Evidence That the Insulin-like Activities of Concanavalin A and Insulin Are Mediated by a Common Insulin Receptor Linked Effector System†

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ABSTRACT: Various lines of evidence comparing the insulin-like bioactivities of concanavalin A (Con A) and insulin and their effects on insulin binding support the concept that the actions of the lectin and hormone are mediated by a common effector system. The observed effects of Con A on glucose oxidation, lipogenesis, 3-*O*-methylglucose uptake, and stimulated lipolysis in fat cells appeared indistinguishable from those of insulin. That the ratio of Con A's to insulin's half-maximal effective concentrations required for effects on these biological processes was essentially constant (about 7×10^2) suggests a close linkage between Con A's effector system and the insulin receptor. In mixing experiments, the effects of Con A were additive to those of the anti-insulin receptor antibody and insulin at submaximal doses; at maximal doses, the mixture evoked the same maximal response as that of insulin. Con A also stimulated, like insulin, diaphragm muscle glycogenesis and adipose tissue lipogenesis in vivo. Cytochalasin B inhibited Con A stimulated glucose oxidation in fat cells in a dose-dependent manner indistinguishable from the inhibition of the insulin-stimulated process, whether the substrate was [1-^{14}C]-, [6-^{14}C]-, or [U-^{14}C]glucose. This indicates that Con A acts like insulin in the process of stimulating carrier-mediated hexose transport and metabolism. The effectiveness or potencies of several other inhibitors of hexose transport, some disruptors of microtubules and microfilaments and a few other inhibitors, were the same for Con A as they were for insulin-stimulated glucose oxidation. Divalent succinyl-Con A exhibited 10% of Con A's potency, although it evoked the same maximal response as insulin, indicating that receptor cross-linking may be as important in the action of Con A as had previously been shown for insulin. The finding of an interesting correlation between the reported ability of the insulin receptor to bind to various lectins on lectin affinity columns and the insulin-like potencies of these lectins supports the concept of a close coupling between Con A's action and insulin receptors. Con A was able to inhibit completely, and reverse, the binding of insulin–agarose beads to intact fat cells. However, inhibition of free [^{125}I]insulin binding to the cells required prior incubation of the cells with Con A for up to 1 h, and no greater than 75% inhibition could be seen. These and other studies [Katzen, H. M., Soderman, D. D., & Green, B. G. (1981) *Biochem. Biophys. Res. Commun. 98*, 410] suggest that binding of the lectin to insulin receptors may not necessarily explain the observed inhibitions of [^{125}I]insulin binding to intact cells.

Concanavalin A (Con A)[1] and several other plant lectins that are commonly used as cell surface membrane probes are well-known to exhibit a variety of insulin-like activities in vitro. Some characteristics of Con A and wheat germ agglutinin, the most extensively studied lectins, are their abilities to stimulate glucose oxidation and hexose transport and inhibit epinephrine-stimulated lipolysis in isolated adipocytes and to inhibit [^{125}I]insulin binding to intact adipocytes and liver cell membranes (Czech & Lynn, 1973; Cuatrecasas & Tell, 1973; Cauatrecasas, 1973a; Czech et al., 1974; Livingston & Purvis, 1980). In addition, Con A–Sepharose affinity chromatography has been used to extract the insulin receptor from detergent-solubilized membrane preparations (Cuatrecases & Tell, 1973).

However, the mechanism(s) responsible for the insulin-like effects of the lectins remains unknown and even controversial. Indeed, it is unclear whether the various effects of Con A are linked to each other by processes stemming from a common receptor or mediator or are isolated events resulting from a variety of unrelated receptors or mediators. Cuatrecasas & Tell (1973) proposed that Con A might exert its insulin-like actions by interacting directly with glycoprotein insulin receptors. It was speculated that Con A and insulin might even share a structural similarity in their binding regions (Cuatrecasas & Tell, 1973; Reeke et al., 1975). On the other hand, it was concluded by Czech et al. (1974) and Carter-Su et al. (1980) that because bioactivities of insulin and insulin binding to fat cells are sensitive to trypsin treatment of the cell while those properties of Con A are not, the lectin's activities are mediated by receptor moieties distinct from those involved in insulin's actions. In more recent studies designed to explain our findings of saccharide inhibitors of Con A that act as agonists and antagonists of the hormone, we proposed that the lectin might act by competitively interrupting an insulin receptor linked interaction between a cell membrane lectin and a separate membrane saccharide-containing moiety (Katzen, 1978, 1979).

Despite the various reports, noted above, few studies have focused on any comprehensive comparison between the lectin's and insulin's effects. The present studies were designed to compare directly, in a quantitative manner, a variety of responses of the fat cell to Con A with those responses to insulin, as well as to confirm and extend some of the earlier findings on the insulin-like properties of Con A on these cells. The present findings support the concept that the multiple insulin-like activities of Con A are mediated by the same effector system as that responsible for insulin's actions. They also suggest a close linkage between the receptors for insulin and the membrane pathway involved in Con A's action. A preliminary report of these findings has been presented (Katzen & Mumford, 1976).

Materials and Methods

Intact, isolated fat cells were prepared by the method of Rodbell (1964) and derived from the distal half of epididymal

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[1] Abbreviation used: Con A, concanavalin A.

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fat pads taken from 140–190-g male albino Charles River (CD) rats fed Purina Chow ad libitum. The fat cell membranes ("ghosts") were prepared as described by Rodbell (1967). For solubilized insulin receptor preparations, membrane ghosts were washed once by centrifugation for 30 min at 45000*g*, and the resultant pellet was mixed well with 0.5% Triton X-100 in Krebs–Ringer phosphate buffer, pH 7.5, and sonicated for 5 min at 22 °C. Insulin, recrystallized (25.9 units/mg), was obtained from Schwarz/Mann Laboratories. Concanavalin A (affinity chromatography purified, and designated homogeneous by acrylamide electrophoresis) was product of Pharmacia. The other lectins were obtained from Vector Laboratories. Concanavalin A–Sepharose (radiolabeled with tracer amounts of ^{125}I-labeled Con A) and insulin–Sepharose with a five-carbon length connecting arm (tracer labeled with [^{125}I]insulin) were prepared as previously described (Soderman et al., 1973; Katzen & Soderman, 1975). Succinylated Con A, lot no. 80923, was obtained from Vector Laboratories. Its specifications declare that up to a concentration of 5 mg/mL it will not agglutinate sheep red blood cells, while the lot of Con A from which it was prepared (designated "homogeneous") agglutinates these cells at a lectin concentration of 68 ng/mL. Cytochalasin's A, B, C, D, and E and nocodazole were obtained from the Aldrich Chemical Co.

The norbornyl inhibitor (diethyl hexahydro-4,8-methanobenzo[1,2-*d*:4,5-*d'*]diisoxazole-3,7-dicarboxylate) and the glycoside inhibitor (6-aminohexyl 6-azido-6-deoxy-1-thio-α-D-mannopyranoside) were kindly provided by Drs. A. Patchett and P. L. Durette (Merck, Sharp & Dohme Research Laboratories), respectively. Anti-insulin receptor sera [sample "B-2" (Kahn et al., 1977)] and cholera toxin were generous gifts of Drs. C. R. Kahn of the National Institutes of Health and R. A. Finkelstein of Dallas, TX, respectively. Anti-bovine insulin antibody (binds 0.90 milliunits of insulin per mL after reconstitution with 1 mL of water) and anti-Con A antibody (1.4 mg/mL) were products of Miles-Yeda, Israel.

[^{125}I]Insulin (containing 1 atom of iodine per molecule) was obtained from New England Nuclear and repurified by DE-23 ion-exchange chromatography, dialysis, and Sephadex G-100 gel fltration. The final product was found, according to the fat cell glucose oxidation assay used to determine insulin-like activity (see below), to be fully biologically active. 3-*O*-[^{3}H]Methylglucose and [1-^{14}C]-, [6-^{14}C]-, and [U^{14}C]glucose were also products of New England Nuclear. All other materials are as previously described (Katzen, 1979; Katzen & Soderman, 1975; Kanfer et al., 1976) or were obtained from usual commercial sources.

The bioassay used for measuring Con A and insulin-stimulated radiolabeled glucose oxidation to $^{14}CO_2$ by the fat cells was performed essentially by the method by Gliemann (1965) as described previously (Katzen, 1979). Other details are given in the text. The production of ^{14}C-labeled total lipids from [U-^{14}C]glucose in the lipogenesis assay for insulin-like activity was determined essentially by the procedure of Rodbell (1964) as previously described (Cascieri et al., 1979). The lipolysis assay was based upon the method of Garland & Randle (1962), also as previously described (Kanfer et al., 1976). Other assays and procedures are described in the legends in the text.

Results

Insulin-like Biological Activities of Con A on Fat Cells. For comparison of the insulin-like action of Con A to that of insulin, several in vitro, as well as in vivo, biological assays that measure effects characteristic of the actions of the hormone were utilized. As seen in Figure 1, the shape of the dose–response curve for the effect of Con A on glucose oxidation appears virtually indistinguishable from that of insulin's, and the maximal stimulatory effects of the ligands were identical with each other. Insulin's half-maximal effect was at about 8.7×10^{-11} M (12.5 microunits/mL) while that for Con A was about 6×10^{-8} M (6 μg/mL). Methyl α-D-mannoside blocked the activity of the lectin. The time-course profiles of the effects of Con A and insulin at relatively low concentrations, as shown in Figure 2, were also essentially the same. The extent of the lag period varied with the concentration of either agent. When tested at concentrations at and above their half-maximal effective concentrations (where the lag period was observed to decrease with increasing ligand concentration), their time-course curves were also indistinguishable from each other (not shown). At concentrations of the lectin and hormone that maximally stimulated glucose oxidation during the incubation period (e.g., at 100 μg/mL and 100 microunits/mL, respectively), linear time-course responses were seen for both agents (e.g., see Figure 7).

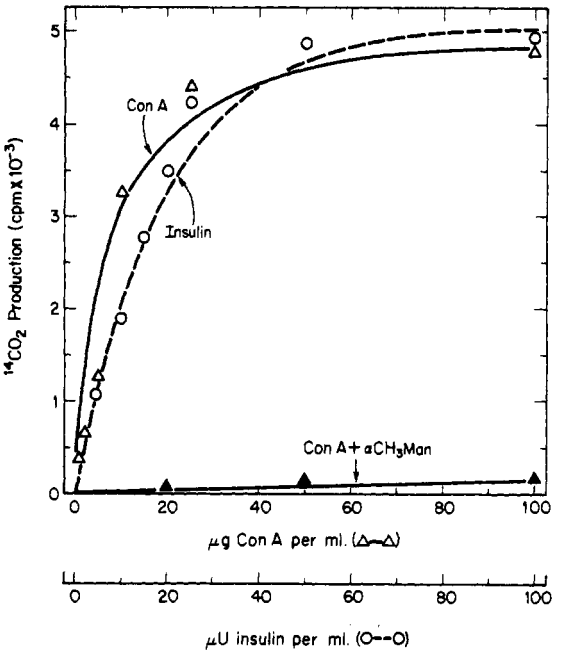

FIGURE 1: Comparison of dose–response effects of Con A and insulin on [U-^{14}C]glucose oxidation to $^{14}CO_2$ by fat cells. The bioassay (utilizing about 5×10^4 cells/mL) was performed as previously described (Katzen, 1979) except that Krebs–Ringer phosphate (instead of bicarbonate) buffer, pH 7.5, was used. All values, averages of three replications, represent $^{14}CO_2$ production above basal values. Methyl α-D-mannoside (αCH$_3$Man) concentration was at 0.1 M. This experiment was repeated 3 times, yielding, in each case, dose–response curves for the lectin and hormone that were indistinguishable from each other and that usually overlapped with each other over most of the dose ranges. Because of variations in the stimulatory effects seen from dat to day with the same ligand, results of only a single day's assay are averaged in this representative experiment, rather than averaging all five experiments. However, despite day-to-day variations in percent stimulatory effects, there were no significant day-to-day variations in half-maximal effective concentrations.

Con A also stimulated lipogenesis in fat cells, as did insulin (Figure 3). The shapes of the dose–response curves for the lipogenic effects of insulin and Con A were indistinguishable from each other. It should be noted that the ratio of the half-maximal effective concentration of Con A on lipogenesis (2.5 μg/mL or 2.5×10^{-8} M) relative to that concentration for insulin (5 microunits/mL or 3.5×10^{-11} M) was virtually the same as that ratio of concentrations for their effects on glucose oxidation (i.e., about 7×10^2) (compare Figure 3 with Figure 1).

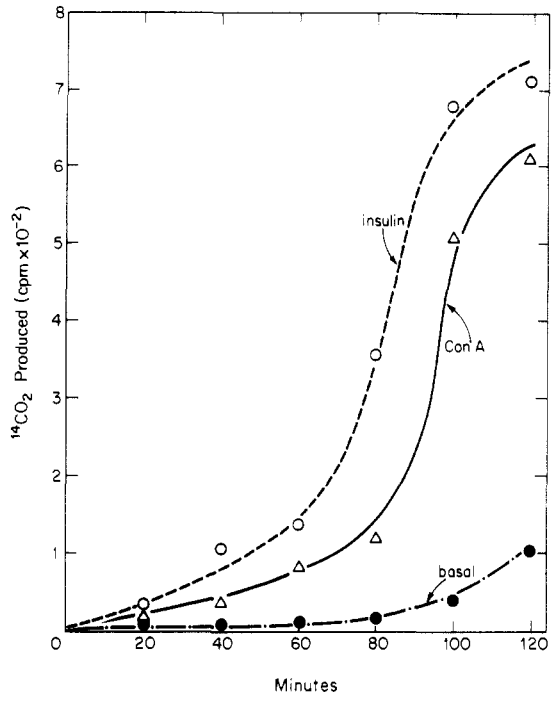

FIGURE 2: Comparison of time courses of Con A and insulin-stimulated [U-^{14}C]glucose oxidation in fat cells. Each ligand was incubated for the designated periods of time, and the $^{14}CO_2$ produced was measured as previously described in Figure 1. Con A concentration was 3 μg/mL (3×10^{-8} M) and insulin concentration 8 microunits/mL (5.6×10^{-11} M). This is a representative experiment of four replicate experiments as discussed in Figure 1.

The ability of Con A to stimulate the uptake of 3-O[^{3}H]-methylglucose into the adipocyte was tested and compared with the effect of insulin (Figure 4). Not only did Con A stimulate hexose transport as previously reported (Czech et al., 1974) but also the shapes of the dose–response curves for the two agents were identical with each other. Their maximal levels of stimulation were also the same. However, the ratio of the half-maximal effective concentration of Con A on sugar transport (about 50 μg/mL or 5×10^{-7} M) relative to that for insulin (about 0.6 microunits/mL or 4×10^{-9} M) (Figure 4) was only about 1.3×10^2 compared to about 7×10^2 for glucose utilization (see Discussion).

Insulin and Con A were also tested in an assay that does not involve glucose uptake in its mechanism, i.e., the inhibition of epinephrine-stimulated lipolysis (Figure 5). Again, the shapes of the dose–response curves for the two ligands were virtually identical with each other, and methyl α-D-mannoside inhibited the insulin-like activity of Con A. It is important to note that the ratio of the half-maximal effective inhibitory dose of Con A on lipolysis (about 7.5×10^{-8} M) relative to that for insulin (about 1×10^{-10} M) was nearly the same (7.5×10^2) as it was for glucose oxidation and lipogenesis. The same concentration of cells was used in all three assays. At higher concentrations of Con A and insulin, their maximal inhibitory effects were both between 95 and 100% (not shown).

The lectin and insulin were tested by utilizing several other lipolytic agents (not shown)[2] to ensure that the antilipolytic effect of Con A is not an isolated action limited to only blocking epinephrine-stimulated lipolysis. Accordingly, Con A was found to act like insulin by inhibiting cholera toxin, ACTH-, and theophyllin-stimulated lipolysis at the same concentrations of lectin as were required to inhibit epinephrine-stimulated lipolysis, and to the same extents.

[2] These data were presented to the scrutiny of the reviewers and can be forwarded to the interested reader by writing directly to the authors.

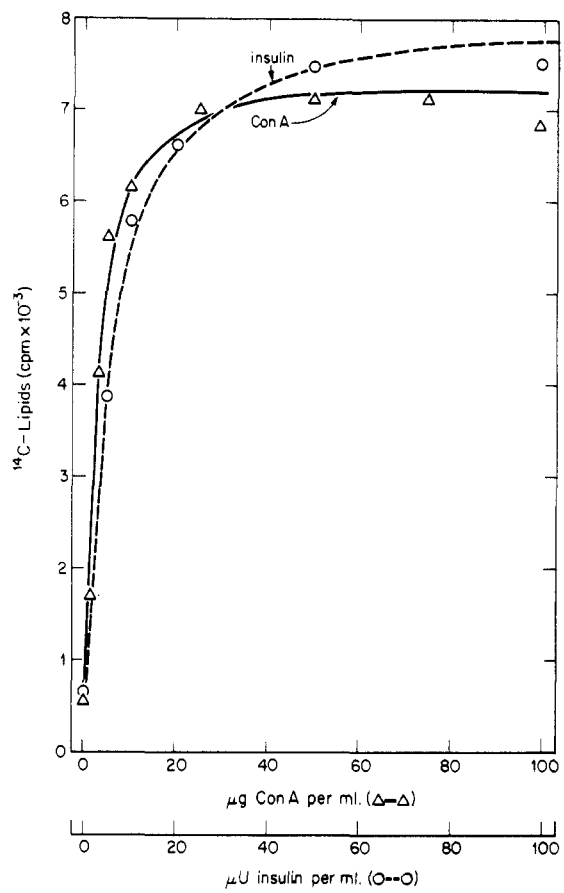

FIGURE 3: Comparison of dose–response effects of Con A and insulin on conversion of [U-^{14}C]glucose to total ^{14}C-labeled lipids in fat cells. Total extractable lipids were determined as described by Rodbell (1964). Each value represents the average of three replications. Incubations are identical with those described in Figure 1. This is a representative of four replicate experiments as discussed in Figure 1.

Table I: Combined Effects of Con A, Insulin, and Anti-insulin Receptor Antibodies on [^{14}C]Glucose Oxidation

supplements[a]	$^{14}CO_2$ produced[b] (cpm ± SEM)
none	136 ± 11
insulin (10 microunits)	1735 ± 132
insulin (100 microunits)	2996 ± 195
insulin (1000 microunits)	3429 ± 286
Con A (10 μg)	1861 ± 162
Con A (50 μg)	3058 ± 265
ARS (1:10 000)	1908 ± 66
insulin (10 microunits) + Con A (10 μg)	2719 ± 175
insulin (100 microunits) + Con A (50 μg)	3215 ± 281
insulin (100 microunits) + ARS (1:10 000)	3594 ± 194
Con A (50 μg) + ARS (1:10 000)	3491 ± 173
Con A (50 μg) + ARS (1:10 000) + insulin (100 microunits)	3508 ± 159

[a] ARS refers to anti-insulin receptor antibody serum (see Materials and Methods). All designated quantities are per milliliter, except ARS which is given as dilution of sera. Other details are as in Figure 1. [b] All values are the average of three replications.

Mixing experiments were conducted (Table I) to determine the combined effects of insulin, Con A, and another insulin receptor effector on glucose oxidation in fat cells. The addition of Con A, at a concentration exhibiting submaximal effects, to incubation mixtures containing submaximal doses of insulin or anti-insulin receptor antibody resulted in essentially additive

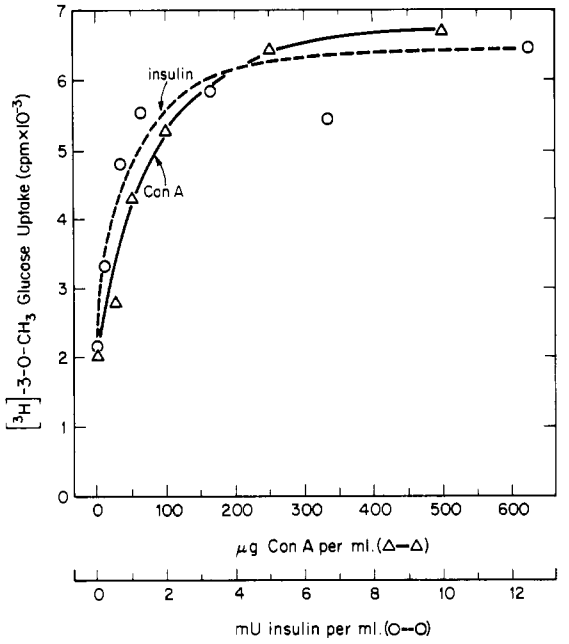

FIGURE 4: Comparison of dose–response effects of Con A and insulin on uptake of 3-*O*-[^{3}H]methylglucose into fat cells. Uptake (utilizing about 5 × 10^6 cells/mL) was determined by the method of Czech (1976). Each value represents the average of three replications. This is a representative experiment of four replicate experiments as discussed in Figure 1.

Table II: Effects of Con A and Insulin on Diaphragm Glycogenesis and Adipose Tissue Lipogenesis from [U-^{14}C]Glucose in Vivo[a]

injected agent (per kg of rat wt)	[^{14}C]glucose incorporated [(cpm/g of tissue) ± SEM] into[b]	
	glycogen	lipids
saline	3631 ± 163	3966 ± 190
insulin (200 milliunits)	72019 ± 4129	77385 ± 6905
Con A (13 mg)	8180 ± 752	5921 ± 328
Con A (67 mg)	15464 ± 1453	15196 ± 954
Con A (335 mg)	23844 ± 3481	37392 ± 3402

[a] Omental adipose tissue lipogenesis and diaphragm muscle glycogenesis were determined essentially by the method of Rafaelsen et al. (1965) and Rafaelsen (1964), as previously described (Durette et al., 1978). Insulin or Con A dissolved in 2 mL of 0.15 M saline containing 2 μCi of [U-^{14}C]glucose was injected intraperitoneally into each female Charles River (CD) rat weighing 145–155 g and fasted overnight. After 2 h, the rats were sacrificed and the tissues removed, weighed, and assayed for ^{14}C radioactivity incorporated per gram of tissue into the alkali-extractable ethanol-precipitable glycogen fraction and the total saponifiable ethanol–petroleum ether extractable lipids, from the muscle and adipose tissues, respectively, as previously described (Durette et al., 1978). [b] Each value represents the average of three experiments, each experiment the average of five replications (15 rats per recorded value).

effects. The antibody had previously been demonstrated by Kahn et al. (1977, 1978) to act like insulin by interacting with the insulin receptor.[3] Con A could not, in the presence of high concentrations of insulin or antibody (Table I), enhance the production of $^{14}CO_2$ beyond the maximal effect of the hormone or antibody.

Con A is also capable of acting like insulin in vivo by stimulating both glycogenesis and lipogenesis (Table II). By

[3] In a confirmation of the activity of the "B-2" sample of the antibody used in the present study (see Materials and Methods), we have independently found it to be indistinguishable from insulin in its ability to inhibit [^{125}I]insulin binding to fat cells and to inhibit stimulated lipolysis and stimulate glucose oxidation.

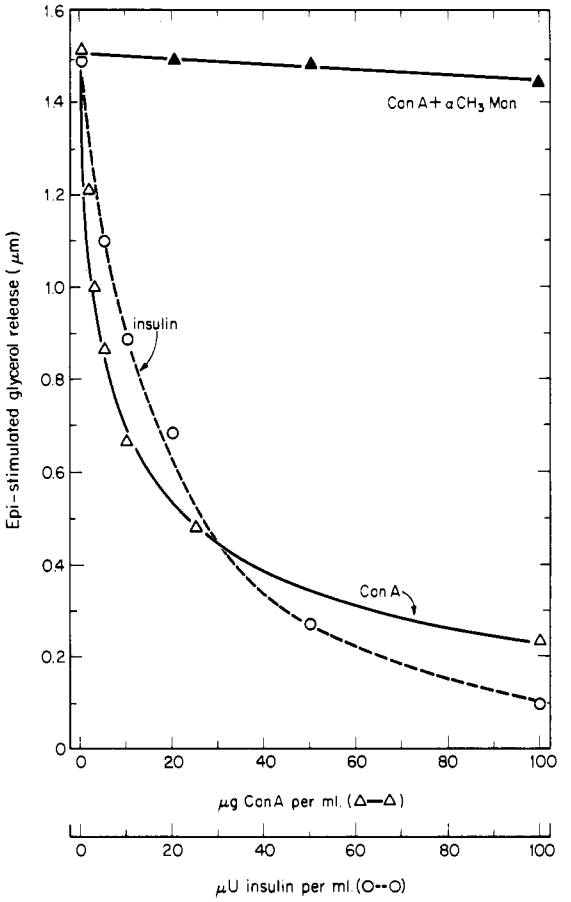

FIGURE 5: Comparison of dose–response effects of Con A and insulin on epinephrine-stimulated (epi-stimulated in figure) lipolysis in fat cells. Values are averages of three replications and represent the amounts of glycerol released, above basal release, per 5 × 10^4 cells during a 1-h incubation period. The bioassay was performed as previously described (Kanfer et al., 1976), except that Krebs–Ringer phosphate (instead of bicarbonate) buffer, pH 7.5, was used. Cells were prepared, and the assay was conducted in the absence of added glucose. Methyl α-D-mannoside (αCH_3Man) concentration was 0.1 M. As described in Figure 1, this is a representative experiment of four replicate experiments.

use of the technique developed by Rafaelsen et al. (1965) and Rafaelsen (1964), which measures the ability of insulin, injected intraperitoneally, to stimulate the conversion of [U-^{14}C]glucose (also injected intraperitoneally) into diaphragm muscle glycogen and epididymal adipose tissue total lipids, the lectin was found to mimic insulin in both tissues in a dose-dependent manner. Due to the limited solubility of Con A, and its toxicity, concentrations capable of exhibiting maximal effects could not be tested.

Comparative Effects of Membrane Inhibitors on the Actions of Con A and Insulin. The effects of cytochalasin B, a glucose-transport inhibitor, on glucose oxidation in fat cells stimulated by Con A and insulin are illustrated in Figure 6. Clearly, the dose–response effects of this inhibitor on [U-^{14}C]glucose oxidation by fat cells in the presence of Con A and insulin were indistinguishable from each other. The concentrations of cyclochalasin B required for half-maximal inhibitions were essentially the same for both Con A and insulin (i.e., about 0.3 μg/mL). Despite inhibitions of basal glucose oxidation of up to 77% by cytochalasin B, no effect on the percent stimulatory activities (i.e., the ratio of stimulated to basal activities) of either insulin or Con A could be observed over the entire range of cytochalasin B concentrations tested. Thus, fat cell glucose oxidation responds similarly to insulin and Con A stimulation during cytochalasin B's inhibitory

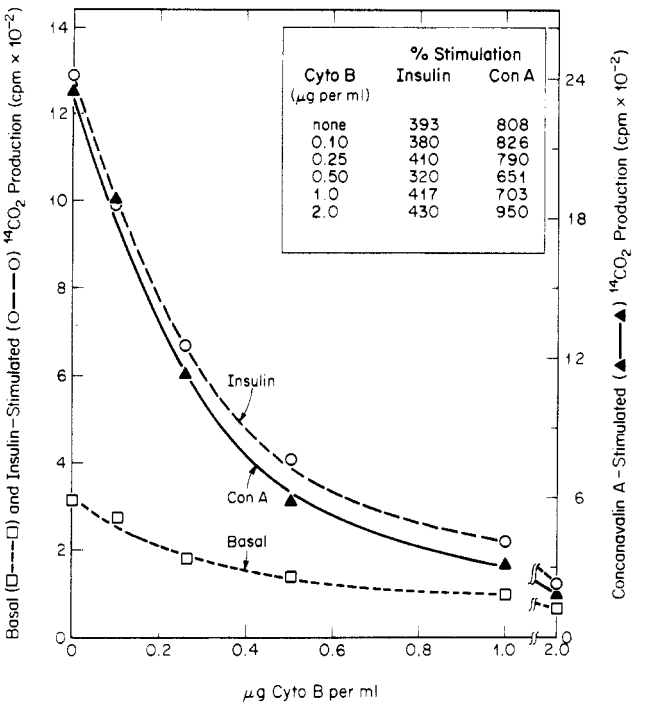

FIGURE 6: Dose-response effects of cytochalasin B (Cyto B) on [U-^{14}C]glucose oxidation in the presence and absence of Con A and insulin. Con A concentration was 10 μg/mL and insulin concentration 15 microunits/mL, each at approximately their half-maximal effective concentration (see Figure 1). Percent stimulation is determined by comparing the stimulated $^{14}CO_2$ production with a basal production after subtracting from both values the oxidation resulting from diffusion-mediated [U-^{14}C]glucose uptake. The latter was determined by measuring basal $^{14}CO_2$ production in the presence of a maximum inhibitory concentration of cytochalasin B (20 μg/mL) (Czech, 1976; Czech et al., 1973; Jarett & Smith, 1979). This experiment was repeated 2 times, yielding virtually identical results.

action. The lack of effect of submaximal concentrations of cytochalasin B on the action of insulin was previously reported by others (Kahn et al., 1978; Jarett & Smith, 1979). In the present study, we found that at a concentration of cytochalasin B high enough, presumably, to inhibit completely the glucose-transport system (about 20 μg/mL), $^{14}CO_2$ production in the presence of either Con A or insulin did not exceed basal levels (not shown).

The inhibitory effects of cytochalasin B on insulin- and Con A stimulated $^{14}CO_2$ production were compared after fat cells were incubated with glucose radiolabeled uniformly or in the carbon-1 or carbon-6 positions (Table III). At doses of cytochalasin B, Con A, and insulin that exhibited approximately half-maximal responses, the effects of cytochalasin B on the fat cell production of $^{14}CO_2$ in response to insulin and Con A were identical with all three radiolabeled substrates. Consistent with the finding in Figure 6, Table III shows that the percent stimulations of $^{14}CO_2$ production (stimulated/basal activity ratio) by either insulin or Con A were unaffected by cytochalasin B even when the 6-^{14}C- and 1-^{14}C-labeled substrates were used.

From a study of other membrane effectors, Table IV compares the concentrations required for half-maximal inhibition of glucose oxidation in the presence of Con A with those concentrations required for effects in the presence of insulin. From a previous random survey (not shown) of various lipids and glycolipids, sphingosine and galactosylsphingosine (phychosine) were found to inhibit $^{14}CO_2$ production in the presence of insulin in a dose-dependent manner. The half-macimal inhibitory concentration in the presence of insulin was found to be essentially the same as that in the presence of Con A

Table III: Comparison of Effects of Cytochalasin B on Insulin- and Con A Stimulated Glucose Oxidations Using Different ^{14}C-Labeled Substrates[a]

supplements[b]	$^{14}CO_2$ production from labeled glucoses[c] (cpm ± SEM) 1-^{14}C	6-^{14}C	U-^{14}C
basal	580 ± 30	273 ± 7	622 ± 75
insulin	1694 ± 106	476 ± 30	2427 ± 117
Con A	2785 ± 265 (2.0X)	719 ± 72 (2.2X)	3901 ± 205 (1.8X)
cyto B	289 ± 18	134 ± 4	238 ± 6
insulin + cyto B	447 ± 37	216 ± 14	633 ± 36
Con A + cyto B	625 ± 41 (2.1X)	304 ± 17 (2.1X)	1001 ± 63 (1.9X)

[a] Details of the assay are as described in Figure 1. [1-^{14}C]-, [6-^{14}C]-, and [U-^{14}C]glucose (0.1 μCi/mL) were each incubated with glucose at a final concentration of glucose of 0.75 mM as described earlier (Katzen, 1979). [b] Insulin is at 15 microunits/mL, Con A at 10 μg/mL, and cytochalasin B (cyto B) at 0.6 μg/mL. [c] Values in parenthesis represent the ratios of the percent stimulation by Con A relative to the percent stimulation by insulin.

Table IV: Comparative Effects of Membrane Inhibitors on [U-^{14}C]Glucose Oxidation Stimulated by Con A and Insulin[a]

inhibitor[b]	molar concn for 50% inhibition during stimulation by[c] Con A	insulin
sphingosine	8.6×10^{-5}	8.5×10^{-5}
galactosylsphingosine	3.2×10^{-5}	3.6×10^{-5}
AHADT-mannoside	4.0×10^{-4}	4.1×10^{-4}
norbornyl inhibitor	7.0×10^{-5}	6.8×10^{-5}
phloretin	4.8×10^{-5}	4.7×10^{-5}
phlorizin	1.0×10^{-3}	1.1×10^{-3}
colchicine	NE	NE
colcemid	NE	NE
vinblastine	NE	NE
nocodazole	5.0×10^{-6}	5.0×10^{-6}
cytochalasin A	6.3×10^{-5}	6.2×10^{-5}
cytochalasin B	7.9×10^{-7}	8.0×10^{-7}
cytochalasin C	NE	NE
cytochalasin D	NE	NE
cytochalasin E	1.1×10^{-4}	1.0×10^{-4}

[a] Details of the assay are as described in Figure 1, and structures of some of the inhibitors are given under Materials and Methods. [b] AHADT-mannoside refers to aminohexyl azidodeoxythiomannoside (see Materials and Methods). [c] Values are concentrations of inhibitors required to half-maximally inhibit $^{14}CO_2$ production in the presence of Con A at 10 μg/mL or insulin at 10 microunits/mL. NE means no observable effect.

(Table IV). Both compounds were separately found to be capable of decreasing $^{14}CO_2$ production to basal levels. Aminohexyl azidodeoxythiomannoside (see Materials and Methods for structure), an inhibitor derived from a previous search for saccharides capable of acting as insulin agonists and antagonists (Katzen, 1979; Durette et al., 1978) and subsequently found to also be capable of inhibiting fat cell 3-*O*-methylglucose uptake,[4] inhibited oxidation in the presence of Con A and insulin to similar degrees (Table IV). Another inhibitor of 3-*O*-methylglucose transport into fat cells,[5] a "norbornyl" compound (see Materials and Methods for structure), also was equally inhibitory. Phloretin and phlorizin, known to markedly inhibit glucose transport in intestine (Sanford, 1967) and fat cells (Czech et al., 1973; Kuo et al., 1967), were also tested. Although phloretin was about 500 times more potent than phlorizin, their respective concentrations required to half-maximally inhibit insulin were the same as those required to

[4] M. A. Cascieri, P. L. Durette, and H. M. Katzen, unpublished experiments.

[5] D. Soderman and H. M. Katzen, unpublished experiments.

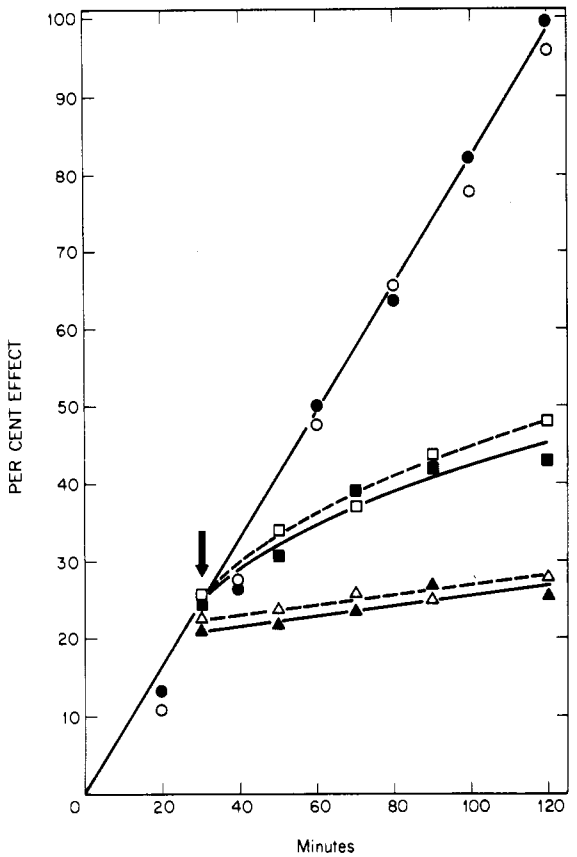

FIGURE 7: Comparison of effects of inhibitors of Con A and insulin-stimulated [^{14}C]glucose oxidation added 30 min *after* the start of stimulated oxidation. Con A (100 μg/mL) (○) and insulin (100 microunits/mL) (●) were included as described in Figure 1 for 30 min, at which time (at the arrow) the norbornyl inhibitor (to 3×10^{-4} M) was added to the incubation containing Con A (△) and to that containing insulin (▲), and aminohexyl azidodeoxythiomannoside (to 2 mM) was added to the incubation containing Con A (□) and to that containing insulin (■). Separate incubations were performed for each time point; all values are averages of three replications. Structures of inhibitors are given under Materials and Methods.

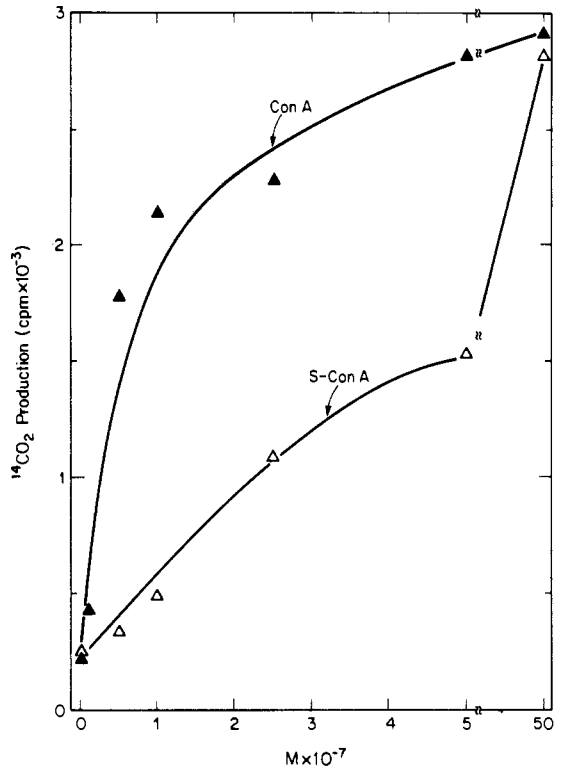

FIGURE 8: Dose–response effect of divalent succinyl-Con A (S-Con A), compared to Con A, on [^{14}C]glucose oxidation in fat cells. Details are as in Figure 1.

inhibit Con A. A variety of microtubule-disrupting agents such as colchicine, colcemid, vinblastine, and nocodazole (Edelman et al., 1973; Hoebeke et al., 1976) were also equally effective or ineffective, as the case may be, on Con A compared to insulin-stimulated glucose oxidations. It is noteworth that cytochalasin D had no affect on glucose oxidation in the presence or absence of insulin or Con A at concentrations over 150 times greater than the inhibitory concentrations of cytochalasin B (compared Figure 6 with Table IV and see Discussion). The concentrations of the other cytochalasins (A, C, and E) required to half-maximally inhibit Con A stimulated glucose oxidation were found to be the same as those required for the insulin-stimulated effect (Table IV).

Figure 7 shows an example of the effects of adding several glucose-transport inhibitors[4,5] to the cells 30 min *after* the cells were treated with Con A and insulin. The Con A stimulated process responded to these inhibitors at the same time (instantaneously) and in the same time-course manner as the insulin-stimulated process. In a similar study of the effects of anti-Con A and anti-insulin antibody sera (not shown), the response of the Con A stimulated cells was also the same as that of the insulin-stimulated ones.

For examination of the effect of reducing the binding capacity ("valency") of Con A on its insulin-like activity, the ability of divalent succinylated Con A (Gunther et al., 1973) to stimulate glucose oxidation was compared wih that ability of the Con A tetramer (Figure 8). Succinyl-Con A retained about 10% of the potency (on a molar basis) of the native tetramer. It was also able to stimulate $^{14}CO_2$ production to the same maximal extent as Con A. In separate experiments (not shown), succinyl-Con A was found also to exhibit 10% of the anti (epinephrine-stimulated) lipolytic activity of the native lectin.

Effects of Con A on Insulin Binding. Con A, when added together with [^{125}I]insulin to incubation media containing intact fat cells, exhibited no inhibitory effect on the specific binding of the hormone to the cells (Figure 9, "zero time"). Indeed, enhanced [^{125}I]insulin binding was frequently seen at the higher Con A concentrations. The unexplained variability of this increase from experiment to experiment made it difficult to study. In accordance with previous, but limited, observations (Cuatrecasas & Tell, 1973; Cuatrecasas, 1973a), incubation of the cells with Con A for 30 min *prior* to the addition of [^{125}I]insulin did result in inhibition of binding (Figure 9). However, shorter preincubation times resulted in lesser degrees of inhibition, while longer times only slightly enhanced the inhibitory effect. At no concentration of Con A or length of preincubation time could we observe inhibitory effects greater than about 75%. Methyl α-D-mannoside completely abolished the effects of Con A.

For examination of the effect of Con A on insulin binding in a different assay system and for comparison of these effects with, conversely, any effect of insulin on Con A binding, the affinity binding buoyant density assay, which utilizes ligand covalently bound to agarose beads (Soderman et al., 1973; Katzen & Soderman, 1972, 1975), was employed (Table V). Previous studies in our laboratory established the validity of this assay to measure insulin binding to fat cell surface receptors that exhibited properties virtually identical with those when free [^{125}I]insulin was employed as the ligand (Katzen & Soderman, 1972; Soderman et al., 1973). We also demonstrated the effectiveness of this assay system, utilizing Con A–agarose beads, to study Con A receptors on the fat cell

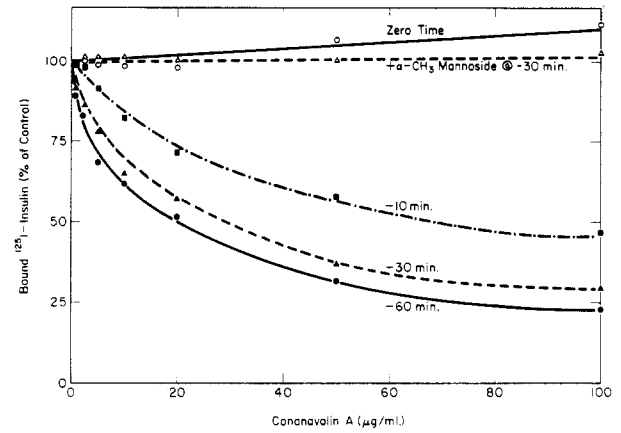

FIGURE 9: Dose–response effects of Con A on binding of [^{125}I]insulin to intact fat cells. Con A, at the designated concentrations, was incubated with the cells (5×10^5 per reaction) suspended in Krebs–Ringer phosphate buffer, pH 7.5, containing 2% bovine serum albumin, in a final volume of 1 mL, at 23 °C, for the designated periods of time prior to the addition of [^{125}I]insulin (to 2×10^{-9} M). The effect on Con A was also tested when the [^{125}I]insulin was added together with the lectin ("zero time"). When included, methyl α-D-mannoside (to 0.1 M) was added with Con A. After addition of the [^{125}I]insulin, cells were incubated for 35 min at 23 °C, and specific binding was determined by the oil density separation method of Gliemann et al. (1972) as previously described (Katzen, 1979). Recorded values for experiments involving preincubation times represent values obtained after deducting the loss in binding due to the additional incubation time, which, in turn, was determined from separate control experiments.

Table V: Effects of Con A and Insulin on Binding of Insulin–Agarose and Con A–Agarose Beads to Receptors on Intact Adipocytes[a]

inhibitor added[b]	% sedimentation (±SEM)[c] insulin–agarose	Con A–agarose
none	6 ± 1	2 ± 1
5×10^{-7} M Con A	28 ± 3	3 ± 1
5×10^{-5} M Con A	94 ± 4	98 ± 5
5×10^{-5} M Con A + αCH_3Man	12 ± 2	
5×10^{-5} M insulin	61 ± 5	3 ± 1
anti-insulin sera (5×10^{-3} mL)	99 ± 2	2 ± 1
anti-Con A sera (5×10^{-3} mL)	7 ± 1	99 ± 2

[a] Binding of insulin–agarose and Con A–agarose beads to insulin receptors and Con A receptors, respectively, on fat cells was determined by the affinity binding buoyant density assay as previously described (Soderman et al., 1973; Katzen & Soderman, 1975). Insulin–agarose beads, tracer labeled with [^{125}I]insulin, and Con A–agarose beads, tracer labeled with ^{125}I-labeled Con A (see Materials and Methods), when bound to their respective receptors on the cells, float as cell–bead complexes to the top of the incubation vessels. Addition of inhibitors of binding of derivatized beads to cells results in a dose-related sedimentation of free (unbound) radiolabeled beads, leaving the remaining cell-bound beads and free cells floating (Katzen & Soderman, 1972). Percent sedimentation is therefore a reflection of the percent inhibition of binding of the ligands immobilized on agarose beads to their respective receptors on the cells (Soderman et al., 1973; Katzen & Soderman, 1975). [b] Methyl α-D-mannoside (αCH_3Man) concentration was 0.1 M. [c] Each value is the average of three replications.

surface (Katzen & Soderman, 1975). In Table V, it is seen that free Con A is a potent inhibitor of insulin–Sepharose binding to intact fat cells even when added at the time the cells were exposed to the derivatized beads.

Indeed, if the free Con A was added 15 min after the cell–bead complexes had formed, at which time floatation of all of the insulin–agarose beads was complete, similar degrees of inhibition could be seen (not shown). Thus, Con A can

Table VI: Comparison of Insulin-like Bioactivities of Lectins with Their Previously Reported Abilities To Bind to Insulin Receptors

lectin[b]	relative bioactivities[a] (% of insulin's activity) inhibition of stimulated lipolysis	stimulation of glucose oxidation
binders		
Con A	96	100
lentil	100	100
garden pea (*Pisum sativum*)	95	85
ricin I	100	100
ricin II	95	93
wheat germ	100	100
nonbinders		
Bandieraea *Simplicifolia*	5	0
gorse seed	0	0
horse gram	0	1
peanut	0	0
Sophora japonica	7	3
soybean	4	0

[a] Values are recorded relative to insulin's effect when the hormone was at 100 microunits/mL and represent the average of duplicate experiments, each run in triplicate. All lectins were at 100 μg/mL. Lipolysis was stimulated by cholera toxin at 0.5 μg/mL. Details of the lipolysis and glucose oxidation assays are as described in Figures 1 and 5, respectively. [b] The designations of lectins being capable (binders) or incapable (nonbinders) of binding to insulin receptors are from the report of Hedo et al. (1980, 1981).

reverse the binding of cells to beads much like methyl α-D-mannoside had previously been shown to reverse the binding of Con A–agarose beads to fat cells (Katzen & Soderman, 1975). The slight inhibitory effect persisting after adding methyl α-D-mannoside to block Con A's effect (Table V) may reflect the previously demonstrated ability of this glycoside to inhibit insulin binding in this as well as in the [^{125}I]insulin binding assay (Katzen, 1979). Reasons for the poor effect of free insulin on the binding of insulin–agarose were previously discussed (Soderman et al., 1973; Katzen, 1979).

While Con A readily inhibited both insulin–Sepharose and Con A–Sepharose bindings, no effect of insulin on Con A–Sepharose binding could be seen (Table V). Inasmuch as there exists only about 5×10^4 insulin receptors per adipocyte (Gammeltoft & Gliemann, 1973), as compared to about 4×10^8 total Con A binding sites per fat cell (Cuatrecasas, 1973b), any effect of free insulin on the putative binding of Con A–Sepharose to insulin receptors would be expected to be negligible compared to the total number of Con A–Sepharose binding sites.

Insulin-like Activities of Various Lectins. In Table VI are shown the biological activities of a variety of different lectins relative to those activities of insulin. Interestingly, a close correlation is observed between the reported abilities of these lectins to retain solubilized insulin receptors on lectin affinity columns and the insulin-like bioactivities of the lectins. It can be seen that all six lectins that were recently reported by Hedo et al. (1980, 1981) to bind to (retain) insulin receptors on lectin affinity columns are also capable of expressing insulin-like bioactivities to virtually the same degrees as insulin. All of the six lectins that reportedly did not bind the receptors lacked any significant insulin-like activities.

Discussion

These studies provide strong support for the concept that the many insulin-like activities of the plant lectin and cell membrane probe concanavalin A are mediated by the same

membrane effector as that responsible for insulin's actions. The results also lend themselves to the view that the insulin receptor is closely linked to the membrane mechanism involved in Con A's actions.

Con A is shown to stimulate glucose oxidation, lipogenesis, and 3-*O*-methylglucose uptake as well as to inhibit epinephrine-, ACTH-, theophylline-, and cholera toxin stimulated lipolysis in isolated fat cells in ways virtually indistinguishable from those of the hormone. The observed effects of combining Con A and insulin and Con A and the anti-receptor antibody in mixing experiments are also consistent with a common effector. Of importance in regard to a common mechanism is the finding that the ratios of Con A's to insulin's half-maximal effective concentrations required to stimulate glucose oxidation, lipogenesis, and lipolysis are essentially constant (about 7×10^2; compare Figures 1, 3, and 5). That the ratio required to stimulate 3-*O*-methylglucose uptake is not identical with the common ratio for the other metabolic processes measured is not surprising, in view of the significantly different cell concentrations employed for the uptake studies. It was previously shown (Czech et al., 1974; Czech, 1976), and confirmed in the present study, that unusually high concentration of insulin are required to stimulate 3-*O*-methylglucose uptake into fat cells. This is likely due to a need to compensate for the high rate of insulin degradation encountered at the high cell concentrations employed in this assay (compare with cell concentrations used for assays in Figures 1, 3, 4, and 5). The observed decreased Con A/insulin ratio found for 3-*O*-methylglucose uptake, therefore, could reflect a greater rate of degradation of insulin relative to that of Con A.

Con A is also shown to act like insulin in vivo by stimulating muscle glycogen synthesis and epididymal adipose tissue lipogenesis when injected intraperitoneally. The effect of Con A on glycogen synthesis is consistent with the previous demonstration of Con A stimulated glyogen synthetase in vitro (Lawrence & Larner, 1978).

Czech et al. (1974) had previously reported virtually identical dose–response curves for the inhibitory effects of neuraminidase treatment of fat cells on the action of Con A compared to insulin on glucose oxidation. In the present study, the similar effects and potencies of a variety of inhibitors of cell membrane associated components on the biological actions of Con A compared to insulin-stimulated cells add further support for a common effector for the actions of the lectin and hormone. The finding that cytochalasin B [a potent inhibitor of carrier-mediated glucose transport that can also disrupt microfilaments (Kletzien et al., 1972; Czech et al., 1973; Cushman & Wardzala, 1980; Aldrich Chemical Co., 1975; Loton & Jeanrenaud, 1974)], but not cytochalasin D [a microfilament-disrupting agent that has little affect on glucose transport (Aldrich Chemical Co., 1975; McDaniel et al., 1975)], inhibits both Con A and insulin-stimulated glucose oxidations suggests that microfilaments play no important role in the activities of Con A or insulin. Kahn et al. (1978) drew the same conclusion for insulin's action from a similar study of the effects of cytochalasins A and D on the activities of the hormone only. It also supports the conclusion that Con A, like insulin, acts by stimulating a specific carrier-mediated glucose-transport system, rather than by enhancing diffusion-mediated uptake.

That Con A stimulates the oxidations of [1-^{14}C]–, [6-^{14}C]-, and [U-^{14}C]glucose to the same extents, respectively, as does insulin, and the lectin- and hormone-stimulated oxidations respond equally to cytochalasin B, emphasizes the similarities in action between Con A and insulin during the processes of hexose transport and metabolism. The effects of other glucose-transport inhibitors (see Table IV) add additional support for these similarities. It is important to note that while the percent stimulatory activities (i.e., the stimulated/basal activity ratio) of both Con A and insulin remained essentially unaffected by concentrations of cytochalasin B that only partially inhibited glucose uptake, very high cytochalasin B concentrations (which presumably blocked all transporters) resulted in complete inhibition of both Con A and insulin stimulatory activities. Thus, the presence of at least some functional glucose carriers is absolutely required for both Con A's and insulin's stimulatory actions on glucose utilization.

Inasmuch as neither colchicine, colcemid, nor vinblastine had any observable effect on either Con A's or insulin's activities, microtubules would not appear to be involved in the actions of either the lectin or the hromone. Surprisingly, nocodazole, a more recently discovered microtubule inhibitor (Hoebeke et al., 1976; Atassi et al., 1975; DeBrabander et al., 1976), was a potent inhibitor of Con A and insulin-stimulated glucose oxidations. Further studies will be required to determine its mode of action.

Succinyl-Con A, a divalent derivative of he tetravalent Con A tetramer (Gunther et al., 1973), was found to be one-tenth as potent as Con A. This significant degree of retention in activity as well as the retention of the ability to maximally stimulate glucose oxidation is interesting in view of succinyl-Con A's extensive loss in abilities to agglutinate sheep red blood cells and virtual complete loss in ability to induce cap formation on mouse spleen cells (Gunther et al., 1973). This stresses the importance of binding to a cell-surface saccharide as a primary requirement for its insulin-like activities. Although its significance, if any, is unclear, it is also interesting how well succinyl-Con A's insulin-like potency correlates with its retention of about 10% of the native lectin's ability to agglutinate mouse spleen lymphocytes (Hadden et al., 1976). In view of the reduced valency of succinyl-Con A and its corresponding reduced ("moderate") ability to cross-link surface receptors (Gunther et al., 1973), receptor cross-linking could play an important role in Con A's insulin-like actions. From studies with the monovalent Fab fragment of the anti-insulin antibody, which Kahn et al. (1978) have shown is devoid of insulin-like activity despite being able to effectively inhibit insulin binding, the latter workers concluded that receptor cross-linking or aggregation is also important in insulin's action. Alternatively, the reduced potency of succinyl-Con A may be due to a chemical modification, rather than valency.

Perhaps the most provocative correlation observed in the present study is the apparent relationship between the reported abilities of Con A and a variety of other lectins to bind solubilized insulin receptors on lectin affinity columns (Hedo et al., 1980, 1981) and the insulin-like bioactivities of these lectins (Table VI). The previous report (Hedo et al., 1980, 1981) also noted the ability of sugars to elute the receptor from the affinity columns. Such a correlation between lectin binding to the insulin receptor and biological activity suggests that the insulin receptor may represent the primary site of action of these lectins. Further studies will be required to extend this correlation and characterize the nature of the binding specificity. However, even if lectin binding proved to result in bioactivity, it would not necessarily follow that such binding should block insulin binding (see below).

Although we can confirm earlier findings of the ability of Con A to inhibit specific insulin binding to the intact cell (Cuatrecasas & Tell, 1973), the mechanism of this inhibition is unclear. In the present studies, we were only able to observe

inhibition of [^{125}I]insulin binding if the cells were treated with Con A for at least 30 min prior to incubation of the cells with [^{125}I]insulin. Even then we could observe no greater than about 75% inhibition. DeMeyts et al. (1974, 1976) reported that rather than being able to inhibit insulin binding to intact monocytes, Con A was only capable of linearizing the curvilinear insulin-binding Scatchard plots. We recently speculated, from studies with some Con A inhibitors that antagonize and act like insulin, that the lectin may inhibit insulin binding by interacting with a membrane carbohydrate that might not necessarily be structural part of the insulin receptor (Katzen, 1979). Maturo & Hollenberg (1978) had reported the existence of a nonreceptor glycoprotein capable of interacting with the insulin receptor and forming a higher molecular weight insulin-binding complex with altered binding properties. The possibility is thereby raised that the observed inhibitory effects on insulin binding could be due to a glycoconjugate distinct from the insulin receptor.

Cuatrecasas & Tell (1973) earlier presented evidence suggesting that high concentrations of Con A could partially inhibit [^{125}I]insulin binding to detergent-solubilized insulin receptors from liver membranes [according to the poly(ethylene glycol) precipitation assay]. This was taken to mean that Con A might exert its insulin-like effects by interacting directly with the insulin binding site. Subsequent studies in our laboratory (Katzen et al., 1981) have shown that filtration of Con A treated solubilized fat cell membrane insulin receptors (prior to incubation with the labeled hormone) is required to observe complete losses in the soluble bound insulin fraction. The latter studies demonstrated that treatment of solubilized receptors with Con A results in an insoluble lectin–receptor complex that can be removed by filtration or centrifugation. Moreover, it was shown that Con A and insulin can cobind to the solubilized insulin receptor without each ligand inhibiting the other (Katzen et al., 1981). Therefore, the observed decreases in bound insulin were likely due to precipitation of Con A occupied receptors from solution rather than to any inhibition of insulin binding. Therefore, the observed inhibition by Con A of insulin binding to *intact cells* may not be due to direct interactions of the lectin with insulin receptors.

In contrast to the results described above, Con A was able to inhibit completely, as well as to reverse, the binding of cells to insulin–agarose beads in the buoyant density assay. The unique experimental situation of measuring the binding of immobilized insulin to fat cells in this assay may require an interpretation quite different from that used to explain the results from the use of free insulin molecules in solution. Thus, effects on cell-surface receptors (e.g., from cross-linking, redistribution, or aggregation of receptors) that may not be detectable when measuring the binding of free insulin may be readily demonstrated when the hormone molecules immobilized to beads and bound to floating cells are determined.

The weight of the present accumulated evidence provides strong support for the notion, but admittedly does not yet prove, that the effector system responsible for the insulin-like activities of Con A is the same as that for insulin. The results do not allow for distinguishing between whether or not insulin receptors function as mediators in triggering the effector system for the lectin-stimulated responses. Further studies will be required to help answer this question. In either case, however, the results suggest a close linkage between the effector(s) and the insulin receptor. Further support for a common effector or mediator system was recently provided by the demonstration of Seals & Jarett (1980) that Con A as well as the antibody to the insulin receptor completely mimic insulin's action in generating, from fat cell plasma membrane preparations, a second messenger-like factor capable of stimulating mitochondrial pyruvate dehydrogenase. A similar insulin-generated substance has been chromatographically fractionated from extracts of insulin-treated rat skeletal muscle and shown to inhibit a cyclic AMP dependent protein kinase and stimulate glycogen synthase phosphatase (Larner et al., 1979) as well as to stimulate adipocyte pyruvate dehydrogenase (Jarett & Seals, 1979). In view of the dissociation of insulin stimulation of glucose transport from glycogen synthesis (Larner, 1972) and the finding of two separate biochemical mechanisms for the activation of glycogen synthase in the absence and in the presence of transportable hexose (Lawrence & Larner, 1978; Oron et al., 1979), it is likely that the insulin- and Con A generated substance affects the intracellular processes without affecting hexose transport. That Con A so closely mimicks insulin action on both processes further supports the view that a close linkage exists between Con A's effector system and the insulin receptor.

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Mechanisms of Hydrolysis of Adenosine 5′-Triphosphate, Adenosine 5′-Diphosphate, and Inorganic Pyrophosphate in Aqueous Perchloric Acid†

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ABSTRACT: The acid-catalyzed hydrolysis of adenosine 5′-triphosphate (ATP) has been found to give rise both to adenosine 5′-diphosphate (ADP) and inorganic phosphate and to adenosine 5′-phosphate (AMP) and inorganic pyrophosphate. Kinetic and isotope studies on the mechanism of hydrolysis of ATP therefore depend on a knowledge of the mechanism of hydrolysis of the polyphosphate products, ADP and inorganic pyrophosphate. The latter reactions have been studied over the acidity range 1–5 M perchloric acid at 25 °C while the more complex problem of the hydrolysis of ATP has been followed at a single acidity (3 M perchloric acid). The positions of bond fission have been determined for both ATP and ADP.

It has been known for a long time that monoesters of phosphoric acid are extremely resistant to nonenzymic hydrolysis. This is, of course, consistent with their common function as intermediary metabolites. The monoesters of the polycondensed phosphoric acids, although still relatively stable chemically, are more labile and undergo hydrolysis under conditions where the products (the parent monoesters of phosphoric acid) are further hydrolyzed only very slowly. For example, it has been shown that ADP,[1] ATP, and adenosine 5′-tetraphosphate are hydrolyzed rapidly in 1 M sulfuric at 100 °C whereas the product, AMP, is not (Liebecq, 1957).

From about 1950 onward determined efforts were made by a number of groups to establish the mechanisms involved in

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[1] Abbreviations used: AMP, adenosine 5′-phosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; P_i and PP_i, inorganic phosphate and pyrophosphate.

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