4-1986

Yeast Mannans: Use as a Transport Vehicle for Peptides

Jacqueline Cesario Lee

YEAST MANNANS: USE AS A TRANSPORT VEHICLE FOR PEPTIDES

by

Jacqueline Cesario Lee

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Biology and Biomedical Sciences

Western Michigan University
Kalamazoo, Michigan
April 1986

YEAST MANNANS: USE AS A TRANSPORT VEHICLE FOR PEPTIDES

Jacqueline Cesario Lee, M.S.

Western Michigan University, 1986

Yeast mannans are phosphorylated glycopeptides found in various species of yeast. Their similarity to the mannose-phosphate signal for intracellular lysosomal uptake led mannans to be considered as a possible vehicle for increasing cellular affinity for peptides. Insulin was used as a model peptide and was conjugated to mannans by two coupling methods. The conjugated mannan-insulins were compared to insulin on the basis of cellular binding and ability to induce typical insulin biological effects.

ACKNOWLEDGEMENTS

Working on this thesis project has been one of the most enjoyable aspects of my time at Western Michigan University. I am grateful to both Dr. Darwin Buthala and Dr. Leonard Beuving for serving on my committee and for lending their expertise to the project. Special thanks to my major advisor, Dr. Leonard Ginsberg, who has been a source of enthusiasm, scientific knowledge, and infinite patience. How to maintain a good sense of humor through it all is something Dr. Ginsberg is certainly qualified to teach.

I would also like to thank Beatrice Wyse, Jerry Colca, and Barbara Gilchrist of The Upjohn Company for their help with the radioimmunoassay and adipocyte assays. All three were always willing to lend assistance and answer questions.

Thanks to my colleague, James Monticello, for preparing the PDP-conjugate and keeping the things around the lab from ever being dull.

Most of all, I would like to acknowledge the support from my husband, John, who now appreciates how much work is involved in completing a master's degree. His helpfulness, understanding, and Chinese cooking were instrumental in getting me through the whole ordeal.

This work is also dedicated to the memory of my father, who instilled in us the value of an education and the love of learning. Special thanks to my mother, who was always there when I needed her.

Jacqueline Cesario Lee

Lee, Jacqueline Cesario

YEAST MANNANS: USE AS A TRANSPORT VEHICLE FOR PEPTIDES

Western Michigan University M.S. 1986

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages _____
2. Colored illustrations, paper or print _____
3. Photographs with dark background ✓
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages ✓
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Dissertation contains pages with print at a slant, filmed as received _____
16. Other _____

University Microfilms International

TABLE OF CONTENTS

Table of Contents--Continued

LIST OF TABLES

INTRODUCTION

Since the turn of the century when Paul Ehrlich originated the concept of using chemical modification of antibiotics as a means to sharpen the aim at bacterial targets, the search for "magic bullets" to improve delivery of drugs to specific tissue and cellular sites has been an area of vigorous and imaginative research (Ehrlich, 1908). The aim of current research is to optimize therapeutic doses for a variety of drugs, primarily by directing the drug to an identifiable target in the body (site specific delivery) or away from sites where it may produce toxic effects without necessarily increasing the drug at the target site (site avoidance delivery) (Mufson, 1985). The target site can be broad (organ targeting) to encompass individual organs and tissues, or it can be directed to particular cells within the tissue (cellular targeting), even reaching organelles or compartments within the cell (subcellular targeting).

Strategies for delivering the drug to target sites can be divided into three areas: anatomical, physical, and chemical. A good carrier must be able to provide protection for its drug cargo during transit, as well as allow controlled transfer of the drug once the target is reached. The carrier should be biodegradable, nonimmunogenic, nontoxic to the system, and as nearly chemically inert as possible.

The physical approach involves changes in the microenvironment. One interesting concept is the use of magnetism to direct drug transport. The carrier in this case consists of microspheres composed of albumin

which are magnetically responsive, ranging in size from 0.2 - 1.5 um. The microspheres are injected into an artery supplying the target area, over which a magnetic field has been placed. The drug-containing microspheres are then concentrated in the target area and localized at the capillary level, serving as a natural timed-release system. This system was effective in an experimental rat tumor model and shows promise for treatment of tumors and chronic inflammatory conditions like arthritis (Widder, 1985).

Strategies taking advantage of natural screening systems in the body usually rely on size of the carrier, vascularity of the area, and the interaction with the reticuloendothelial system (RES) to direct the drug to the desired organ. Particles less than 0.1 um spread throughout the vasculature, while those between 0.1 and 1.0 um are taken up by the RES (macrophages in the liver, spleen, bone marrow, and monocytes in the blood). The route of injection does not seem to influence the efficacy of the RES in clearing the particles from circulation. Typically, 80 - 95% of the particles introduced end up in the RES. Larger particles, in the range greater than 3.0 um, tend to lodge in the lungs. Carrier systems which have been used in this context include liposomes, microspheres, and red blood cells.

Liposomes are vesicles composed of phospholipids and may be single-layered (unilamellar) or multi-layered (multilamellar). The ability of the liposome to encapsulate water-soluble drugs while maintaining a compatible nature with the plasma membrane make them an attractive vehicle. To expand targeting potential beyond size-screening, liposomes have been coupled to various molecules including antibodies and glycolipids

(Mufson, 1985). Disadvantages with liposome systems include poor drug capture, leakage during transport, stability problems with storage, and difficulty in controlling particle size (Poste & Kirsh, 1983). The problems with leakage of aqueous contents of liposomes may be ameliorated by the production of a new type of liposome (stable plurilamellar vesicle) composed of the same phospholipids as multilamellar vehicles, but having a more homogeneous distribution of aqueous compartments and more evenly distributed osmotic pressure (Gruner, Lenk, Janoff, & Ostro, 1984).

Another novel drug transport method is the use of red blood cells. Erythrocytes immersed in a drug solution tend to absorb some of the drug. If the cells are returned to the body, some of the drug is slowly released. Surface modification with glutaraldehyde or sialidase will encourage the RBC's to lodge in the spleen, while extensive modification tends to direct the cells to the liver. This system has two major disadvantages: the organ specificity is limited and the preparation of RBC's from each individual patient requires withdrawal of the blood, encapsulation of the drug, and reinjection of the treated cells back into the body (Sanders, 1985).

The anatomical aspect of targeted drug strategies has also included the search for new sites for drug administration, in addition to the more traditional intravenous, intrarterial, intraperitoneal, and oral routes. The skin and mucous membranes have both proved effective means of introducing drugs into the body. Transdermal systems are useful with potent drugs which often are accompanied by undesirable side effects like nitroglycerin and the anti-motion-sickness agent scopolamine. These systems are designed to deliver their contents into the general circulation

and are not targeted to a particular organ. The skin serves as the primary determinant of diffusion rate, depending on both skin permeability and the type of complex in which the drug is contained. Transdermal systems are divided into two types: the monolith, a matrix containing a semisolid solution of the drug and relying solely on the diffusion rate of the skin; and the membrane, which incorporates the matrix with a permeability membrane having an upper rate limit about twice the normal permeability of the skin. Both systems are applied as patches (wearing time depends on the type of system) and may cause local skin irritation and hypersensitization, especially when the patient is re-exposed to the drug by another route. Transdermals have the advantage of bypassing most first-pass hepatic metabolism, as well as a high degree of user compliance. Female reproductive hormones, cardiovascular drugs, analgesics, and antihistamines are all being considered as possible candidates for transdermal administration (Good, 1985).

The other promising site for nontraditional delivery is the nasal mucous membrane. Transnasal administration of drugs has been used since ancient times, as in the inhalation of psychotrophic hallucinogens by South American Indians. More recent exploration of the nasal route has included cardiovascular drugs (propranolol, nitroglycerin), antimicrobials (especially those poorly absorbed orally), and a number of hormones. Nasal administration may be a good alternative for lipophilic drugs like propranolol, which are easily absorbed. It also has the benefit of avoiding hepatic first-pass metabolism, while producing blood levels similar to those obtained with intravenous administration in some studies. Compliance levels are good, especially when multiple

daily dosing is required. Nasal conditions (nasal obstruction due to polyps, atrophic rhinitis, excessive secretion from irritants, nasal allergies) may affect delivery. The administration of the rapidly absorbed lipophilic hormones are not adversely affected by colds or rhinitis. Peptides, however, are poorly absorbed and require adjuvants like bile salts (Chien, 1985).

Chemical strategies of site specific delivery all involve the attachment to a drug or carrier surface of some ligand which will direct the drug to the desired target. Candidates for the ligand include monoclonal antibodies, carbohydrates specific for cell surface receptors, toxinbinding subunits, hormones, and vitamins. Monoclonal antibodies have been studied as ligands for liposomes. In one conjugation system, the attachment to the liposome is made through an activated sulfhydryl reagent binding covalently to a sulfhydryl group on the antibody. The high specificity of the cell surface antigen to the liposome antibody system makes it an attractive targeting device, but may be offset by the small number of antigens on the surface of the cell. Monoclonal antibodies have been suggested as ideal carriers for tumor targeting and have been used successfully to locate the tumor site when the antibody is attached to radioactive compounds that allow tumor imaging (Widder, 1985). Establishing markers on tumor cells and the shedding of tumor antigens into the circulation are obstacles to the implementation of monoclonals as a site-specific system. In the mouse tumor model, sequestering of the delivery system by the RES was also a problem, but was reduced by pretreatment with non-drug containing liposomes prior to injection of drug carrying vesicles (Mufson, 1985).

Plant and microbial toxins are known to bind to specific receptors on the surface of cells. These toxins are typically composed of two subunits, one responsible for toxicity while the other is essential for cell surface binding. The binding subunits of the diphtheria toxin and the castor bean toxin have been explored as possible targeting agents. Glycolipids (lactosylcerebroside and dimannosyldiglyceride) were used successfully in vivo to direct liposomes to the liver in mice (Szoka and Mayhew, 1983). The success of these in vivo drug targeting ligands may lead to their use in the future treatment of human disease.

Peptides such as oxytocin, enkephalin, and insulin have been increasingly important as a class of pharmaceutical agents. Although the lipophilic peptides are readily absorbed, nonlipophilic, large peptides like insulin require the use of an adjuvant to gain entry into cells. When administered orally, the peptide is subjected to degradation by proteolytic enzymes. Several routes have been explored to deliver insulin into the body, including the rectum, vagina, lung epithelium, and liposome therapy, but all have met with variable success. Nasal delivery of insulin was found to be more effective than subcutaneous injection of insulin in human studies, however, the surfactants used to gain transnasal entry (usually sodium glycolate) produces irritation (Pontiroli, 1982).

The aim of this study is to explore the phosphorylated carbohydrate as a carrier for the peptides. Insulin was chosen as a model peptide because it is easily obtainable, well characterized, and a number of biological parameters of its activity have been described. The plasma membrane provides a highly impermeable barrier to entry of charged

molecules or large, uncharged polar molecules into the cell. This barrier can be breached by specialized transport proteins which allow passage through the membrane (Wilson, 1978). Large molecules like proteins, polysaccharides, and polynucleotides are unable to use transport proteins as a route into the cell. These macromolecules enter the cell primarily by endocytosis, either as the constitutive process of fluid-phase endocytosis which allows generalized entry or through receptor-mediated endocytosis of specific types of macromolecules (Silverstein, 1977). Elucidation of the process of receptormediated endocytosis resulted from the work of Goldstein and associates on the uptake of cholesterol by animal cells and the subsequent discovery of the LDL receptor and its intracellular fate, for which they were awarded a Nobel prize in 1985 (Goldstein, Anderson, & Brown, 1979). They found that cholesterol binds to a specific extracellular receptor on the plasma membrane after which the complex is collected into coated pits which are internalized as coated vesicles and routed to lysosomes, where the complex is separated into ligand and receptor. The receptor is then free to be recycled to the plasma membrane. The transport of macromolecules through coated pits has been found to include polypeptides such as insulin, lectins, and toxins from plant and bacterial sources (Sharon & Lis, 1972). Although the pathways taken by these polypeptides once inside the cell are not fully understood, most appear to involve transport to lysosomes or endosomes as an initial step in intracellular processing (Marnell, Shie, Stookey, & Draper, 1984; Bergeron, Cruz, Kahn, & Posner, 1985).

Natowicz, studying enzymes produced intracellularly but residing

primarily in lysosomes after dispersal from the Golgi apparatus, found that molecules carrying a high content of phosphorylated mannose were preferentially taken up by lysosomes (Natowicz, Chi, Lowry, & Sly, 1979). This implies that lysosomal enzymes which are exocytosed may return to the cytosol through mannose-phosphate specific receptors on the plasma membrane, which aggregate into coated vesicles and are endocytosed. Mannose-6-phosphate was identified as the high lysosomal uptake signal on fibroblast lysosomal hydrolases (Von Figura & Klein, 1979). Kaplan and others, also using human fibroblasts noted that the form of lysosomal enzymes that are rapidly internalized ("high uptake form") are more acidic that forms which are internalized more slowly. He proved that the acidic group responsible for the high uptake form was the phosphate group associated with D-mannose (Kaplan, Achord, & Sly, 1977). He later used the human spleen enzyme B-glucuronidase to identify the specific phosphohexose related to high lysosomal uptake as mannose-6-phosphate. Mannose-6-phosphate (or its analogues) are recognized by receptors on the plasma membrane, initiating the receptor-mediated endocytosis of the enzyme carrying the signal (Natowicz et al., 1979).

The idea of attaching the carbohydrate recognition signal to polypeptides which did not normally carry it was explored by Lee, who found these peptides could bind to the receptor and gain entry to the cell through the mannose-6-phosphate moiety (Lee & Stowell, 1978). The castor bean toxin, ricin, has also been used to produce chimeric forms with mannose-phosphate. Ricin consists of two subunits: the enzymatically active A chain, which affects the function of Elongation Factor 2 of

protein synthesis, but is incapable of entering the cell and the binding subunit (B chain) which is not toxic alone, but binds to the plasma membrane receptor allowing adsorptive endocytosis of itself and the associated A chain (Gilliland, 1978). Youle and associates were also successful in attaching the enzymatic portion of the ricin toxin to mannose-phosphate and delivering the ricin A chain intracellularly through the mannose phosphate receptor of the lysosome. This was proved by the exhibition of the toxic effects of the ricin on protein synthesis (Youle, Murray, & Neville, 1979).

The successful use of mannose-phosphate as a vehicle for intracellular transport of toxins suggested further experimentation on the feasibility of mannose-phosphate as a carrier for other polypeptides into cells which do not have specific receptors for the polypeptide or for gaining entry by receptor-mediated endocytosis for peptides which do not normally gain entry by this route.

In the present study, a mannose-phosphate obtained from yeast is employed as a carrier system for insulin. Phosphorylated oligosaccharides have been obtained from various genera of yeast including Candida, Hansenula, Saccharomyces, and Kloeckera. (Kocourek & Ballou, 1969; Thieme & Ballou, 1971) Polymannose isolated from fungal sources is usually associated with proteins and is known by the generic term "yeast mannan". These mannans vary both in mannose content and amount of protein attached to the polysaccharide chain. The main unifying characteristic of mannans is the high D-mannose content, in which they are unique as a group of naturally occuring products. In his excellent review on yeast mannans, Ballou (1979) ascribes the diversity in contri-

bution of protein to the mannan both to genus of yeast from which it is isolated and to the cellular location of the mannan. Yeast mannans are covalently linked polymannose-protein complexes, found coating the cell and interspersed with the cell wall. They can also be found in the periplasmic space associated with enzymes. The protein content varies from 5% in the "structural" cell wall associated mannan to 30-50% in the enzyme-linked periplasmic types of mannans. Some mannans line the exposed cell wall and probably function as sexual agglutination factors. The polymannose portion of the mannan is usually in the form of a long chain of D-mannose in the alpha(1-6) linkage, extending up to 150 mannose units. From the backbone chain, side chains linked-alpha(1-2) or 1-3) or mono- to pentamannosides are found. The mannan isolated from Saccharomyces cerevisiae has a backbone chain of 150 residues and side chains of up to four mannosides, with more that half of the mannose present as side chains. Glycopeptides from the mannans are characterized by an unusually high content of serine and threonine, up to 45%, but otherwise are not distinctive in amino acid composition. The peptides are primarily linked to the mannoside through asparagine via N-acetylD-glucosamine, although some oligosaccharides are linked through serine or threonine. The total complex varies in molecular weight from 25,000 to 250,000 daltons (determined by gel filtration).

The mannans used in this study were obtained commercially and treated with a protease to reduce the native peptide. They were covalently bound to insulin in hopes of producing a carrier system allowing entry of the insulin through the mannose phosphate receptor on the plasma membrane. Rat adipocytes were used as an in vitro system to test the

efficacy of the transport and the degree of retention of biological activity of insulin entering the cell through this pathway.

MATERIALS AND METHODS

The yeast mannans used in this study were obtained from Sigma Chemical Co., St. Louis, Missouri (from Saccharomyces cerevisiae, lot number 33F-3878) and were subjected to analysis for carbohydrate, protein, and phosphate content, as well as gel filtration to estimate molecular weight. All chemicals used in this study were obtained from Sigma unless otherwise indicated. All radioactive compounds were from New England Nuclear, Boston, Ma.

Carbohydrate analysis was done according to the Phenol-Sulfuric Acid method of DuBois. Samples were read at 487 nm and compared to a standard curve of mannose (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956).

Protein content was determined by the method of Lowry using crystalline Bovine Serum Albumin standards (Lowry, Rosebrough, Farr, & Randall, 1951).

Phosphate content was measured by the method of Gatt, in which hydrolyzed and unhydrolyzed mannan were compared (Gatt & Borman, 1966). The phosphate content of unhydrolyzed mannans were subtracted from the hydrolyzed to give the bound phosphate content.

To estimate molecular weight, mannans were applied to a Sephadex G100-40 column (47 cm x 1.9 cm) having a bed volume of 100 ml in 0.02 M Tris buffer, pH 7.4. The elution of the mannan (25 mg/ 200ul) was compared to standards of Dextran Blue (ca. 2,000,000 MW), pepsin (34,700

MW), albumin (66,000 MW), lysosome (14,300 MW), and Potassium permanganate (75 MW) to obtain an estimate of the molecular weight. The column was run at a rate of 5 ml / hour and collected at 12 ml/ tube. The yeast mannans elution peak was located by absorbance at 280 nm and by carbohydrate analysis.

The ability of three proteases (papain, pepsin, and chymotrypsin) to reduce the protein content of the mannan was compared by Lowry analysis. Subsequently, agarose-bound chymotrypsin was chosen as the best enzyme for this purpose. Chymotrypsin (100 mcg) was incubated overnight with shaking at room temperature with 10 mg of native mannan in 1 ml of 10mM Tris buffer with 0.1 M $CaCl_2$, pH 7.8. The enzyme was removed by centrifugation and the mannan was concentrated by precipitation with 50% ethanol.

Coupling Methods

Two methods of coupling insulin to the mannans were investigated. The cyanogen-bromide method was followed according to Hitchcock-DeGregori with some modifications (Hitchcock-DeGregori & Yienger, 1984). Under a hood, one milligram of cyanogen bromide in 1 ml of acetonitrile was allowed to react at 20° with 10 mg of chymotrypsin-treated yeast mannan. The pH was maintained between 10-11 for approximately 20 minutes or until the pH stabilized by addition of small amounts of 6N NaOH. (Cyanogen bromide should be used with caution since it can produce an extremely toxic cyanide gas.) After the pH of the reaction mixture stabilized, the solution was dialyzed against ice cold water for 1 hour and against cold acetate buffer for another hour. The insulin

was added in excess (1 mg with 20% as Fluorescein isothiocyanate-labeled insulin) to the mannans in Sodium Bicarbonate coupling buffer (0.2M) and incubated overnight with shaking in the cold. The mannan-insulin was concentrated by ethanol precipitation, and washed twice with coupling buffer. The precipitated mannan-insulin was resuspended in 1M ethanolamine buffer pH 8.0 overnight to block unreacted groups. The complex was precipitated in 50% ethanol, resuspended in coupling buffer and vortexed, then precipitated again with 50% ethanol, followed by resuspension and precipitation with ethanol, alternating between coupling buffer and acetate buffer (three times each), finally resuspended in 10mM Tris-HCL (pH 7.5) buffer for storage.

A disc gel electrophoresis (PAGE-SDS) was prepared according to the instructions of LKB Bromma (LKB Application note 320), using a 7.5 % acrylamide in 0.375 M Tris-Glycine (pH 8.3) and run at 4 mA/ tube for 90 minutes. Both ethanol precipitated and non-precipitated mannan-insulin were compared with FITC-insulin and with native yeast mannan.

The SPDP-conjugated mannan-insulin was prepared by James Monticello (Biomedical Sciences Dept., Western Michigan University) by crosslinking the lysine 29 of insulin (B chain) with the heterobifunctional agent Succinimidyl-3 (2-pyridyldithio) Proprionate (SPDP). The alpha amino groups of the insulin were first blocked with citraconic anhydride which was removed after SPDP treatment by reducing the pH. The PDP-insulin was then exposed to yeast mannan previously treated to expose sulfhydryl groups and allowed to react. Extensive dialysis followed to help remove free insulin from the coupled mannan-insulin (Roth, Maddux, Wong, Iwamoto, & Goldfine, 1981).

Radioimmunoassay

The amount of insulin bound to the mannans in each method was quantitated by radioimmunoassay using the cellulose powder method of Zaharko (Zaharko & Beck, 1968). The anti-insulin antibody was supplied by the Upjohn Company as was use of the Tracor Analytic Gamma Trac (Model 1191) gamma counter. Briefly, dilutions of the mannan-insulin were allowed to react with anti-insulin antibody (200ul of 80,000 dilution) for 2 hours at room temperature in the presence of 10uU of 125-Iodinated Insulin. After incubation, the antibody-bound mannan-insulin was separated from the unbound mannan-insulin by mixing with an excess of cellulose (to which unbound insulin becomes attached.) The cellulose-bound mannan-insulin was then separated by centrifugation for 4.5 minutes at 3200 rpm, after which the supernatant was decanted and the cellulose pellet counted on a gamma counter. To quantitate the insulin present, a standard curve of known amounts of insulin (1uU to 512uU) was run concurrently. These values were compared using the Rodbard Statistical Program for radioimmunoassay (Rodbard & Hutt, 1974).

Adipocyte Preparation

Rat adipocytes were isolated by a modification of Rodbell's method (Rodbell, 1964). Only plastic or siliconized glassware should be used to avoid disrupting the fat cells. Adult male Sprague Dawley rats were used. Adipocytes were liberated from stromal cells and basement membrane with Type II collagenase. Silicone oil (bis 3,5,5,trimethylhexyester) was obtained from AG Fluka (Hauppauge, NY). Two types of

population were used: large adult male rats (weight averaging 400 grams) and young adult male rats (weight averaging 120 - 150 grams). The rats were sacrificed by CO_2 suffocation and epidydimal fat pads were asceptically removed. These pads were suspended in Fat Cell Buffer (see Appendix) and treated with collagenase (2mg /ml) in a 37° C. water bath with occasional agitation. The total amount of collagenase varied with the number of rats being processed, typically using 6 mg. for 5 small rats. Treatment usually required about 45 minutes, the extent of digestion of collagen being estimated by clouding of the solution as fat cells were freed. The adipocytes were washed three times in warm buffer to remove the enzyme, centrifuged at 400 rpm for 2 minutes each time, and were then filtered through nylon hosiery to separate stromal cells from the fat cells. The cells were resuspended in buffer lacking albumin and counted on a haemocytometer. Approximately 300,000 to 500,000 cells are needed per tube in the assay. To insure a homogeneous suspension of fat cells during dispensing, enough buffer was added to make twice the volume needed for the entire assay. The cells were then aliquoted to 200-300ul / tube while being rapidly but gently stirred on a magnetic stirrer.

Insulin Binding Assays

Cells were incubated with fluorescein labeled native mannans for 45 minutes at 37° C. and in the cold. Another group was pretreated with unlabeled mannans for 20 minutes prior to incubation with the labeled mannans.

The ability of the isolated adipocytes to recognize and bind insulin was assessed by observing the binding of radio-labeled insulin over time. Large adult rats were used in this study. The ^{125} I-Insulin (25uU/tube) was added to aliquots of approximately 300,000 cells/tube. Incubation times were 30, 60, and 120 minutes at 37°C with occasional shaking. The cells were separated from the buffer by centrifugation with silicone oil (AG Fluka), which results in a layer of adipocytes floating on the oil layer over the buffer. After washing in fresh buffer, the cell layer was removed and counted in a gamma counter.

The adipocytes were used in a cell binding assay to compare the mannan-insulin conjugates with native insulin for competitive inhibition with ^{125} I-Insulin. Each assay tube contained 300,000-500,000 cells/tube and radiolabeled insulin (10uU/tube). Either native insulin (60uU) or mannan-conjugated insulin (cyanogen bromide conjugate= 25uU, PDP= 50uU) was added. In addition, the mannan-insulins were also combined with native mannans (200mcg/tube). A nonspecific binding tube with 100,000uU of unlabeled insulin was included. The assays were done in triplicate, unless otherwise noted. All tubes were incubated at 37° C for 45 minutes, then washed with fresh buffer. After separation with oil, the cells were counted on a gamma counter to detect ^{125} I-Insulin bound.

Glucose Uptake Assays

To determine whether the mannan-insulin retained the capacity to induce characteristic insulin biological effects, ^{14} C-glucose was used to measure uptake into the adipocytes. Radiolabeled glucose (0.1

uCi/tube) was added to approximately 500,000 cells/ tube, and either insulin (60uU or 120uU), mannan-insulin (25uU or 50uU), or no insulin was also added. Native mannans were added in high concentration (200 mcg) or low concentration (20ug) instead of insulin in some tubes. In an attempt to block cell mannan binding sites, the native mannans (200uU) were also added in combination with the both types of mannan-insulin conjugates. After incubation for 45 minutes at 37°C with occasional shaking, the cells were separated with oil as described above and counted in Scintisol on a Tracor Isopac beta counter.

Statistical Analysis

Statistical analysis was done using Student's T-test (onetailed) with p values at least $p = 0.01$ unless otherwise indicated.

RESULTS

Characterization of mannans from Saccharomyces cerevisiae

The mannans have a molecular weight of 60,000 daltons, were 50 % carbohydrate, 20 % protein, and 7 % phosphate by weight (Table 1). Ethanol precipitation proved to be an effective means of separating the mannan from aqueous solution. After treatment with a protease (chymotrypsin), precipitation of the mannan with an equal volume of ethanol (50% aqueous solution) removed most of the carbohydrate from the solution. However, 13.5 mcg of protein remained in solution while 12.8 mcg was precipitated with the carbohydrate. This implies the protein remaining in solution was removed from the glycoprotein by the protease.

Coupling the Mannan to Insulin

The Lowry method was used to compare the protein content before and after chymotrypsin treatment. The concentration of protein following insulin conjugation by the cyanogen bromide method was also analysed. To get an estimate of the carbohydrate to protein ratio, the native, proteasetreated, and conjugated mannans were subjected to carbohydrate analysis (Table 2).

The mannan-insulin was run on disc gel electrophoresis. Native mannan, Fluorescein isothiocyanate-labeled insulin, and Protein Standards (Sigma Dalton VI) were also run. Both ethanolprecipitated and nonprecipitated mannan-insulin were included. Appendix B shows the bands visible

after staining with Coomassie blue R250. The nonprecipitated mannan-insulin shows three bands, one which co-migrated the same distance as the FITC-Insulin. The ethanol-precipitated mannan-insulin shows only 1 band, nearer the migration of the native mannan band. This implies that ethanol precipitation removes free insulin from the mannan-insulin conjugate.

Radioimmunoassay

Radioimmunoassay showed the cyanogen-bromide mannan-insulin contained 5000uU/ml, while the PDP mannan-insulin contained 480uU/ml. The cyanogen bromide method conjugates about 10 times as much insulin as does the PDP method as previously described.

Adipocyte Preparation

Appendix C contains photographs of rat adipocytes following the digestion of stromal cells and collagen. The cells vary widely in size, but the younger (smaller) rats appear to have cells of a more homogeneous distribution than the older (more obese) rats. Adipocytes are primarily vacuolated with triglyceride storage vesicles, restricting the cytoplasm to the periphery of the cell. Figure 2 shows the fluorescent labeling acquired by cells incubated at 37° C. No binding of fluorescence was seen in the cells incubated in the cold or in cells pretreated with unlabeled mannans. This implies a saturable binding of the mannan to the cell surface receptors.

Competitive Inhibition With Insulin

Epididymal adipocytes showed an increase in binding of radioactive insulin over the 120 minute sampling period (Table 3). A sample was taken at 30 minute intervals for up to 120 minutes. The cells showed rapid uptake of the ^{125} I-Insulin. Competitive binding between the mannan-insulin and radiolabeled insulin for cell receptors was compared. Nonspecific binding was about 7 per cent. The results are given in Table 4. Each mean counts/minute is the result of at least three trials. Maximum binding was determined by exposing the cells to ^{125} I-Insulin alone. Fifty microunits of insulin or 60 uU of the mannan-insulin (PDP method) conjugate reduced the radioactive insulin bound by 55%, while half that amount of mannan-insulin (cyanogen bromide method) reduced the binding significantly by 68 per cent. Excess native mannans were added to the mannan-insulin conjugates in an attempt to bind the cellular phosphomannose sites and preclude access through the mannan portion of the glycopeptide (single trials only). An average reduction of inhibition in radioactive binding to 36 % was shown when mannans were added to the mannan-insulin conjugates (Table 4).

Glucose Uptake

Since the adipocytes were able to recognize and bind the mannan-associated insulin, the next assay examined the biological activity of the conjugates. This was measured by the increase in glucose uptake using ^{14}C-Glucose. There was a linear increase in glucose uptake with increasing insulin concentration. Fifty microunits of the cyanogen

bromide mannan-insulin conjugate gave similar results to the same concentration of native insulin (Table 5). Native mannan was also tested for its ability to affect glucose uptake. The lower concentration (which represents 10 times the quantity of insulin in ug present in 50 uU) had little effect, but the excess mannan (200 ug) had a statistically significant increase in uptake (Appendix D). The reduction of glucose uptake when excess mannan is competed with the mannan-insulin conjugate is statistically significant, as is the increase in the presence of mannan-insulin alone when compared to the glucose uptake when no native insulin was added.

The data from the same protocol with the PDP-mannan-insulin is shown in Table 6 and Appendix E. All means are calculated from at least three trials.

Table 1

Characterization of the Mannans

Protein content	20 %
Carbohydrate content	50 %
Phosphate content	7 %
Molecular weight	60,000 daltons

Table 2

Protein and Carbohydrate Content

Mannan	Protein	Carbohydrate
	ug/ml	ug/ml
YM-Chymotrypsin	107	10800
YM-Insulin (CnBr)	151	1640

Table 3

Binding of Radiolabeled Insulin to Rat Adipocytes

Time (minutes)	Counts per minute
0 (Background)	150
30	1235
60	2071
120	2327

Table 4

Competitive Inhibition of Noniodinated insulin and Mannan-Insulin Against ^{125}Insulin in Rat Adipocytes

Insulin Type	Mean Counts/Minute	Per Cent Inhibition
Radiolabeled insulin only	2682	0
Noniodinated insulin (60uU)	1217	55 *
Mannan-insulin Cyanogen Br. (25uU)	849	68 *
Mannan-insulin PDP (50uU)	1205	55
Mannan/Mannan insulin (CnBr) (25uU)	1790	34
Mannan/Mannan insulin (PDP) (50uU)	1639	39

* Statistically significant

Table 5

Adipocyte Uptake of ^{14}C-Glucose
in the Presence of Insulin and Mannan-Insulin (Cyanogen Bromide)

Insulin (amount)	Mean cpm	
No insulin	414	A
Native insulin (100uU)	746	B
Native insulin (50uU)	1271	
Mannan insulin (50uU)	1384	A,C
Mannan (200ug)/Mannan-insulin (50uU)	551	C,D
Mannan only (200ug)	1129	A,B,D,E
Mannan only (20ug)	671	C,E

Explanation of statistical significance designated by letters: the value at each letter is significantly different from other values with that same letter.

Table 6

Adipocyte Uptake of ^{14}C-Glucose (PDP)

Insulin (amount)	Mean cpm
No insulin	773
Insulin (50uU)	1202
Mannan-insulin (50uU)	1689
Mannan (200ug)/Mannan-Ins.(50uU)	263

DISCUSSION

Mannans are a heterogenous group of proteoglycans, unique in having phosphorylated mannose as their primary carbohydrate. The values obtained for carbohydrate and protein constituents and molecular weight are within the range for Saccharomyces cerevisiae as found by Ballou in his review of the structure and composition of yeast mannans (Ballou, 1974). However, Ballou found that the degree of measurable phosphorylation depended on the method of acetolysis used in preparing the mannans from yeast cells, since cell wall isolated mannan differ in phosphorylation levels from mannans isolated from whole cell homogenates. He found ratios of carbohydrate : phosphate varying from 50 to 150. The 7% found in this experiment would indicate a lower level of phosphorylation than in Ballou's review, but in another analysis of cell wall mannan chemotypes by Cawley, the possibility of two chemotypes for Saccharomyces cerevisiae is discussed. One is the highly phosphorylated mannotriose side chain containing mannan, while the other variety contains a tetra-mannose side chain and lower levels of phosphorylation (Cawley & Ballou, 1972). To determine if high and low phosphorylated forms could be found in our mannans, the mannan was separated by DEAE Sephadex (A-50-120) chromatography (in 0.025M Tris-HCL, pH 8.0, with a 0.05M - 0.5M NaCl gradient). A single peak was eluted, implying only one level of phosphorylation.

Prior to coupling insulin to the mannan, some of the native peptide was cleaved by treatment with the protease chymotrypsin, resulting in

about a fifty per cent reduction in resident protein on the mannan. This was done in an attempt to lower the antigenicity of the carrier and to reduce peptide content prior to the addition of insulin (MW 6000).

Two methods of coupling mannan to insulin were employed in order to create carriers with varying amounts of insulin. The cyanogen bromide method involves reaction at alkaline pH of the cyanogen bromide with hydroxyl groups on the carbohydrate, resulting in the production of cyanamides with the alpha amino groups of proteins (Axen, Porath, & Ernback, 1967). When used with a glycoprotein with a high carbohydrate content as in yeast mannan, multiple insulin binding sites should be available on the mannose. The SPDP method, however, is a peptide-to-peptide heterobifunctional crosslinker, and requires the presence of sulfhydryl groups on one of peptides. SPDP contains a hydroxysuccinimide ester group on one end which reacts with groups of peptides to form amide groups, and also contains a pyridylsulfide group capable of reacting with thiols to form disulfide bonds (Jou, Mazzaferro, Mayers, & Bankert, 1983). The protocol used to produce this PDP-insulin blocked the alpha amino groups of insulin, allowing binding to lysine residue 29 of the B chain (Roth et al., 1981). This reaction has the advantage of proceeding under conditions of neutral pH and may be less destructive to biological molecules than the alkaline pH required with cyanogen bromide coupling. Since the peptide component of the mannan provided only 10% of the glycopeptide, the supposition was that less insulin would be bound by this method. Radioimmunoassay results of comparable amounts of both mannan-insulin conjugates show the cyanogen bromide mannan has

incorporated about 10 times the amount of insulin as the PDP method. Validity of the radioimmunoassay for the mannan-insulins compared to native insulin was difficult to determine. Since it was not possible to establish concentrations of insulin in the mannan-insulin directly, binding curves for the conjugates could not be made. The amount of insulin bound to the conjugate was found by Lowry analysis to be about 44mcg/ ml or 1100uU/ ml. The RIA method is considered to be ten times as sensitive for measurement in this range, so the RIA value for insulin in the mannan-insulin of 5000uU is reasonably close. Since the RIA is probably the more accurate value, all concentrations are expressed as measured by RIA. After conjugation of insulin and mannan was accomplished, the problem of removing unreacted insulin still in solution remained. Affinity chromatography with the lectins Concanavalin A and Lentil lectin was explored, as well as the production of an anti-insulin affinity column. The lectin columns preferentially bind the mannose residues, allowing free insulin to escape in the void volume. After elution of mannans (both insulin-bound and mannan not coupled to insulin) with methylmannoside, the mannans were applied to the anti-insulin column. Elution was accomplished by lowering the pH. The presence of mannan insulin was detected by carbohydrate and protein analysis (as previously described). Although this scheme did produce a good separation of free from bound insulin, only 2 mcg of a starting total of 10mg. of mannan remained. This may have been due to a variety of factors, including less potent than specified anti-insulin antibody (used to make the column), destruction of the antibody when binding to the column, less than complete elution from the lectin columns, are among the more likely.

A faster, more productive way to separate the mannan-insulin from free insulin was found by precipitating the mannan-insulin with 50% ethanol. Mannan-insulin before and after ethanol precipitation was compared by polyacrylamide electrophoresis. No free insulin band was visible in the ethanol precipitated band. In subsequent experiments, conjugates were prepared by the ethanol precipitation method.

One of the major questions addressed in further experimentation was how binding of the insulin attached to mannans would compare to binding of native insulin by the adipocytes. If mannan-insulin was able to bind the fat cells, the ability of the conjugated insulin to initiate a characteristic insulin effect (the increase of uptake of glucose into the cell) would also be investigated. The in vitro assay for efficacy of the chimeric insulin was tested using a modification of Rodbell's rat adipocyte system.

The mannan-insulin was able to compete with the radiolabeled insulin for cellular receptors at a significant level. Since the supply was limited, the PDP conjugated mannan-insulin was only done in two trials. This may be the reason it is not statistically significant, even though at the same concentration it has the same per cent of inhibition as the native insulin. In an attempt to block cellular mannose-phosphate binding sites, the mannan-insulin was added to the cells in combination with a large quantity of native mannan. This resulted in decreased inhibition of the ^{125} I-Insulin, which may have been due to competition for mannose-phosphate binding sites, but does not prove that mannose-phosphate receptors were the binding site. Pretreatment with unlabeled mannans prevents cellular binding when adipocytes are subsequently

treated with fluorescent-labeled mannans. This suggests the presence of functional, saturable mannan receptors on the adipocytes. Blocking the exposed insulin with anti-insulin antibody or blocking cellular insulin receptors with anti-receptor antibody might be tried to eliminate binding through the insulin moiety. The question still remains whether the mannan-insulin is binding the mannose-phosphate receptors, the insulin receptors, or both.

The ability of the mannan-insulin to initiate a biological effect was examined. Insulin is known to affect fat cells by increasing the uptake of glucose from the surrounding medium, a parameter which is easily measured with ^{14}C-Glucose. Rodbell (1964) found the glucose uptake was most effectively increased by addition of insulin in the 10 uU to 100uU range in animals fasted overnight. He observed an increase in the glucose uptake of 1.6X when 100uU of insulin was added to the cells for 2 hours at 37o. Similar results were obtained with 100 uU of insulin (1.8X increase). The conjugated insulins were able to produce results that correspond to, but are not significantly greater than, an equivalent amount of native insulin. With the mannan-insulins and the 50uU insulin, the increase in glucose uptake was three times the uptake when no insulin was added the cells. The reduced effect at the higher insulin concentration may have been due to the influence of negative cooperativity or down regulation of the cellular receptors. The phenomenon of down regulation, i.e., the loss of cell surface receptors in response to exposure to insulin, has been analyzed by a number of researchers (Berhanu et al., 1982; Marshall & Olefsky, 1981; Garvey, Olefsky, & Marshall, 1985). Insulin is also known to regulate binding by altering receptor

affinity (Czech, 1985).

Another interesting aspect of the effect on glucose uptake was the interaction of the native mannan with the adipocyte (with no insulin added) and when mannan-insulin is present. Mannans in 20mcg concentration, which is about 10X the amount of insulin in 50uU, caused no significant increase in glucose uptake. However, when the mannan was added in excess (200mcg), an insulin-like activity was initiated. The high concentration of mannan was initially used to attempt to block cellular mannose-phosphate receptors and added to the fat cells in combination with the mannan-insulin conjugate. When added in conjunction with the mannan-insulin, the combined effect was not significantly different from the basal level of glucose uptake. Compared to the glucose uptake from the mannan-insulin or the high concentration mannan alone, it was significantly different. The PDP method mannan-insulin produced similar responses. The inhibition of mannan against mannan-insulin conjugate was less marked in these trials. The native mannan (high concentration) may be exerting its effect through crosslinking cellular receptors. The production of insulin-like activity has been reported with trypsin, lectins like Concanavalin A, antibody-mediated (anti-receptor antibody), and with the use of crosslinking agents like disuccinimyl suberate (Kono & Barham, 1971; Pilch & Czech, 1979; Keilacker, Knospe, Ziegler, & Whittaker, 1985).

The native mannan-induced increase in glucose uptake could be explained in terms of the crosslinking theory, but the inhibition of mannan-insulin responses in the presence of native mannan still remains unresolved. It could be the result of mannan-induced crosslinking

preventing the action of the insulin bound to the conjugate, but it could also be the result of blockage of cellular mannose-phosphate receptors by the excess mannan, thus preventing the binding by the conjugate.

It would be helpful to extend the experiment to some type of cells known to have reduced numbers of insulin receptors or deficient insulin receptors. Insulin-like effects under these circumstances might be the result from binding at mannosephosphate receptors. This would still leave questions concerning just how the insulin-like activity was being initiated, i.e., whether the mannan was acting to bring insulin in proximity to its receptors, accelerating aggregation and internalization.

The role of internalization is still ambiguous in the literature. Some researchers believe it is necessary for insulin down regulation (although the fate of the receptor is widely debated), others point to the insulin receptors at intracellular sites like the Golgi and nuclear membrane and question the relationship to internalization and plasma receptor binding (Fehlman, Carpentier, Van Obberghen, Freychet, Thamm, Saunders, Brandenburg, & Orci, 1982; Nakao, Kagawa, Shimizu, Jha, & Matsuoka, 1984; Bergeron et al., 1985).

This controversy points out one possible use for conjugates which allow entry of insulin through other receptors to elucidate the role, if any, of the intracellular insulin receptors. Mannan-insulin conjugates could also be used to deliver insulin to cells with defective receptors, like those found in patients suffering from diabetes mellitus type II (NIDDM).

While insulin was chosen as a model peptide for the carrier in this study, the mannan carrier was envisioned as a general peptide carrier, able to be used for a wide variety of peptide transport problems. For example, it might be useful to carry antimicrobial agents into the macrophage, since some pathogenic organisms (e.g., Brucella species) can be sequestered there. Peptides might be introduced through mannose-phosphate receptors into cells which they normally can not enter. This might be useful in helping to understand the mechanisms of peptide activity within cells. For peptides administered orally, the mannans might provide protection from breakdown in the gastrointestinal tract.

APPENDIX A

Buffer for Adipocyte Assays

HEPES Stock Buffer (500 ml)

131mM Sodium chloride	19.16 grams
4.8mM Potassium chloride	0.90 grams
1.3mM Calcium chloride	0.48 grams
0.9mM Potassium phosphate	0.41 grams
1.45mM Magnesium Sulfate (heptabasic)	0.74 grams
25mM HEPES	14.9 grams

Use sterile H_2O, adding one ingredient at a time with stirring. Bring to 500 ml with H_2O. To use, take 1 part of the stock above to 4 parts sterile H_2O, then add 2.0 grams of Bovine Serum Albumin and 20 mg/ 100 ml D-glucose. Bring to pH 7.4 with NaOH.

If kept refrigerated, the stock buffer is good for 30 days.

APPENDIX C

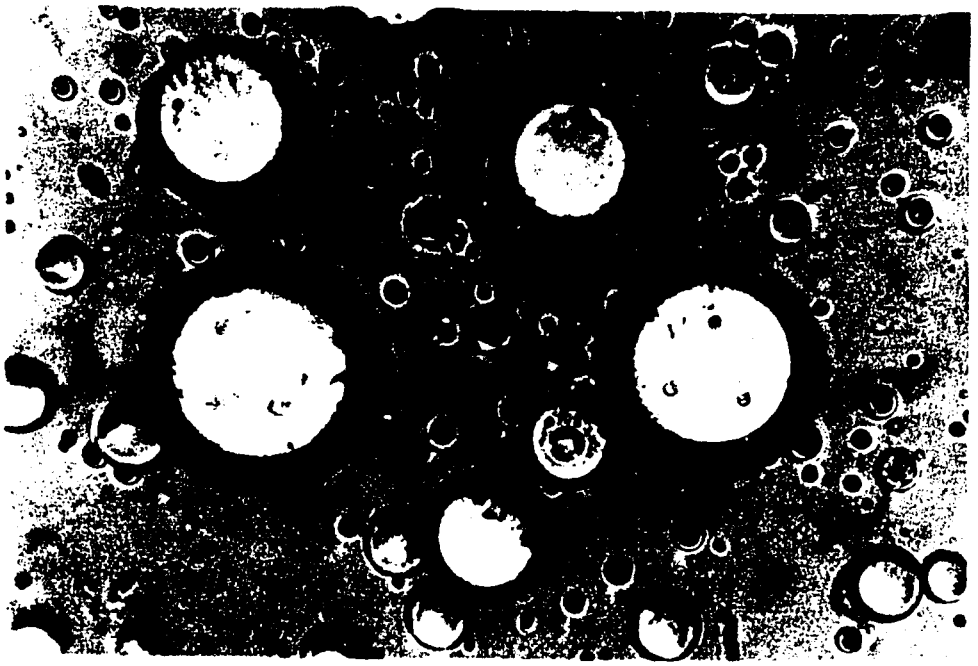


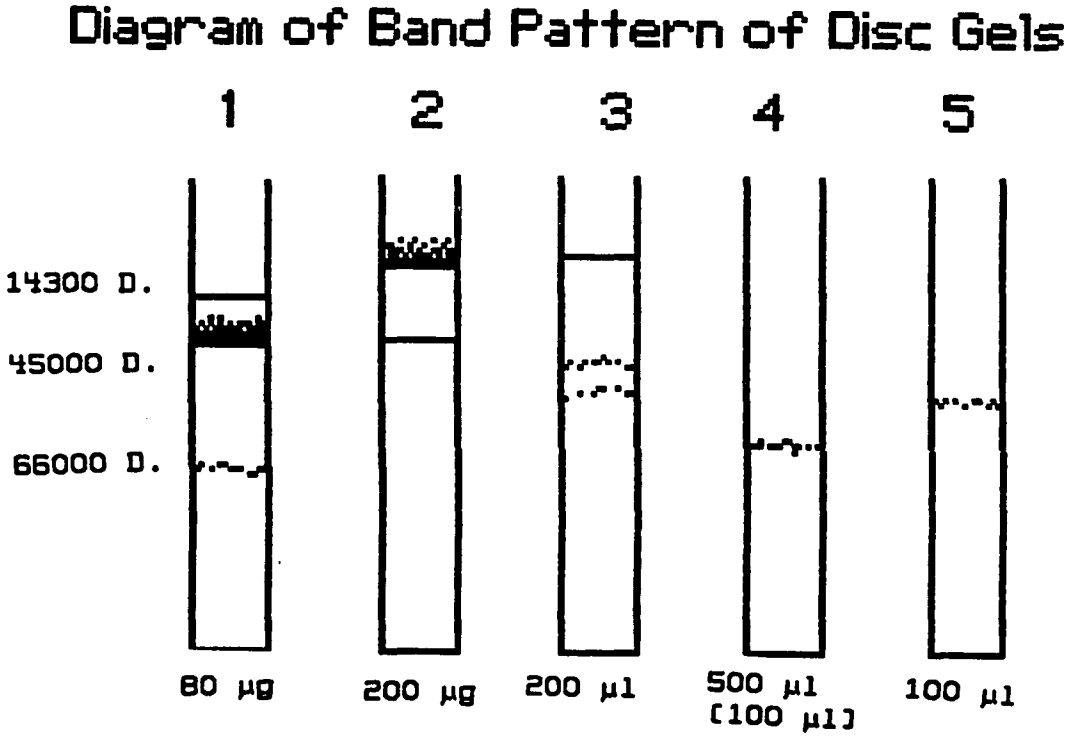
Figure 1. Isolated rat epididymal adipocytes (100X)

Figure 2. Adipocytes after exposure to Fluorescein isothiocyanate labeled yeast mannans (400X)

APPENDIX B

Polyacrylamide Gel Electrophoresis

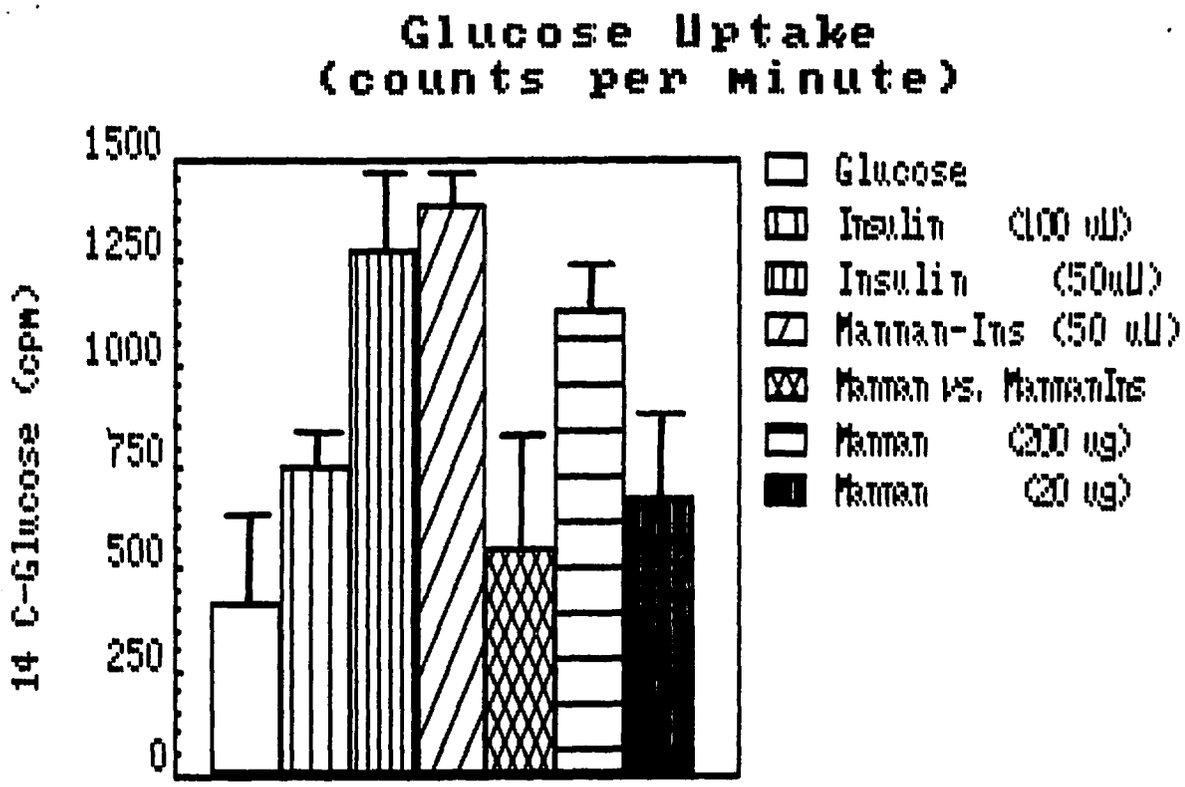
Protein standards (1), FITC-Insulin (2), Non-ethanol precipitated Mannan -Insulin (3), Ethanol precipitated Mannan-Insulin (4), and Native Mannan (5) were electrophoresised on 7.5% Polyacrylamide gel with SDS for 90 minutes at 4 mA/tube. After fixation and staining with Coomassie blue R250, these bands were visible. Note the multiple bands in 3 and single band in 4 and 5.



APPENDIX D

Glucose Uptake With Cyanogen Bromide Method

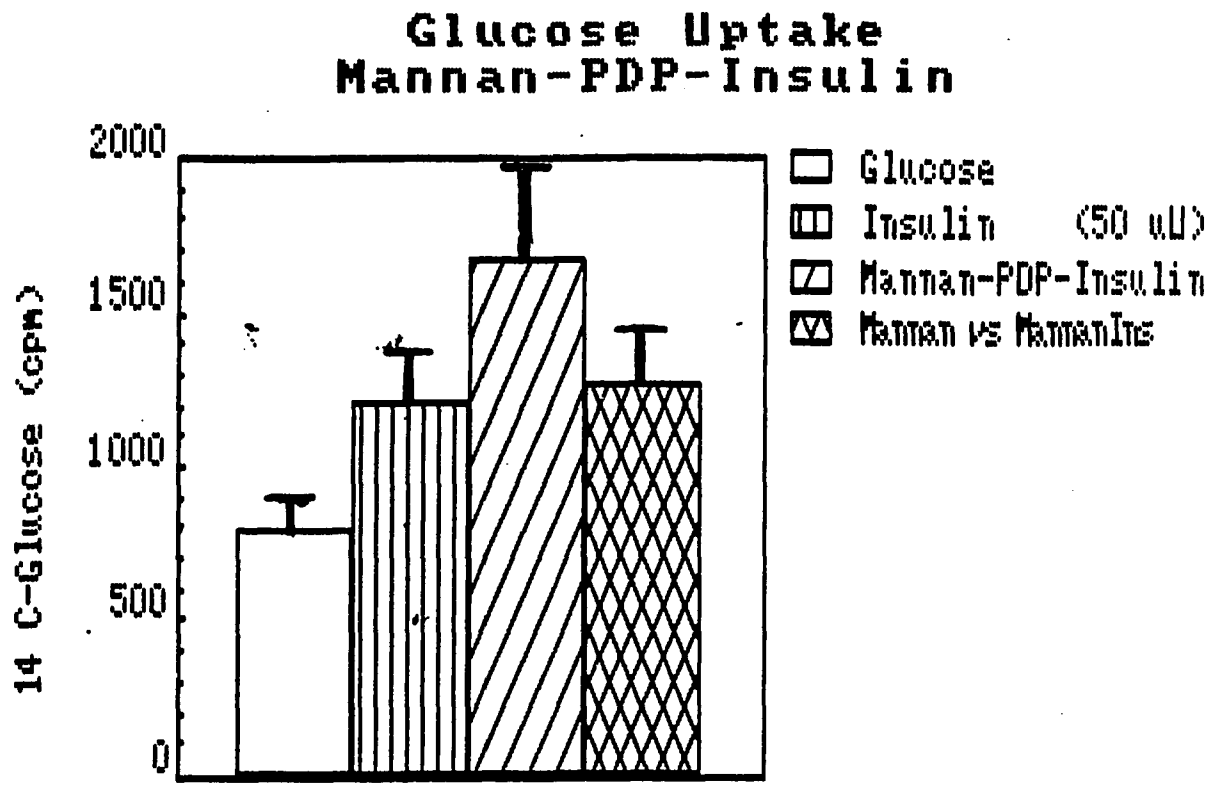
The capacity to increase uptake of glucose into the adipocytes was measured as counts per minute of 14C-Glucose taken into the cells after 45 minutes in the presence of insulin, mannan-insulin, or native mannans.



APPENDIX E

Glucose Uptake With PDP Method

This graph represents the comparison of glucose uptake when Mannan-insulin using the PDP conjugation method is tested.



BIBLIOGRAPHY

Axen, R., Porath, J., & Ernback, S. (1967). Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. Nature, 214, 1302-1304.

Ballou, C. E. (1974). Some aspects of the structure, immunochemistry, and genetic control of yeast mannans. Advances in Enzymology, 40, 239-270.

Bergeron, J. J. M., Cruz, J., Khan, M. N., & Posner, B. I. (1985). Uptake of insulin and other ligands into receptor-rich endocytic components of target cells: The endosomal apparatus.Annual Review of Physiology, 47, 383-403.

Berhanu, P., Olefsky, J. M., Tsai, P., Thamm, P., Saunders, D., & Brandenburg, D. (1982). Internalization and molecular processing of insulin receptors in isolated rat adipocytes. Proceedings of the National Academy of Sciences (U.S.A.), 79, 4069-4073.

Cawley, T. N., & Ballou, C. E. (1972). Identification of two Saccharomyces cerevisiae cell wall mannan chemotypes. Journal of Bacteriology, 111, 690- 695.

Chien, Y. W. (1985). Transnasal Systemic Medications. Amsterdam: Elsevier Science Publications.

Czech. M. P. (1985). The nature and regulation of the insulin receptor: structure and function. Annual Review of Physiology, 47, 357-381.

DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 28, 350-355.

Ehrlich, P. (1908). Modern Chemotherapy. In T. Brock (Ed.), Milestones in Microbiology (pp. 176-185). Washington, D. C.: American Society for Microbiology.

Fehlman, M., Carpentier, J., Van Obberghen, E., Freychet, P., Thamm, P., Sauknders, D., Brandengurg, D., & Orci, L. (1982). Internalized insulin receptors are recycled to the cell surface in rat hepatocytes. Proceedings of the National Academy of Sciences (U.S.A.), 79, 5921-5925.

Garvey, W. T., Olefsky, J. M., & Marshall, S. (1985). Insulin receptor down-regulation is linked to an insulin-induced postreceptor defect in the glucose transport system in rat adipocytes. Journal of Clinical Investigation, 76, 22-30.

Gatt, R., & Borman, E. R. (1966). Phosphate analysis and hydrolysis. Analytical Biochemistry, 15, 167-169.

Gilliland, P. (1978). Chimeric Toxins: toxic, disulfide-linked conjugate of concanavalin A with fragment A from Diphtheria toxin. Proceedings of the National Academy of Sciences (U.S.A.), 75, 5319-5323.

Goldstein, J. L., Anderson, R. G. W., & Brown, M. S. (1979). Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature, 279, 679-684.

Good, W. K. (1985). Transdermal drug delivery systems. The Latest Developments in Drug Delivery Systems, 2, 40-48.

Gruner, S. M., Lenk, R. P., Janoff, A. S., & Ostro, M. J. (1984). Novel multilayered lipid vesicles: comparison of physical characteristics of multilamellar liposomes and stable plurilamellar vesicles. Biochemistry, 24, 2833-2841.

Hitchcock-DeGregori, S. E., & Yienger, B. E. (1984). Ligand independent binding of serum proteins to sepharose 4B activated using cyanogen bromide and blocked with ethanolamine. Biotechniques, 2 (5), 326-334.

Jou, Y., Mazzaferro, P. K., Mayers, G. L., & Bankert, R. B. (1983). Methods for the attachment of haptens and proteins to erythrocytes. Methods in Enzymology, 92, 257-276.

Kaplan, A., Achord, D. T., & Sly, W. S. (1977). Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. Proceedings of the National Academy of Sciences (U.S.A.), 74, 2026-2030. 6-2030.

Keilaker H., Knospe, S., Ziegler, M., & Whittaker, J. (1985). Effect of antibody-mediated crosslinking of insulin on its bioactivity and receptor binding. Experimental Clinical Endocrinology, 85(1), 38-46.

Kocourek, J., & Ballou, C. E. (1969). Method of fingerprinting yeast cell wall mannans. Journal of Bacteriology, 100(3), 1175-1181.

Kono, T., & Barham, F. W. (1971). Insulin-like effects of trypsin on fat cells. The Journal of Biological Chemistry, 216(20), 6204-6209.

Lee, Y. C., & Stowell, C. P. (1978). The binding of D-glucosyl-neoglycoproteins to the hepatic asialoglycoprotein receptor. The Journal of Biological Chemistry, 253(17), 6107-6110.

LK Bromma. (1983). Polyacrylamide gel electrophoresis. L. K. Bromma Application Note 320. New York: author.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Determination of protein content. The Journal of Biological Chemistry, 193, 265-275.

Marnell, M. H., Shie, S., Stookey, M., & Draper, R. A. (1984). Evidence for penetration of Diphtheria toxin to the cytosol through a pre-lysosomal membrane. Infection and Immunity, 44(1), 145-150.

Marshall, S., & Olefsky, J. M. (1981). Characterization of insulin-induced receptor loss and evidence for internalization of the insulin receptor. Diabetes, 30, 746-753.

Mufson, D. (1985). The Application of liposome technology to targeted delivery. The Latest Developments in Drug Delivery Systems, 2, 16-21.

Nakao, K., Kagawa, S., Shimizu, S., Jha, B., & Matsuoka, A. (1984). A possible role for insulin-receptor subunit in degrading receptor-bound insulin. Hormone and Metabolic Research, 16, 319-320.

Natowicz, M. R. , Chi, M. M., Lowry, O. H., & Sly, W. S. (1979). Enzymatic identification of mannose-6-phosphate on the recognition marker for receptor-mediated pinocytosis of beta-glucuronidase by human fibroblasts. Proceedings of the National Academy of Sciences (U.S.A.), 76, 4322-4326.

Pilch, P., & Czech, M. P. (1979). Interaction of cross-linking agents with the insulin effector system of isolated fat cells. The Journal of Biological Chemistry, 254(9), 3375-3381.

Pontiroli, J. (1982). Transnasal delivery of insulin in humans. British Journal of Medicine, 284, 303-307.

Poste, G., & Kirsh, R. (1983). Site-Specific (targeted) drug delivery in cancer therapy. Biotechnology, 12, 869-876.

Rodbard, D., & Hutt, D. (1974). Statistical analysis of radioimmuno-assays and immunoradiometric assays. In RIA and Related Procedures in Medicine (pp. 165-192). Vienna: International Atomic Energy Agency.

Rodbell, M. (1964). Metabolism of isolated fat cells. The Journal of Biological Chemistry, 239(2), 375-380.

Roth, R. A., Maddux, B. A., Wong, K. Y., Iwamoto, Y., & Goldfine, I. D. (1981). Insulin-ricin B chain conjugate. The Journal of Biological Chemistry, 256(11), 5350-5354.

Sanders, H. J. (1985). Improved drug delivery. Chemical and Engineering News, 63(13), 30-48.

Sharon, N., & Lis, H. (1972). Lectins: cell agglutinating and sugar specific proteins. Science, 177, 949-959.

Silverstein, S. C., Steinman, R. M., & Cohn, Z. A. (1977). Endocytosis. Annual Review of Biochemistry, 46, 669-722.

Szoka, F. C., & Mayhew, E. (1983). Alteration of liposome disposition in vivo. Biochemical and Biophysical Research Communications, 110(1), 140-146.

Thieme, T. R., & Ballou, C. E. (1971). Nature of the phosphodiester linkage of the phosphomannan from yeast. Biochemistry, 10(22), 4121-4129.

Von Figura, K., & Klein, U. (1979). Isolation and characterization of phosphorylated oligosaccharides from alpha-n-acetylglucosaminidase that are recognized by cell-surface receptors. European Journal of Biochemistry, 94, 347-354.

Widder, K. J. (1985). Magnetic targeting of drugs. The Latest Developments in Drug Delivery Systems, 2, 21-27.

Wilson, D. B. (1978). Cellular transport mechanisms. Annual Review of Biochemistry, 47, 933-965.

Youle, R. J., Murray, G. J., & Neville, D. M. (1979). Ricin linked to monophosphopentamannose binds to fibroblast lysosomal hydrolase receptors, resulting in a cell-type-specific toxin. Proceedings of the National Academy of Sciences (U.S.A.), 76, 5559-5562.

Zaharko, D. S., & Beck, L. V. (1968). Studies of a simplified plasma insulin immunoassay using cellulose powder. Diabetes, 17, 444-457.