5650 Ellis Avenue

April 28, 1952

Dr. E. L. Willett, Research Director
American Foundation for the Study of Genetics
Route 5
Madison, Wisconsin

Dear Dr. Willett:

This is just a short note to say that the bull sperm which you sent us arrived in good order and we did a few simple experiments with it. We now are planning another experiment which will take some time to prepare and I shall contact you and tell you more about it at a later date.

With best wishes,

Sincerely yours,

Leo Szilard

LS/sds

5650 Ellis Avenue

May 13, 1952

Dr. E. L. Willett, Research Director
American Foundation for the Study of Genetics
Route 5
Madison, Wisconsin

Dear Dr. Willett:

The two substances which you might try for your centrifuge plan in order to adjust the density of the suspension which we discussed when you were in Chicago are gum acacia and methyl cellulose. The first of these you can get practically in any pharmacy; the second, you can obtain from the Dow Chemical Company. They have various types graded according to the viscosity of a 2% solution at 20° C. which are as follows:

Type	Viscosity in Centipoises
15	13-18
25	20-30
50	40-60
100	80-150
400	350-550
1500	1200-1800
4000	3000-5000

With best wishes,

Sincerely yours,

Leo Szilard

LS/sds

Methocel

2% solutions at 20°

Type	centipoises
15	13-18
25	20-30
50	40-60
100	80-120
400	350-550
1500	1200-1800
4000	3000-5000

Ford

5650 Ellis Avenue

June 5, 1952

Dr. E. L. Willett, Research Director
American Foundation for the Study of Genetics
Route Five
Madison, Wisconsin

Dear Dr. Willett:

Enclosed is a copy of a very interesting article which appeared in Nature. I have marked the most important passage for your convenience.

I expect to have luncheon with Mr. Prentice today, and on this occasion I shall give him a copy also.

With kind regards,

Sincerely yours,

Leo Szilard

LS/sds
Enclosure

Please reply to Madison office

AMERICAN FOUNDATION FOR THE STUDY OF GENETICS

RESEARCH LABORATORY: ROUTE 5, MADISON, WISCONSIN
Telephone 4-0748

Chicago Office: 325 NORTH WELLS STREET, CHICAGO 10, ILLINOIS
Telephone SU perior 7-9756

January 13, 1953

Dr. Leo Szilard,
Institute of Radio Biology & Biophysics,
University of Chicago,
5650 Ellis Avenue,
Chicago 37, Illinois.

Dear Dr. Szilard:

In our conference last week you asked me to send you the reference giving the motility of sperm under different temperature conditions. The only paper I know which has made a specific study of this problem with bovine spermatozoa is that by Allen Bane entitled: A Study on the Technique of Hemocytometric Determination of Sperm Motility and Sperm Concentration in Bull Semen. Cornell Vet. 42, 518, 1952.

Yours very truly,

E. L. Willett

ELW:RG

E. L. Willett

AMERICAN FOUNDATION FOR THE STUDY OF GENETICS

TO: J. R. Prentice

DATE April 23, 1953

FROM: E. L. Willett

ANS'D .....

FILE .....

SUBJECT: Report on visits with Luyet and Brown

On the morning of April 16, 1953, I spent approximately two and one-half hours with Father Luyet at the St. Louis University and an additional one hour with two of his graduate students. His staff consists of two full-time nuns and a number of graduate students.

The first thing that Father Luyet did was to put on a simple demonstration of devitrification. He demonstrated the principles which were discovered by Tammann in 1898. These principles are as follows. When a liquid is gradually cooled it finally reaches its freezing point. Most liquids, when cooled to a temperature below their freezing point, form crystals if they are cooled slowly. They thus become crystalline in form and remain in that state no matter how cold they are cooled. If frozen very rapidly, however, they do not form crystals but become vitreous, non-crystalline, or amorphous. To remain in this state, however, they have to be cooled to a temperature much below the freezing temperature. They have to be cooled to a temperature below the devitrification point. If kept for any length of time between the devitrification point and the freezing point, the material will gradually change from the amorphous to the crystalline state. In order to be kept permanently in the amorphous state, therefore, as stated above, the material has to be kept below the devitrification point. He pointed out that the devitrification point of a one or two molar solution of glycerol and water has a devitrification point at -60°C. This fact may explain why -60°C. seems to be near the breaking point above which we can not store frozen semen.

Luyet's demonstration consisted of placing a drop of 50% glucose in water on a thin piece of mica, plunging the mica into liquid air, removing it and allowing it to warm slowly in the air. Upon removal the solution is transparent like glass (vitrified). In a few moments the material becomes white (crystalline) and then melts to a liquid.

Luyet's graduate students have begun some work on freeze-drying of tissues and cells. Most of this work has been done with muscle fibers and vinegar eels or paste eels. These eels are small nematodes and are microscopic in size. Up to now they have been working mainly on techniques and have not been successful in retaining life through the dry-freeze process.

In their studies of freezing of eels, chick embryo heart tissue, moss leaves and other materials, the people at St. Louis University have accumulated good evidence that the main function of glycerol in preserving frozen material is that it replaces water inside the cell. With these various materials, the less the intracellular water, the greater the survival. When chick embryo and plant tissue are exposed to a glycerol solution, theyfirst

decrease in size (lose water) and then recover full size again (absorbs glycerol). It appears, therefore, that the benefits of glycerol are derived from its action inside the cell rather than outside.

Luyet and his students have been attempting to find substitutes for glycerol. Among the promising ones are ethylene glycol, propylene glycol, acetamide and methyl urea. Acetamide would lend itself to drying. One of his students is studying the interrelations of levels of acetamide and pH.

It was pointed out that there may be some unsurmountable obstacles in the freeze-drying process. One of these is that, during the drying process, very likely the frozen material will have to be kept below the devitrification point in order to prevent devitrification and destruction of the living material. At these very low temperatures, however, the vapor pressure of water is so very low that drying will be extremely difficult.

Luyet mentioned that Blakslee working with Jimsen, and Salt in Canada working with Drosophila have found no evidence that freezing causes mutations.

It was also pointed out that reconstitution of freeze-dried material might be extremely difficult. The sudden and abrupt addition of distilled water to dried material might cause its destruction. This is one problem which will need to be worked upon.

In the course of our discussions it became apparent that there are two methods of approach to the freeze-drying problem. One is the ultra-rapid (very rapid or sudden) freezing of tissue. Luyet says that this is almost impossible. He is able to approach this by freezing thin films or droplets. It is conceivable, I suppose, that some good engineering might evolve some mechanical means of doing this on a large scale. The second approach is the search for some compound which will serve as a substitute for glycerol (thereby enabling slow freezing of material) and also which can be dried. As indicated above, acetamide may possibly serve this purpose.

Another problem in the freezing of material without glycerol by ultra-rapid methods is that the material has to be thawed through the devitrification zone as rapidly as it is frozen. Otherwise crystals will form and the material damaged.

I spent about two and one-half hours with Dr. Ivan Brown at Duke University on April 17th. I found him to be an extremely keen young fellow. He has an extensive research program under way and has quite a large staff working under him. His main project is that of freezing red blood cells. This project has great importance in relation to medicine. He has also worked a little on the freezing of skin, cornea, and blood vessels. Dr. Brown has an M.D. degree with specialization in surgery but has also taken a great deal of additional training in pathology and has a good background in physiology. He is much more interested in research work than in the practice of medicine. He is a member of the department of surgery in Duke University and spends considerable time in surgery.

Brown reports that he has had fairly good results using propylene glycol instead of glycerol. He points out that these various polyhydric alcohols

(propylene glycol, ethylene glycol and glycerol) work because they are naturally vitrifying substances. They vitrify readily and do not form crystals except with great slowness. He states that even a solution of 60% glycerol will gradually devitrify at -70°C. if left long enough. He states that if one takes a tube containing 60% glycerol in water and lets it stand at -70°C., in two months some water will freeze out and the contents of the tube will become opaque. He points out, therefore, that the devitrification point is no set point but is in part a function of time as well as of temperature.

He feels that the secret of preservation by freezing is the removal of water from the cell and substitution with polyhydric alcohols.

He has tried various carbohydrates as a substitute for glycerol including monacetin, diacetin, dulcitol and mannitol. The first two were not as good as glycerol because they do not penetrate the cells readily. The last two were no good.

He states that there is no evidence (except with bacteria) that tissues or cells can be preserved by freeze-drying. He is rather critical of the work on freeze-drying of skin and other tissues which has been reported in the literature. When I remarked that we were interested in freeze-drying he stated: "You are licked."

He pointed out in accordance with his work with red blood cells that one can use high concentrations of glycerol if the glycerol is removed very soon after thawing. With red blood cells this has to be done by repeated centrifugings.

He pointed out that much of the intra-cellular water remaining after most is replaced by glycerol is bound water, that is, water that is strongly attached to intra-cellular material and that can be removed only with great difficulty. This water is in such a state that it is extremely difficult or impossible to crystallize.

He pointed out that dilution aids in the preservation of concentrated suspensions of red blood cells during the freezing process. He originally worked with very concentrated suspensions, much more concentrated than undiluted semen. He now dilutes out about 1:4 or more. His theory or explanation for this phenomenon is that with great concentrations of red blood cells there is not enough space in the interstices between the crystals of ice. It can be seen in the English film that the red blood cells tend to accumulate in the spaces between the crystals of ice. These spaces are filled with glycerol.

Brown pointed out that the greater the concentration of glycerol, the warmer the temperature at which frozen red blood cells can be stored.

Brown has had the problem of removing the glycerol from the red blood cells immediately after thawing, for glycerol can not be injected intravenously. He has found that solutions of sodium lactate, sodium citrate, or sodium sulphate is better for removing glycerol than most other materials. Of these three mentioned above the lactate salt seems to be least toxic.

He also states that proteins in any liquid are very beneficial when removing the glycerol after thawing. He states that ~~the~~ lactate, citrate, and sulphate, and also egg white and other proteinaceous materials are completely non-penetrating and enable the production of a high osmotic pressure (which one has with high concentrations of glycerol) with minimum effect on the cell because they do not penetrate the cell or do to a small extent and therefore have little osmotic effect upon the cell. Apparently, also, proteinaceous materials have some protective action in presence of glycerol. This fact may explain the results that have been obtained by Hank Dunn with egg white.

Brown also pointed out that glutathione or cystine enhance the equilibration process. This is attributed to the presence of sulfhydral groups which have the property of increasing cell wall permeability. There is a possibility that, with the use of these or similar compounds, glycerol could be added very quickly to the medium and equilibration of the cell would take place in a very short time. He pointed out that equilibration at room temperature would take place much more rapidly than at refrigeration temperature due to the greater activity of the molecules at warmer temperature. Brown also pointed out that copper works in the opposite way to that of glutathione or cystine. The copper ion reduces the permeability. He also expressed the opinion that diffusion of molecules through the cell wall is an active rather than a passive process. He thinks that some mechanism acts to hasten the process and thinks that the process might be enzymic in nature.

Impressions and conclusions

Luyet and Brown have been working for many years on this problem of the freezing of living matter. In many ways they are way ahead of us. They have a much greater background of experience and fundamental information. It will behoove us to keep in close touch with them, for they will undoubtedly be uncovering information which will be of great help to us. The chief difficulty with the information that they might obtain is that we do not know whether or not it will apply to bovine spermatozoa because of the great differences in these biological materials. The information they can obtain for us will simply serve as guides or leads that will have to be tested by us with bovine spermatozoa.

It is my opinion and that of others with whom I have talked that, to get the most useful information of a fundamental nature which will be helpful to us, we need to have fundamental work done <u>with bovine spermatozoa</u>. Work with other species or materials may be helpful and may not be. If we should farm out any work to be done for us, it would seem to me to be best, therefore, that it be specified that this work be done with bovine spermatozoa. Even spermatozoa of other species are so different from bovine spermatozoa that information obtained with them still may not be applicable to the species with which we work. Any laboratory doing such work for us should, therefore, have a ready source of spermatozoa.

I feel that Luyet and Brown and other men working in this field with other species can be most useful to us by our keeping in close contact with them and even calling them in occasionally on a consultation basis. Their consultations with us will throw fresh ideas and viewpoints into the picture as far as our work is concerned. These people can guide us in fundamental

approaches to our problems. There may arise certain fundamental problems as we work along which we do not have the facilities, abilities or time to attack ourselves and which might be studied just as well by men such as Luyet with the use of other species. When we confront certain specific problems of this nature, it would then be wise for us to farm out the work. I think that farming out the work in this way should be done only when we have certain rather specific problems of this nature which need to be solved which we can not solve ourselves.

My visits with Luyet and Brown were certainly extremely stimulating and interesting and worthwhile. At both places I had the feeling that I wish I could stay much longer -- even several days at each place. Both men are extremely busy, however, and I felt that the time I spent with them was all I should impose upon them. In fact, after my session with Luyet he practically asked me to leave because he had some work that had to be done before noon. Brown, also, is under considerable pressure of work and I felt that I actually spent more time with him than I should have.

I might also add that I spent a couple of hours with Professor R. E. Comstock of the statistical department at North Carolina State College. Several years ago he made a study of the effect that egg transplantation with farm animals might have upon progress in improvement of farm animals by breeding, especially dairy cattle. I wanted to get first-hand information from him which I will need in the preparation of the paper which I am to present in the symposium at Iowa State College in July.

I realize that the above account of this trip is not very well organized. The report was dictated in large part from my notes, and I have not taken the time to reorganize the material. I am mainly interested in subject matter.

Sincerely,

Elwin

E. L. Willett

ELW:RG