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TAGGING INSULIN IN MICROGRAVITY

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STATEMENT OF THE PROBLEMS TO BE ADDRESSED BY THE G-399 PROJECT

Declining Enthusiasm

A major problem seen today is the declining enthusiasm for technology among American college students. There are certainly many students which are attuned to science and technology, but their numbers are declining every year.

Declining Number of High Tech Tools

There is a declining number of high tech tools that are available to college professors which can be used to teach students the motivation to acquire knowledge in applied science. Sierra College's mission is to give students skills in applying scientific tools to industry needs. This project is to demonstrate to the students the whys and wherefores of practical and applied science, and the creation and value of teamwork in the solution of technical and scientific problems.

Diabetes Mellitus Treatment

Major advances have been made in the treatment of diabetes by the fine tuning of treatment methodologies. Up until ten years ago, all blood sugars were done in the lab monthly or less frequently with the patient only checking his urine for sugar; this resulted in poor control of glucose and a poorly controlled patient. Now, individual patients can check their own blood sugars, thus achieving better control and prolonging for a few years the complications that will surely develop.

Knowing the exact subcellular sites of action of insulin in the body has the potential to give basic science investigators a basis from which a cause and cure for this disease can be approached. The goal of this project is to create a test reagent that can be used to visualize these subcellular sites.

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SOLUTION - THE GET AWAY SPECIAL PACKAGE FOR THE SHUTTLE

High Tech Environment

The shuttle program by itself creates a unique high tech environment which gives "infectious enthusiasm" to students, researchers, and the professors. The realistic demanding requirements that are the epitome of perfection which require the patience of Job, are inherent in the program and are part and parcel of a good business. The most rewarding personal goals of life are usually those attained by following the rules set down by someone else; working in the Shuttle environment teaches the student this lesson thus preparing them for the real business world of applied science.

Shuttle Micro Gravity Environment

The unique micro gravity environment of the Shuttle will allow the creation of a reagent that has the possibility of elucidating the subcellular sites of action of insulin.

If The Experiment Is Successful

If the procedure does indeed create the desired reagent for insulin, the same procedure modified slightly, could be used to create many other tagged entities. The list of uses for this procedure would be endless and could extend from hormones to organisms to drugs.

DIABETES AND INSULIN

Diabetes mellitus can be defined as a disease wherein the physiologic control mechanisms for the regulation of glucose levels by insulin are measurably outside of defined normal levels. This is a very complicated disease to grasp in concept since at one end of the spectrum an individual has lost his ability to make any measurable insulin, because of the destruction of the insulin producing cells from the apparent effects of an auto immune phenomenon or acute pancreatitis to the woman who only manifests the disease when she is pregnant.

The common denominator for all the diabetic states is a deficiency in tissue response to insulin. At one end of this continuum is a no-insulin state with a predictably no control over glucose to the other end of the continuum where there is an excess of perfectly good insulin that apparently is unable to influence the tissue receptors. Tissue receptors have been counted on a cellular basis and have been found to vary from tissue to tissue and between disease states. The exact location of these receptors on the cell wall or other structures is unknown.

A basic unknown is whether insulin can exert its effects from outside the cell, i.e. near it or attached to its membrane, or does it penetrate the cell and exert its actions from within the cell. Possibly, it acts at different sites with different tissues or a combination of sites.

Autoradiography

Several techniques have been utilized in an attempt to isolate the sites of action of items such as insulin. One of these is autoradiography in which the test item is obtained from animals fed radioactive materials. The test item is then radioactive and is given to another animal. After an appropriate length of time the target tissues are harvested and thin slices obtained for the microscope. A photographic emulsion is placed over the tissue allowing the radioactive material to expose the emulsion. By looking for silver granules one can infer that the target item had appeared at that point. This technique is capable of showing which cells absorb the test item, but is not even close to being accurate enough to disclose the exact subcellular site of attachment.

The Need To Visualize The Sites Of Action Of Insulin

What is clearly needed is to visualize individual insulin molecules at their sites of action. Insulin molecules themselves do not possess any attributes that would enable their visualization. A tag is needed that can be placed on the insulin molecule which will enable its localization while it is attached to the insulin receptors at its sites of action. This tag has to satisfy a number of qualifications, two of the more important are that it must not significantly alter the action of insulin, and that it must present a unique appearance on electron microscopy to allow its reliable identification.

Ferritin

Ferritin is a large molecule having a molecular weight of approximately 900,000 with iron atoms arranged in a characteristic tetrad formation which is easily identified on a molecule to molecule basis in tissues at a 160,000 magnification. Insulin with a molecular weight of approximately 6,000 has no distinguishing features in the electron microscope. The following figures show ferritin, both in its pure state and after its conjugation.

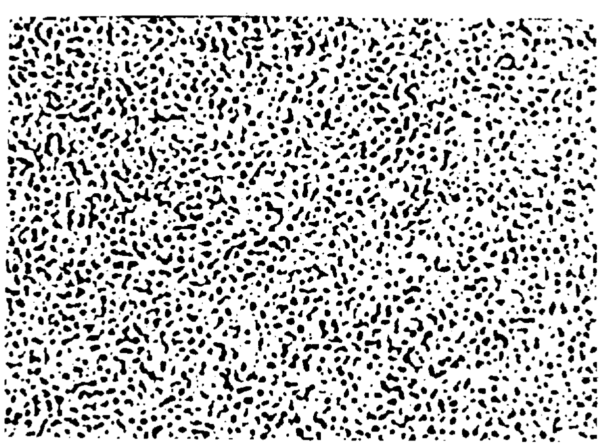

Figure 1.
Pure Ferritin 160,000 X
Note: Uniform Pattern

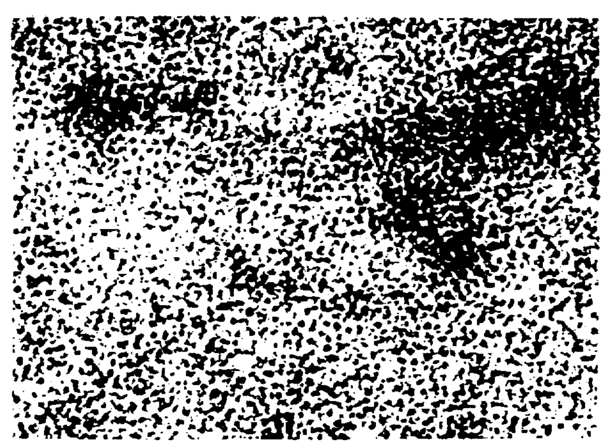

Figure 2.
Ferritin-Insulin conjugate
160,000 X, Clumpped Pattern

INSULIN TAGGING

Tagged Insulin Reagent

In summary, the insulin tagging process to be used on G-399 involves the conjugation of insulin molecules with ferritin molecules to create a reagent that will be used back on Earth in an attempt to elucidate the sites of action of insulin.

During 1969 and 1970, co-author of this paper, Dr. Ronald Nelson tagged insulin with ferritin and injected it into diabetic laboratory rats. Preliminary results were obtained, but the creation of the test reagent was difficult because of the disparity in molecular weights between the insulin and its iron containing tag. The photomicrographs above clearly show that the solution of ferritin itself is very homogeneous, while the ferritin-insulin conjugate is very lumpy, even after considerable processing steps. It is hoped that in the microgravity environment of space that this disparity in molecular weights will not be a factor in the reaction that conjugates insulin molecules with ferritin.

The conjugating process that will take place on the Shuttle and its past history is as follows:

THE TAGGING PROCESS

The original process utilized in 1969 and 1970 for the creation of the test reagent occurred in a glass container stirred by a standard glass encapsulated magnet. The reaction required 24 hours at a temperature of 4 degrees Celsius. During this time the insulin molecules were conjugated (tagged) with the iron containing molecules of ferritin. After conjugation, the mixture was dialysized against normal saline to remove any remaining traces of the very active conjugating agent difluoro-dinitro-phenyl-sulfone (DFDNDPS). Further purification steps involving filtration and fractionation through sephadex columns removed unconjugated insulin and overly conjugated insulin and ferritin complexes. A major problem with the process was the migration of the heavier iron containing ferritin to the bottom of the reaction vessel which resulted in unwanted conjugation of ferritin to ferritin, and at the top insulin to insulin.

The reactants will be mixed in the GAS container by being pumped around a continuous loop for 24 hours in the microgravity environment of the Shuttle thus avoiding the settling out of the reactants and ensuring a uniform mixture for the entire 24 hour period. The process in the Space Shuttle will end after the dialysis phase with the reactants being stored. Further purification and separation of the various conjugates will be done back on earth where the normal gravity gradient will be needed for the operation of the sephadex columns.

Post flight usage of the reagent will involve its injection into the arterial circulation of test animals that have been made diabetic.

After various lengths of time the animals will be sacrificed and electron microscopy used to search out the location of the ferritin which will be presumed to be attached to insulin. A series of controls will be utilized to make sure that the data is meaningful.

If the process proves useful for the hormone insulin, other hormones and important proteins could be tagged with ferritin and their sites of action determined.

Photomicrographs below show some of the original data which appears to indicate that at least one of the sites of action are the pinocytotic vessicles in the walls of the capillaries found in diaphragm muscle. A major site of diabetic lesions is in large blood vessels. A break through in protecting these structures would be most welcome in the medical community. Little is known about the function of insulin in glandular tissue and ferritin tagged insulin has the potential for isolating its action in these very complex and also very important tissues.

Figure 3. Orientation Picture of Tissue 50,000 X
Red blood cell lower right surrounded by endothelial cells containing pinocytotic vessicles.
(Rat pacreatatic tissue capillary)

Figure 4.

Figure 5.

Insulin-Ferritin Conjugates within pinocytotic vessicles
(160,000 X Rat diaphragm muscle capillary endothelial cell)

INSULIN TAG EXPERIMENT HARDWARE

Experiment Apparatus

The hardware for the insulin tag experiment consists of 6 containers which hold the fluids required to complete the experiment, 7 solenoid controlled valves that keep the fluids separated prior to the start of the experiment, two fluid pumps for mixing and one air pump for fluid transfer (see figure 6).

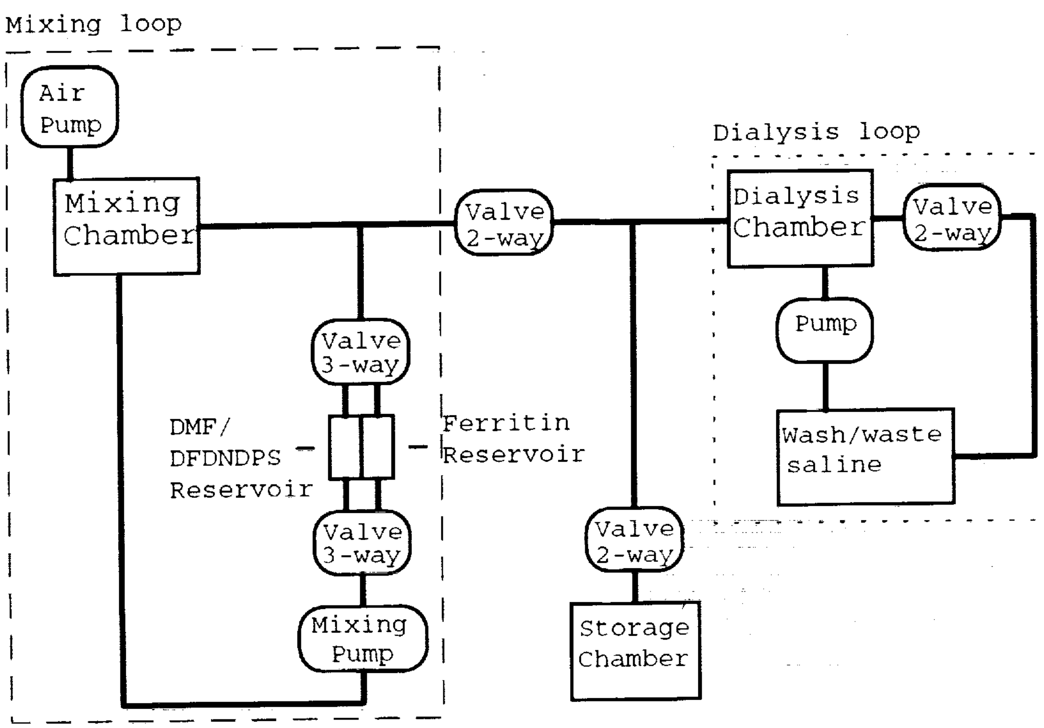

Figure 6. ITE Schematic

Mixing Phase

The insulin tag experiment (ITE) begins with a temperature check of the ferritin reservoir, mixing chamber, and DFDNDPS/DMF reservoir. If the temperature is in the 1 to 6 degree C range, the mixing phase of the ITE begins.

The goal of the mixing phase is to combine the DFDNDPS/DMF solution, which is held in the DFDNDPS/DMF reservoir, with the ferritin/ringers solution, which is contained in the ferritin reservoir, and the insulin/deionized water/sodium carbonate solution, which is held in the mixing chamber (see table 1). All three solutions are isolated by solenoid controlled valves with Teflon wetted parts. Once the proper temperature is reached, the ferritin side of the two three-way valves will open, and the mixing pump will start and run for 4 minutes. At the end of the 4 minute ferritin mix, the pump will shut down and the ferritin side of the three-way valves will close. The

DFDNDPS/DMF side of the three way valves will then open, and the pump will start and run for 4 minutes.

Table 1: ITE Solutions

Component	Volume	Launch Location
DMF	27 ml	
DFDNDPS	961 mg	DFDNDPS/DMF reservoir
ringers	69 ml	
ferritin	6 g	Ferritin reservoir
insulin	1.4 g	
DI water	101 ml	
sodium carbonate	3.4 g	Mixing chamber
wash saline	500 ml container	Wash/waste saline

The anodized 6061-T6 mixing chamber has an internal latex bladder connected to the inside top of the chamber with a tubing clamp. The top of the mixing chamber is penetrated by two 0.125 inch NPT 316 stainless steel compression fittings which enter through the neck of the bladder. One of the fittings is connected to the multiplexed port on the 3-way valve leading into the ferritin and DFDNDPS reservoirs (see figure 6). The other fitting is connected to one side of the mixing pump. Because DMF is a fairly active organic solvent, all of the connections between the chambers, valves, and pumps are made with Teflon tubing.

Wrapped and glued to the outside of the mixing chamber is a 2.5 Watt kapton insulated electric resistance heater. The microcontroller will use the heater to maintain the temperature in the 1 to 6 degree C range.

On the end opposite the pump and valve connections, is a single 316 stainless steel compression fitting. This fitting is connected to a small air pump which will pressurize the mixing chamber during the fluid transfer phase.

The ferritin and DMF/DFDNDPS reservoirs are each of the same basic design. Each reservoir is constructed of a piece of aluminum tubing with an aluminum end cap welded on one end, and a o-ring sealed screw in cap on the other. Penetrating the end of each cap is a 316 stainless steel 0.125 inch NPT compression fitting. These fittings are connected to the 3-way valves with Teflon tubing. Fastened to the wall inside each reservoir is a flexible bladder filled with air. The air bladders leave room for fluid expansion should the chambers freeze.

Transfer Phase

After the mixing phase is complete, the fluid in the mixing chamber must be transferred to the dialysis chamber (see figure 6). To accomplish this transfer, the two-way valve between the mixing chamber and the dialysis chamber will open, and a small air pump will pressurize the mixing chamber to a maximum of 4 psig. The increase in pressure will force the bladder in the mixing chamber to collapse and the fluid in the mixing chamber into the dialysis chamber. The two-way valve that separates the mixing and dialysis chambers will then be closed, and the air pump will be shut down.

Dialysis Phase

The dialysis chamber is constructed of 6061 T6 anodized aluminum. This chamber has a pair of 316 stainless steel compression fittings penetrating the welded end used as entry and exit ports for the dialysis fluid. The o-ring sealed screw cap end has a single 316 stainless steel compression fitting through which the tagged insulin will enter and exit. Inside the dialysis chamber, a single tube on the screw cap end opens into the flexible dialysis membrane.

The dialysis chamber is heated with a 2.5 Watt Kapton insulated electric resistance heater and has a temperature sensor for feedback to the microcontroller.

The wash and waste saline containers are 500 ml I.V. fluid bags contained in a composite structure box. The box is constructed of s-glass over foam core sides, top, and bottom. A 12 Watt heater is mounted on an aluminum plate in the bottom of the box.

The dialysis phase will last for 4 hours. During this time, saline solution will be transferred from the wash saline storage container, by a magnetically coupled gear pump, into the dialysis chamber. As fresh saline is pumped into the dialysis chamber, the waste saline is forced through an open two-way valve into the waste saline storage container.

Storage Phase

The storage phase is the final step in the ITE. The goal of the storage phase is to transfer the solution in the dialysis chamber into the storage chamber. The two-way valve that separates the dialysis chamber from the storage chamber will be opened, and the two-way valve that allows waste saline to flow into the waste saline storage area will remain closed. The wash saline pump will transfer approximately 100 ml of wash saline into the dialysis chamber. This will force the solution contained in the dialysis tubing into the storage container. Finally the storage container two-way valve will be closed.

The storage container is a 250 ml polypropylene chemical storage bottle with a stainless steel tube penetrating the lid. The storage container is connected to a stainless steel "T" fitting which is connected between the dialysis chamber and the mixing chamber. An isolation valve prevents fluid from entering the storage container during the fluid transfer phase.

Safety Considerations

The fluid pumps used in the ITE are magnetically coupled, gear driven. The fluid pumps can generate a maximum of 49 psig in the unlikely event of an overpressure condition. An overpressure condition may occur if a valve failed to open and the pump was switched on. The magnetic coupling on the pump disengages within 0.5 seconds during an overpressure condition and the pressure drops to 0 psig. The ITE has been proof pressure tested to twice the maximum design pressure (100 psig). The pump housing is 316 stainless steel and the internal gears are Delrin.

Because the ITE uses a flammable organic solvent, the entire experiment must be triple contained. The ITE is fully contained in a 0.160 inch 6061-T6 aluminum containment vessel. The containment vessel will be purged with gaseous nitrogen prior to the sealing of the GAS canister.