μCi/μg) to lymphocytes, and Ozanne and Sambrook (1971) examined the binding of concanavalin A and of wheat germ agglutinin, labeled with ^{125}I by a peroxidase procedure (specific activity, 0.001 μCi/μg), to normal and virus-transformed cells. Similarly, Inbar and Sachs (1969) have studied the binding of metal-labeled (^{63}Ni) concanavalin A to cells. In the present studies the availability of radioactive derivatives of very high specific activity (2–6 μCi/μg) has permitted more detailed studies of the nature of the lectin–cell interaction.

The preservation of mitogenic activity in phytohemagglutinin–agarose derivatives (Greaves and Bauminger, 1972) suggests that these plant proteins exert biological effects by cell surface interactions. The present studies indicate that under the conditions of binding used here the plant lectins are binding almost exclusively to the surface of the fat cell. With both plant lectins the equilibrium of binding is approximated very rapidly, and a plateau of binding is maintained at that level for a considerably longer period of time. Simple sugars or macromolecules (ovomucoid) having specificity for a given plant lectin cause immediate and profound dissociation of that lectin–cell complex. The magnitude of the dissociative process is approximately the same whether the sugar is added to the cells before the lectin, whether it is added after the lectin but before equilibrium of binding is achieved, whether it is added 40 min after binding equilibrium has been established, or whether the cells have been incubated with very low or very high (saturating) concentrations of the plant lectin. Furthermore, ovomucoid–agarose derivatives, which cannot enter the cells, are nearly as effective as *N*-acetyl-D-glucosamine in causing dissociation of the wheat germ agglutinin–cell complex. If phagocytosis or intracellular translocation of these plant lectins occurs under the conditions used here, it must represent an insignificant proportion of that which is specifically bound to the surface of the cell.

Although the binding of wheat germ agglutinin and concanavalin A to cells is clearly associated with carbohydrate

[2] The number of binding sites for these plant lectins is considerably greater than that for insulin, which is about 10^4 molecules per fat cell (Cuatrecasas, 1971a).

components of the cell membrane, it is apparent from the kinetics of the lectin–cell interaction, the effects of digesting the cells with various enzymes, and the partial "competition" of binding between lectins that there are multiple and heterogeneous binding "receptors" for these lectins. This heterogeneity, which probably reflects the lack of absolute specificity of the lectins for various complex carbohydrate determinants, is not surprising in view of the ability of even simple saccharides to bind to these plant lectins with varying degrees of affinity (Burger and Goldberg, 1967; Yariv *et al.*, 1968; So and Goldstein, 1967; Sharon and Lis, 1972). It is at present not possible to determine whether changes in lectin–lectin interactions occur during the binding process which might account, at least in part, for the apparent complexities in the binding curves. The observation that a certain proportion of concanavalin A molecules precipitate with time during incubation in NH_4HCO_3 at 37° (Cunningham *et al.*, 1972) suggests that changes in the state of aggregation of this lectin could possibly occur during incubation with cells. More precise or detailed analysis of the available binding data is not yet possible because of the ambiguities resulting from the apparent multiplicity of binding sites, the large proportion of medium lectin which is bound to the cells when low concentrations of the lectin are used,[3] and the likelihood of important interactions between binding sites or between lectin molecules.

The well-known ability of wheat germ agglutinin and concanavalin A to agglutinate a variety of cells (reviewed by Sharon and Lis, 1972), especially neoplastic or transformed cells, is generally believed to be based on the ability of these lectins to cross-link receptor sites by virtue of their divalent structure.[4] The ability of simple sugars to specifically cause rapid and marked dissociation of the lectin–cell complex must reflect the ability of these simple sugars to bind to unoccupied sites on lectin molecules which are already attached to the cell. The dissociation caused by the sugars present in the medium cannot result from the interaction of the sugar with the lectin free in solution, thus acting to prevent reassociation of the lectin with the cell, since the spontaneous rate of dissociation of the lectin–cell complex is negligible under the conditions utilized in these studies. The rates of spontaneous dissociation determined in the present studies are measured under experimental conditions which exclude reassociation of those lectin molecules (labeled) which dissociate from the cell–lectin complex. The lectin molecules attached to the cell must either have totally free and exposed sugar-binding sites, or the free simple sugar in solution must be capable of binding to or interacting with free subsites which are contained in the same overall site which is forming the principal lectin–cell bond. The latter possibility is very unlikely on steric grounds in view of the ability of macromolecules such as ovomucoid (or ovomucoid–agarose) to dissociate the wheat germ agglutinin–cell complex as effectively as the simple sugar. The simplest rationalization of these observations is that the plant lectins possess more than one binding site and that virtually all of the cell-bound lectins have at least one unoccupied carbohydrate binding site which is potentially capable of a "special" kind of interaction with carbohydrate determinants.

The existence of free saccharide binding sites on the cell-bound lectin molecules is not alone sufficient to explain the rapid dissociation which occurs upon addition of the specific simple sugar to the medium. The interaction of the sugar with one or more of these free binding sites must in addition induce a major change in the binding properties, conformation, or quaternary structure of the lectin which results in the rapid release of the cell-bound lectin molecules. This could occur if binding of the sugar to the free lectin site results in a conformational change in the molecule which would in turn result in a major decrease in the affinity, or in an altered specificity, of the site which is already involved in the interaction of the lectin with the membrane. Alternatively, a protomeric lectin complex could possibly dissociate by the binding of the simple sugar to those components of the complex whose carbohydrate binding sites are free. In this case release of the lectin into the medium would not represent a true dissociation of the cell–lectin complex since the monomeric portion of the complex which was originally involved in the interaction with the membrane determinant would still be intact. This explanation is perhaps somewhat less likely than the former since the loss of cell-bound lectin which occurs upon addition of the sugar generally exceeds 75% of the cell-bound radioactivity after a reasonably short period of incubation.

It is known that concanavalin A in solution exists predominantly as stable dimers below pH 6 and as tetramers above pH 7 (Kalb and Lustig, 1968). Recent X-ray crystallographic studies which elucidate the structure of concanavalin A at 2-Å resolution reveal a tetrameric structure consisting of four identical saccharide binding sites (Edelman *et al.*, 1972; Hardman and Ainsworth, 1972). The studies of Edelman *et al.* (1972) also suggest that the orientation of a simple monosaccharide (iodophenyl-D-glucopyranoside) in the binding site of concanavalin A may be very different from that expected to occur with the nonreducing terminal residues of di- and oligosaccharides which presumably would be the natural receptors on the cell surface. The present studies support the view that certain simple sugars may bind quite differently than the cell surface saccharide receptors to concanavalin A. The results would be readily explained within this framework if the special type of active-site occupation which exists with certain monosaccharides, and also possibly other analogous types of oligosaccharides (perhaps present in ovomucoid), give rise to a major change in the molecular properties of concanavalin A such that the nature of the binding properties of the other sites on the molecule would be grossly altered.

The changes in the circular dichroic spectrum of concanavalin A in the near-uv region which occur upon binding of α-methyl-D-mannoside (Pflumm *et al.*, 1971), and the changes in the precipitability of the lectin caused by this sugar (Cunningham *et al.*, 1972) suggest that sugar binding under physiological conditions is accompanied by a conformational change in the protein. The binding of such a sugar to a given site may on this basis be associated with major changes in the binding properties of the other sites of the protomer, or possibly may change the quaternary structure of the protein, thus perhaps explaining the ability of simple saccharides to dissociate the cell-bound lectin, to prevent or reverse agglutinated cells, and to prevent the biological effects of the lectin.

Assuming molecular weights for wheat germ agglutinin (Ozanne and Sambrook, 1971; Nagata and Burger, 1972) and concanavalin A (Sumner *et al.*, 1938; Kalb and Lustig, 1968; Hardman *et al.*, 1971; Edmundson *et al.*, 1971; and Edelman and Millette, 1971) of 25,000 and 100,000, respectively, fat cells can bind as many as 3–4 $\times 10^8$ molecules of either lectin

[3] It is not possible to determine if this results from the presence of very large numbers of binding sites or from the presence of a small group of binding sites of very high affinity.

[4] It has not been possible to demonstrate agglutination or aggregation of fat cells with a variety of concentrations of wheat germ agglutinin or concanavalin A under various conditions of incubation.

per cell. For an average fat cell having a diameter of 35 μm, this represents a density of about 10^5 lectin molecules per μm^2 of cell surface area. This density is approximately the same as described for the binding of concanavalin A to lymphocytes (Edelman and Millette, 1971) and of concanavalin A and wheat germ agglutinin to SV3T3, 3T3, BHK, and Py-BHK cells (Ozanne and Sambrook, 1971), if the smaller size of these cells, compared to fat cells, is considered. The very large number of lectin-binding sites in all these cells can perhaps be appreciated by comparison with the number of 10^4 sites per cells, which is the number of insulin-receptor sites of isolated fat cells (Cuatrecasas, 1971a). For purposes of comparison, it is of interest that the estimated average density of acetylcholine receptors of the muscle end plate (Miledi and Potter, 1971; Barnard *et al.*, 1971) and of the postsynaptic membrane of the electric tissue of *Torpedo marmorata* (Miledi *et al.*, 1971) and of *Electrophorus electricus* (Kasai and Changeux, 1971) can be estimated to be about 10^5 binding sites per μm^2 of surface area.

The present studies indicate that wheat germ agglutinin, when used in low concentrations, binds to isolated fat cells very specifically. These sites must have a very much greater affinity for wheat germ agglutinin than for concanavalin A. Similarly, the fat cell has binding sites which are highly specific for concanavalin A. The specificity for each lectin is perhaps not absolute, as suggested by the small degree of mutual "competition" which exists when high concentrations of one native lectin are used to displace low concentrations of the iodinated lectin. With such high lectin concentrations, however, it is possible that factors other than competition for the same binding site are involved. For example, it is difficult to exclude the existence of interactions between the two lectins, or of nonspecific distortions of membrane structure under these conditions.

It has been demonstrated (Cuatrecasas, 1972) that wheat germ agglutinin and concanavalin A can bind directly to the insulin receptor of fat cells, and both can compete with insulin for binding to this receptor. The small displacement of binding of concanavalin A or of wheat germ agglutinin by insulin, described in the present studies, cannot be a reflection of interactions of this hormone with its specific receptors since the effects are only observed with unphysiological concentrations which are several orders of magnitude greater than are required to saturate the fat cell receptors for this hormone (Cuatrecasas, 1971a). This does not exclude the possibility that insulin can compete with wheat germ agglutinin or concanavalin A for binding to insulin receptors. The total number of lectin-binding sites is very large, and only a small portion of these can be expected to represent binding to insulin receptor regions; loss of this small portion of the binding capacity may not be detectable under these circumstances.

It is of interest that the known biological effects of wheat germ agglutinin and concanavalin A on isolated fat cells, such as stimulation of glucose transport, inhibition of lipolysis, and inhibition of adenylate cyclase activity, are maximal with concentrations of these plant lectins which are very far below those required to saturate the binding sites for these lectins (Cuatrecasas and Tell, 1972). This points to the existence of functionally heterogeneous receptors for these lectins. This is supported further by the observation that whereas concanavalin A inhibits adenylate cyclase activity and is mitogenic at concentrations below 50 μg/ml, higher concentrations cause stimulation of enzyme activity and are toxic to lymphocytes in tissue culture (Cuatrecases and Tell, 1972).

The manner by which the binding of plant lectins to cells initiates biological effects is not yet understood. Although it is quite possible that the simple binding of lectins to certain surface molecules is sufficient to perturb biological functions, the possibility cannot be excluded that aggregation or clustering of surface receptors (Singer and Nicholson, 1972; Nicholson, 1971; Nicholson, 1972) or of lectin molecules (Cunningham *et al.*, 1972) may also be contributory or essential processes. The aggregation of cells does not appear to be essential for at least certain biological effects since the maximal insulin-like effects and the effects on adenylate cyclase of concanavalin A and of wheat germ agglutinin occur with concentrations of the plant lectins which cause no discernible agglutination of cells (Cuatrecasas and Tell, 1972). Regardless of which physical or structural processes may be involved in the interaction of plant lectins with cell surfaces, the ability of these lectins to elicit major biological effects must result from perturbations of specific biochemical events. It is possible, for example, that the inhibition of adenylate cyclase activity demonstrable in subcellular fractions by low concentrations of concanavalin A and wheat germ agglutinin (Cuatrecases and Tell, 1972) may be closely related to the biochemical basis of certain biological effects, such as mitogenicity and insulin-like activity.

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A Nuclear Magnetic Resonance Study of Bovine Pancreatic Trypsin Inhibitor. Tyrosine Titrations and Backbone NH Groups†

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ABSTRACT: The low-field portion of the 250-MHz ^1H nuclear magnetic resonance spectrum of bovine pancreatic trypsin inhibitor has been studied as a function of pH over the range pH 7–13. Of the 12 resonances which are observed downfield from the aromatic region, 11 exchange with the deuterons of the solvent (D_2O) with half-times exceeding 4 months. Analysis suggests that these resonances can be assigned to buried NH protons. The 3,5 protons ortho to the hydroxyl group of the four tyrosine residues have been resolved and titrated yielding pK_A's for the four tyrosines of 10.6, 10.8, 11.1, and 11.6. Denaturation of the protein is observed only above pH 13, or, at pH 7, in 8 M urea with excess β-mercaptoethanol.

The basic bovine pancreatic trypsin inhibitor (BPTI)[1] consists of a single polypeptide chain of 58 amino acids with three disulfide bridges. Although it is the smallest globular protein with known X-ray structure (Huber *et al.*, 1970, 1971), it is large enough to contain hydrogen-bonded sections of β sheet and α helix characteristic of other crystalline proteins. The ease of refolding this molecule following either reduction of the bridges of the native inhibitor (Anderer and Hörnle, 1966; Chauvet and Acher, 1966; Avineri-Goldman *et al.*, 1967; Pospisilova *et al.*, 1967) or deblocking of the cysteines in the synthetic inhibitor (Noda *et al.*, 1971) suggests that BPTI may be a suitable system for monitoring the dynamic aspects of protein folding. Known methods for selective disulfide bridge reduction (Liu and Meienhofer, 1968; Meloun *et al.*, 1968a), selective tyrosine modification (Meloun *et al.*, 1968b; Sherman and Kassell, 1968), and controlled sequential proteolytic cleavage (Wilson and Laskowski, 1971) offer a variety of modified derivatives for experimental observation. Moreover, a theoretical understanding of the BPTI structure is simplified by the relatively small number of variable dihedral angles and the conformational constraints imposed by the high density of disulfide cross-links. Two unusual features of the inhibitor are its retention of activity following exposure to high temperatures or pH extremes (Green and Work, 1953a,b; Sherman and Kassell, 1968), and its role in the trypsin–inhibitor complex which has one of the highest known association constants between two macromolecules (Vincent *et al.*, 1971).

Nuclear magnetic resonance studies of protein structures

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[1] Abbreviations used are: BPTI, basic pancreatic trypsin inhibitor; Me_6Si_2O, hexamethyldisiloxane.