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EFFECT OF GLYCEROL IN PROTECTING TISSUE HOMOGENATES

AGAINST THE EFFECTS OF FREEZING

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CHAPTER I

INTRODUCTION

The preservation of viable tissue preparations and fully active tissue extracts for various studies has long been the nemesis of the cellular physiologist and enzymologist. Tissue and cellular preparations can be preserved at room temperatures for a very short time without loss of viability or activity. If the living material is chilled to and maintained at 4 to 5° C., it will retain most of its original enzymatic activity for several hours without an appreciable loss. Many times, however, the research worker has neither the time nor facilities to undertake a study of a biological specimen immediately. If methods could be devised whereby the material could be preserved and stored for a considerably longer period, it could be used whenever the time for study is available.

The freezing of most biological material results in a loss of cell viability and a concomitant loss of much of its enzymatic activity. However, considering the inverse relationship between the rate of metabolic activity and temperature, preservation at low temperatures would be advantageous if the tissue preparation could be protected against

many of the deleterious effects of sub-zero temperatures.

Despite the fact that much biological material is extremely sensitive to freezing, many organisms are not killed by near 0° C. temperatures. Investigators have reported that bacteria or their spores withstand temperatures of 0° C. (McLean, 1918; Smart, 1935; Tanner and Wallace, 1931; Lipman, 1936). However, other authors describe lethal and injurious effects at these low temperatures (Rivers, 1927; Park, Williams and Krumweide, 1924). Many of the yeasts and algae are able to grow and reproduce at low temperatures (Smart, 1935; Kadisch, 1931). The rhizopods, flagellates, and ciliates normally do not resist temperatures lower than a few degrees below 0° C. (Chambers and Hale, 1932; Jahn, 1933).

It is known that poikilotherms die when body temperature reaches a few degrees below 0° C. Warm-blooded animals cannot normally survive 0° C. body temperatures, except those that are adapted for hibernation (Murigin, 1937).

The spermatozoa of both poikilotherms and homoiotherms normally will withstand 0° C. temperatures (Weber, 1936; Smith and Polge, 1950). This may be due to the relatively small amount of water in the spermatozoa. The eggs of higher animals, such as birds and mammals, were generally found to be killed after having been exposed to a few degrees below 0° C. (Moran, 1925; Smith, 1952b). The eggs of some insects and worms have often been found viable after withstanding a temperature of -30 to -40° C. (Zawadowski, 1926 cited by Belehradek, 1935; Uvarov, 1931).

Schenck had some success in maintaining viable white blood cells of frogs and turtles after they had been subjected to a -70° C. for a short time (Luyet and Gehenio, 1940). Simonin (1931) was able to grow embryonic tissues of mouse, rat, and ox after they had been subjected to temperatures from 0° C. to a -15° C. Nerve cells and liver tissue were found to be more sensitive to low temperatures than heart, lung, and intestine. Adult nerve and muscle fibers are killed at a few degrees below 0° C. Ice crystals were found in the majority of these preparations (Chambers and Hale, 1932; Bahrmann, 1932).

Many forms of plants and animals, upon being dessicated, can survive the lowest temperatures that can be produced in the laboratory (Becquerel, 1932 a, b, c; 1950). He has been able to freeze dessicated bacteria, spores, seeds of plants, and insects' eggs to the temperature of liquid helium (-269° C.) without destroying their viability. Luyet and his associates have made many investigations into the problem of freezing and protecting tissues by a procedure that Luyet terms "vitrification of cells." Luyet, in his experiments, has attempted to dehydrate tissues and then freeze very rapidly in order to avert the deleterious effects caused by the formation of intracellular ice.

Several substances have been utilized to obtain dehydration. Luyet and Thoennes (1938), in their work with plant epidermis, used hypertonic solutions of sodium chloride. Hypertonic solutions of sucrose have been used in an effort to protect frog sperm (Luyet and Hodapp, 1938), and mouse and rat skin (Taylor, 1953) before freezing. Hypertonic solutions of glucose have also been used in experiments with chick embryo

hearts (Luyet and Hodapp, 1938). None of those substances in their various concentrations have increased survival to more than ten to forty per cent.

More recently, polyhydric alcohols such as ethylene glycol, propylene glycol, and glycerol have been used in studies concerning the preservations of organisms, tissues, and cells. The progress made in this field dates from the accidental discovery made by Polge, et al,,in 1949, that glycerol has the almost unique property of protecting the spermatozoa of the domestic cock against the otherwise lethal effects of freezing to -190° C. These sperm after freezing were fully motile and capable of fertilizing eggs from which were hatched normal chicks (Polge, 1951).

Since the discovery of glycerol as a protecting agent, there have been numerous investigations using the glycerol-freezing method of preservation of various types of cells and tissues. Viability of Entamoeba histolytica has been preserved following several days at -190° C. after treatment with glycerol solutions (Fulton and Smith, 1953). Attention also has been directed to the effects of the glycerol-freezing method of preservation of embryonic cells and tissue. Chang (1953), in his investigations, finds that the hearts of rabbit embryos will resume beating after thawing and warming following glycerol treatment and freezing. Nerve fiber explants from embryonic chick brain will continue to grow following thawing after treatment with ethylene glycol and freezing (Luyet and Gonzales, 1953). Chick embryo heart explants will also continue to grow after treatment with ethylene glycol (Luyet and Keane,

1952) or glycerol (Luyet and Gonzales, 1952) and freezing.

Billingham and Medawar (1952) discovered that skin from the rabbit could be frozen after treatment with glycerol; the skin was viable as an autograft after thawing. Taylor (1953) reported good growth in explants taken from cultures of mouse and rat skin after they had been treated with glycerol and frozen to a -195° C. Keeley, et al. (1952) also reported survival (at least 1 year) of dog skin as autografts after having been treated with glycerol and frozen for over two months.

Grand, et al. (1952) found that adrenocarcinomatic tissue which had been treated with ethylene glycol and frozen could be transplanted into healthy rats with a success of over 90 per cent.

Eastscott, et al. (1954) have been able to preserve human corneal grafts by treatment with glycerol and freezing. Survival and results obtained are comparable with fresh grafts.

Polge, Parkes and Smith in England, and Bunge and Sherman in the United States, have been the chief workers using the glycerol-freezing technique to preserve mammalian germ cells. In their experiments with rabbit sperm, Polge, et al. (1949) were not completely successful. However, Sherman (1954) and Bunge, et al. (1954) have perfected methods with which to preserve human sperm for months. These frozen spermatozoa have been used for impregnation and normal full term infants have been delivered (Bunge, et al., 1954).

Investigations into the survival of fertilized rabbit eggs after glycerol treatment and freezing have met with some success (Smith, 1952b).

The preservation of mammalian gonadal tissue by glycerol treatment and freezing has also been attempted. The testes of young rats retain their ability to grow and to develop as homografts. Spermatozoa have been found in scrotal grafts of testes (Deanesley, 1954b). The granulosa cells of rabbit ovarian tissue so treated survive and grow in culture 25 to 50 per cent of the time (Parkes and Smith, 1950; Smith, 1952a).

Immature rat ovary transplants will survive and cause return of the oestrus cycle following similar glycerol-freezing treatment (Deanesley, 1954a).

Effort has been directed toward utilizing the glycerol-freezing technique in an attempt to preserve red blood cells (Smith, 1950; Sloviter, 1951a, 1951b, 1952; Lovelock, 1952, 1953a, 1953b; Chaplin and Veall, 1953; Chaplin and Mollison, 1953; Brown and Hardin, 1953). Earlier work was concerned with attempts to find a method whereby a large volume of red blood cell suspensions could be preserved. As investigations progressed, it was found that as much as 500 ml. of blood could be preserved. Ninety per cent of human red cells stored in glycerol solutions at -70° C. have been recovered. Sixty-four per cent of these cells survived normally (120 day life) after transfusion. Probably more than 64 per cent survived for a shorter period of time. For in vivo survival of these cells the larger portion of the glycerol used in protecting the cells against freezing must be removed. This has been accomplished with great success by a stepwise method of diluting the glycerol.

Many tissue and cellular preparations are inactivated and

killed as they approach the dehydrated state and are useless for many types of *in vitro* studies, especially for enzymatic studies. Some cellular substances are also inactivated rapidly after death before measures such as chilling can be taken. Often *in situ* freezing of the entire carcass of the specimen is necessary to halt these biochemical processes in order to preserve various substances intact (LePage, 1948). This method, although cumbersome at times, is an excellent one for preserving many of the enzymes and substrates normally inactivated to some degree before preservation can be started. On the other hand, many of the enzymes are thermolabile and sub-zero temperatures may destroy them if they are not protected in any way (Hepburn, 1915; Rivers, 1927).

It is known that liver tissues which have been subjected to low temperatures respire at a very much lower rate than do fresh tissues (De Robertis and Nowinski, 1942; Lynen and Burkhardt, 1944).

Albaum, et al. (1952) discovered that there is a significant breakdown of adenine nucleotides in tissues stored from one to four weeks at low temperatures. Also, slow freezing breaks the linkage between most of the thiamin pyrophosphate protein complex of liver tissue. This does not occur if the freezing is done rapidly (Hammond, 1952).

Mondy and Daniel (1954) found that rat liver which had been frozen and homogenized showed a marked decrease in choline dehydrogenase activity.

The present problem was undertaken in an effort to devise some technical procedure to effect longer protection and preservation of tissue preparations and cellular homogenates. Such a protective action is afford-

ed by glycerol when used on some organisms and tissues. Results of foregoing experiments provide a basis for inquiries into further applications of the glycerol-freezing method. Using the amount of oxygen consumption as a criterion of enzymatic activity, experiments were planned in which oxygen consumption measurements would be done on various tissue preparations both before freezing and after freezing and thawing, and with and without glycerol in the tissue media.

CHAPTER II

MATERIALS AND METHODS

Source of Materials

The experimental animals used in this study were albino rats and albino rabbits. The rats were obtained from The Holtzman Rat Company, Madison, Wisconsin. The rabbits were purchased from the Oklahoma Rabbit Market, Oklahoma City. The ages of these animals varied during the course of experimentation from 90 to 120 days.

All of the standard chemicals used were of reagent grade. Adenosine triphosphate (ATP) was purchased from The Matheson Company, Incorporated. Diphosphopyridine nucleotide (DPN), cytochrome c, and alpha-ketoglutaric acid were purchased from Nutritional Biochemicals Corporation. Sodium succinate, sodium malonate, and glycerol were obtained from Eastman Organic Chemicals. The hexokinase was prepared and assayed according to the procedure outlined by Berger, et al. (1946). The water used in the preparation of the solutions was distilled by a Barnstead, Steam Heated, Standard Model, Distilling Apparatus.

Concentration and Composition of Tissue Media

The basic medium used throughout the experiments on tissue chunks or pieces and brain homogenates was the Krebs-Ringer-phosphate buffer as described by Umbreit, et al. (1949). The buffer contained

0.127 M NaCl, 0.005 M KCl, 0.0019 M $CaCl_2$, 0.00119 M $MgSo_4 \cdot 7H_2O$, and 0.01 M KH_2PO_4 and Na_2HPO_4. The final pH was 7.4. The presence of calcium ions in this buffer is justified by the investigations of Elliott and Libet (1942), Elliott and Henry (1946), and Elliott (1948). They found that the presence of calcium decreases the oxygen consumption of rat brain homogenates in both the bicarbonate and the Ringer-phosphate systems. However, calcium prolongs the maintenance of oxygen consumption over relatively long periods of time. Since, in these experiments measurements were desired for periods up to two hours, calcium was included in the buffer. The phosphate buffer system was used in preference to the bicarbonate buffer system because it is easier to prepare and maintain with less variability. Dickens and Simer (1931) found no significant difference in the QO_2 or respiratory quotient of tissue slices in bicarbonate or phosphate Ringer solutions. More recently, Elliott (1948) found no difference in the oxygen consumption of brain tissue slices and suspensions from various mammals when measured in bicarbonate and phosphate Ringer buffers.

In the majority of experiments it was desired to measure the oxygen consumption of concentrated homogenates of brain tissue. These preparations, although diluted to a final concentration of 10 per cent, still contained enzymes, cofactors, activators, and substrates in concentrations high enough to give satisfactory oxygen consumption measurements. Therefore, substances such as DPN, ATP, and cytochrome c were not added, except in those experiments in which it was desired to discover their individual effect on the oxygen uptake of frozen homogenates or to

study individual enzyme systems.

Glucose (0.2 per cent) was added to all these homogenates in order to maintain oxygen consumption at a high level for relatively long periods of time. Elliott and Libet (1942) noted that suspensions of brain tissue prepared by homogenization in an isotonic medium respire as much as 400 per cent more than suspensions prepared in hypotonic media. In initial experiments designed to show the effect of glycerol on fresh tissue and on frozen tissue, 30 per cent tissue homogenates were prepared in concentrations of glycerol ranging from 15 per cent to 30 per cent. In such instances, 30 per cent glycerol in Krebs-Ringer-phosphate buffer was added to the tissue with no allowance being made for the volume of glycerol added and its effect in diluting the ionic content of the medium. If the cell membrane is completely permeable to glycerol it will exert no osmotic effect in the medium; if mitochondrial membranes are involved this might also be true. It seems possible that a solution containing as much as 30 per cent glycerol effectively might be hypotonic. In later experiments the electrolyte concentration of the glycerol solutions was adjusted to be equal to that of Krebs-Ringer-phosphate buffer.

Oxygen consumption measurements were also done on dilute rat brain homogenates (1.67 per cent). These experiments were designed to measure the efficiency of individual enzyme systems; isotonic KCL (0.154 M) was used as a homogenizing medium. All of these homogenates (fresh and frozen) were then pipetted into chilled Warburg vessels containing reaction media which will be listed in another chpater of this thesis.

Oxygen Consumption Measurements

Oxygen consumption measurements were made at 38° C. using the Warburg modification of the Barcroft apparatus. The direct method was employed (Umbreit, et al., 1949). The Warburg vessels were of 15 ml. capacity with one sidearm. The volume of reaction medium in each vessel varied from 2.06 ml. to 2.8 ml. depending upon the study being made. Placed in the center well was 0.2 ml. of 20 per cent KOH. The stroke rate of the apparatus for the initial experiments was 128 per minute. Under those conditions it was not uncommon to find tissue in the center well following oxygen consumption measurements. The mixing of homogenate with KOH in the center well led to variability in results which was prevented by reducing the stroke rate to 108 per minute. After a 15 minute period for gaseous and temperature equilibration, the manometers were closed to the outside atmosphere. Oxygen consumption measurements were made at 10 or 15 minute intervals over a period of one to two hours depending on the study being made. Oxygen uptake is expressed as oxygen consumed per 100 milligrams of tissue, wet weight.

Preparation of Tissues

In the experiments designed to determine the oxygen consumption of brain tissue, the tissue was obtained after the animals were killed by decapitation. The cerebral hemispheres were removed and rapidly washed in chilled Krebs-Ringer-phosphate buffer. The tissue was kept in chilled buffer until a sufficient quantity could be obtained for an experiment. Six rat brains were commonly used in each experiment requir-

ing six to eight minutes for removal of the tissue. Only one rabbit brain was needed for a single experiment. The cerebral hemispheres were then blotted with filter paper and weighed on an analytical balance. The disposition of the tissue differed in various experiments.

Tissue chunks. In the experiments conducted to determine the effect of glycerol in protecting brain chunks from damage at low temperatures, the rat brain hemispheres were chopped into eight roughly equal portions and put in small beakers containing chilled mixtures of glycerol and Krebs-Ringer-phosphate buffer where they were allowed to equilibrate for 15 minutes. They were then placed in an ice-chest containing dry ice for 24 hours. The beakers were placed directly on the dry ice so that it was only a matter of seconds before the tissue reached the temperature of the dry ice (-79° C.). After thawing in a water bath at 40° C., the brain chunks were washed three times in decreasing concentrations of glycerol. The tissue chunks were then treated as fresh brain for the preparation of homogenates.

Tissue homogenates. Tissue homogenates were used in the major portion of the experimental work. The tissues, ready for homogenization, were transferred to Potter-Elvehjem type homogenizing tubes, which were kept in an ice bath. In preliminary experiments the percentage of tissue homogenized and the presence or absence of glycerol plus the concentration of glycerol in the homogenate were varied. The results of these variations will be discussed later. Homogenizing the tissue at a concentration of 20 per cent in chilled Krebs-Ringer-phosphate buffer containing 0.2 per cent glucose was found to be the most satisfactory method

of preparing concentrated brain homogenates.

The 20 per cent homogenates were pooled in an Erlenmeyer flask in an ice bath, and were diluted in the Erlenmeyer flask to 10 per cent homogenates with Krebs-Ringer-phosphate buffer or with a mixture of glycerol and Krebs-Ringer-phosphate buffer. A quantity of 2.8 ml. of the 10 per cent homogenate was pipetted into the Warburg vessels in an ice bath. In other experiments, 1.4 ml. of the 20 per cent homogenates was pipetted into the Warburg vessels which contained 1.4 ml. of Krebs-Ringer-phosphate buffer or glycerol and Krebs-Ringer-phosphate buffer.

For oxygen consumption measurements on fresh tissue the homogenates were allowed to stand in the Warburg vessels in an ice bath for an additional 20 minutes. This action compensated for a similar time interval required in setting up an experiment with frozen tissue.

For oxygen consumption measurements on frozen tissue, Warburg vessels containing 2.8 ml. of a 10 per cent homogenate in Krebs-Ringer-phosphate buffer plus glycerol and Krebs-Ringer-phosphate buffer were placed in an ice-chest containing dry ice. The temperature of the dry ice-chest was -79° C. The ice-chest was kept in a refrigerated room for the freezing time desired, which varied from 24 hours to 28 days. After the freezing period the preparations were thawed by placing the vessels in a water bath at 40° C. They were then put in an ice bath at 4-5° C. until they could be adjusted on the Warburg manometers. In the meantime, 0.2 ml. of 20 per cent KOH solution was pipetted in the center well. The time required for the procedure was approximately 20 minutes. Later it was found that the KOH solution could be placed in the center well before

freezing with no deleterious results.

Tissue was also frozen in Erlenmeyer flasks as approximately 25 ml. of a 10 per cent homogenate in Krebs-Ringer-phosphate buffer plus glycerol or Krebs-Ringer-phosphate buffer. These preparations were also thawed at 40° C. and then placed in an ice bath while 2.8 ml. transfers were made to chilled Warburg vessels.

The Warburg vessels containing fresh or frozen-thawed tissue were then placed on the manometers and, following a 15 minute temperature equilibration period in the Warburg bath, oxygen consumption measurements were begun.

The effect of glycerol and freezing on the oxygen consumption of diluted homogenates, using alpha-ketoglutarate and succinate as substrates, was also determined. The homogenates were prepared by the method of Potter, et al. (1948), using isotonic KCL (0.154 M) as a homogenizing medium. A total of 50 milligrams of tissue (1.67 per cent) was pipetted into the Warburg flasks which contained the various substrates, cofactors, and activators. Sodium malonate (0.033 M) was added to the reaction mixture in which alpha-ketoglutarate was the substrate, to inhibit the further oxidation of alpha-ketoglutarate after it proceeds one step to succinate (Ochoa, 1944). The vessels containing the alpha-ketoglutarate as substrate were allowed an equilibration period of 15 minutes before measurements of oxygen consumption were begun. The vessels containing succinate as a substrate were allowed a 15 minute equilibration period before succinate was added to the reaction mixture from the sidearm. This preliminary equilibration at 38° C. causes the destruction of

other oxidizing enzymes, thus permitting study of only the succinoxidase system (Green, et al., 1948). The vessels were then equilibrated an additional 5 minutes following the dumping of succinate before oxygen consumption measurements were begun.

"Mitochondrial preparations." Homogenates of rat brain were treated by the method of Brody and Bain (1952) which they describe as a means of isolating mitochondria from rat and rabbit brain. This material will be referred to as "mitochondrial preparations" in the remainder of the thesis. Oxygen consumption and phosphorylation were studied using these preparations. The animals were killed by decapitation and the cerebral hemispheres were removed at once to small beakers containing cracked ice. All other preparations were carried out at temperatures very near 0° C. The hemispheres were then homogenized with three volumes of 0.25 M sucrose in a Potter-Elvehjem homogenizing tube. The final preparation was divided into two equal portions. The final solute of one portion was 0.25 M sucrose while that of the other half was 0.25 M sucrose containing 10 per cent glycerol. The two portions were again divided into halves and one part of each was immersed and frozen in a mixture of dry ice and acetone. The other parts were used as fresh controls. The frozen "mitochondrial preparations," after 15 minutes in the freezing mixture, were thawed in a water bath at 40° C. All fractions (fresh and frozen) were then pipetted into chilled Warburg vessels containing the reaction medium which will be listed in another chapter of this thesis.

The vessels were then placed on the manometers which were put into the Warburg bath and flushed for one and one-half minutes with oxy-

gen. They were allowed to equilibrate for 5 minutes after which the vessels were closed to the atmosphere. Oxygen consumption measurements were taken at 10 minute intervals for fifty minutes.

It was desired to test the "mitochondrial preparations" as to their ability to esterify inorganic phosphate at the same time that oxygen consumption measurements were being made. Hexokinase was employed in the reaction medium in order to trap the adenosine triphosphate formed. Glucose in the presence of hexokinase and adenosine triphosphate will utilize the adenosine triphosphate to form glucose-6-phosphate, thus removing the phosphate radical from adenosine triphosphate before it can be broken down by adenosine triphosphatase. This method was devised by Cross (1949). The phosphate uptake was measured as the difference between the inorganic phosphate present at the beginning of the oxygen consumption measurements and that present at the end of these measurements. Trichloroacetic acid was added at the beginning of the experiment to one of duplicate vessels to stop metabolic activity at once and it was added to the other duplicate at the end of the oxygen consumption measurements.

Inorganic phosphate was determined on the protein-free filtrate of all vessels by the spectrophotometric method of Fiske and Subbarow (1925). A ratio of the micromoles of phosphorus utilized to the microatoms of oxygen consumed was then determined. This was done in order to determine if phosphorylation occurred with oxidation in the frozen and glycerol-treated preparations to the same degree that it occurred in the normal fresh preparations.

CHAPTER III

RESULTS

Experiment I

De Robertis and Nowinski (1942) and Nowinski and De Robertis (1943) have shown in their investigations that freezing rat liver tissue prior to homogenization will cause a great decrease in the capacity of the tissue to consume oxygen. Figure 1 illustrates a comparable decrease in the oxygen consumption of rat brain homogenates which have been frozen to -79° C. for a period of 24 hours. The respiration of rat brain homogenates is lowered approximately 50 to 80 per cent by the process of freezing and thawing. Tissues frozen as 40 per cent homogenates respire at a higher rate than do the preparations frozen as 10 per cent homogenates. A possible explanation is given on page 46.

Experiment II

Deanesley (1954a, 1954b) and Parkes and Smith (1950) have had excellent results in the preservation of ovarian and testicular tissue chunks by the use of the glycerol-freezing technique. It was thought that such a method might be utilized to preserve tissues which later would be homogenized after thawing and clearing of glycerol. The degree of glycerol protection against sub-zero temperatures (-79° C.) can be seen in Figure 2. This method of glycerol treatment appears to impart

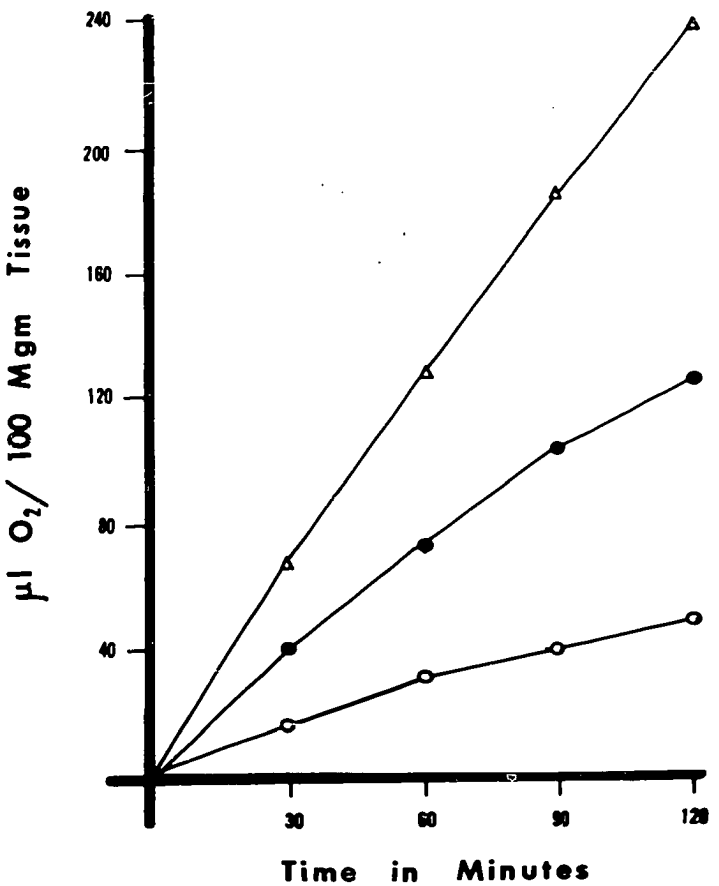

Figure 1. The effect of freezing on the oxygen consumption of rat brain homogenates. Each reaction vessel contained 2.8 ml. of 10 per cent homogenate (280 mgm. tissue) in Krebs-Ringer-phosphate buffer containing 0.2 per cent glucose as substrate. In the center well was placed 0.2 ml. 20 per cent KOH. Total volume was 3.0 ml. Temperature was 38° C. Gas phase was air. Frozen preparations were maintained at -79° C. for 24 hours.

- Δ Fresh homogenates (14 vessels)
- ● Frozen homogenates, frozen as 40 per cent homogenates (22 vessels)
- O Frozen homogenates, frozen as 10 per cent homogenates (11 vessels)

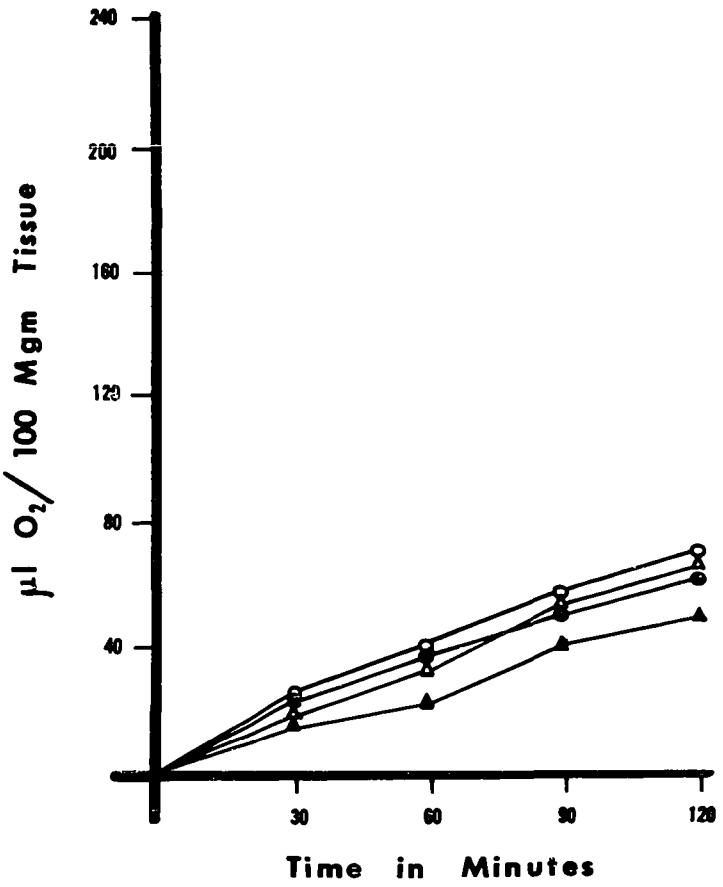

Figure 2. The oxygen consumption of homogenates prepared from chunks of rat brain treated with various concentrations of glycerol and frozen. Each reaction vessel contained 2.8 ml. of 10 per cent homogenate (280 mgm. tissue) prepared from previously frozen rat brain chunks. The reaction medium was Krebs-Ringer-phosphate buffer containing 0.2 per cent glucose as substrate. In the center well was placed 0.2 ml. 20 per cent KOH. Total volume was 3.0 ml. The temperature was 38° C. The gas phase was air. Frozen preparations were maintained at -79° C. for 24 hours.

▲ No glycerol (4 vessels)
● Treated with 30 per cent glycerol (4 vessels)
O Treated with 60 per cent glycerol (4 vessels)
Δ Treated with 100 per cent glycerol (4 vessels)

no significant protection to the small tissue chunks which have been frozen for 24 hours. Therefore, a new approach must be attempted.

Experiment III

In experiments on the preservation of epithelial tissue, red blood cells, sperm, and ovarian tissue, various investigators have failed to take into consideration the alteration of the tonicity of the tissue preparation media caused by the addition of glycerol, especially in large amounts (Keeley, et al., 1952; Deanesley, 1954a, 1954b; Smith, 1950; Sherman, 1954; Bunge, et al., 1954). In most instances, the concentration of glycerol used ranged from 15 per cent to 40 per cent. At those concentrations the ionic content of the media is diluted considerably.

Oxygen consumption measurements were made on fresh and frozen (-79° C. for 24 hours) brain homogenates which had been homogenized in various Krebs-Ringer-phosphate buffer solutions to which had been added various concentrations of glycerol: (a) Oxygen consumption measurements were made on preparations homogenized in solutions containing the total ionic content of Krebs-Ringer-phosphate buffer to which had been added desired concentrations of glycerol. These were designated as "adjusted glycerol-Krebs-Ringer-phosphate buffer solutions." (b) Oxygen consumption measurements were also made on preparations homogenized in glycerol-Krebs-Ringer-phosphate buffer solutions whose ionic content decreased as the glycerol content increased. These solutions were called the "unadjusted glycerol-Krebs-Ringer-phosphate buffer solutions." The results of these experiments (Table I) indicate that the ionic content of the medium is definitely a factor to be considered in oxygen consumption measurements

TABLE I

OXYGEN CONSUMPTION OF FRESH AND FROZEN

GLYCEROL-TREATED RAT BRAIN HOMOGENATES

CONCENTRATION OF GLYCEROL IN MEDIA	TOTAL MICROLITERS O_2 / 100 MGM. TISSUE WET WEIGHT / 2 HOURS							
	ADJUSTED MEDIA				UNADJUSTED MEDIA			
	FRESH	NO. OF VESSELS	FROZEN	NO. OF VESSELS	FRESH	NO. OF VESSELS	FROZEN	NO. OF VESSELS
5%	241	6	207	6	234	8	175	12
10%	237	8	240	22	229	7	210	7
15%	225	6	211	10	216	7	180	6
20%	213	6	187	7	188	7	178	6
30%	163	6	143	6	124	7	123	8

Each reaction vessel contained 2.8 ml. of 10 per cent homogenate (280 mgm. tissue). The reaction medium was adjusted glycerol-Krebs-Ringer-phosphate buffer or unadjusted glycerol-Krebs-Ringer-phosphate buffer which contained 0.2 per cent glucose as the substrate. The glycerol concentration varied as indicated above. Placed in the center well was 0.2 ml. 20 per cent KOH. Total volume was 3.0 ml. Gas phase was air. Temperature was 38° C. Frozen preparations were kept at -79° C. for 24 hours.

both of fresh and of frozen homogenates at the glycerol concentrations tested. The oxygen consumption both of fresh and of frozen homogenates was higher in "adjusted glycerol-Krebs-Ringer-phosphate buffer solutions" than in "unadjusted solutions." In later experiments all of the glycerol-buffer media were "adjusted solutions."

Experiment IV

In previous research using the glycerol-freezing method on intact cells and tissues, it was found that an equilibration period in glycerol (one to two hours) is necessary before freezing to allow the glycerol to permeate the cells and tissues adequately (Sloviter, 1951a; Smith, 1952b; Deanesley, 1954a). Studies were made to discover if such equilibration is necessary when freezing tissue homogenates. Rat brain homogenates were allowed to equilibrate in glycerol for intervals of 5 to 45 minutes before a 24 hour freezing period. Table II shows that a prolonged period of equilibration does not enhance the protective action of glycerol during freezing as measured by the rate of oxygen consumption. However, homogenizing in 10 per cent glycerol seems to be more effective in maintaining the oxygen consumption of frozen-thawed homogenates than does homogenizing in buffer with the addition of glycerol prior to freezing. This occurs regardless of the time allowed for equilibration. An explanation for this is not apparent.

Experiment V

In Table I and Figure 3, the effect of various concentrations of glycerol on fresh and frozen homogenates is illustrated. It was

TABLE II

EFFECT OF EQUILIBRATION IN 10 PER CENT GLYCEROL-KREBS-RINGER-PHOSPHATE BUFFER BEFORE FREEZING ON OXYGEN CONSUMPTION OF FROZEN RAT BRAIN HOMOGENATES

HOMOGENIZING MEDIUM	EQUILIBRATION TIME IN MINUTES	NUMBER OF VESSELS	TOTAL MICROLITERS O_2 / 100 MGM. TISSUE, WET WEIGHT / 2 HRS.
BUFFER	5	3	234
	15	3	234
	30	3	234
	45	3	220
10% GLYCEROL	5	3	255
	15	3	252
	30	3	246
	45	3	235

Each reaction vessel contained 2.8 ml. of 10 per cent homogenate (280 mgm. tissue). The reaction medium was 10 per cent glycerol-Krebs-Ringer-phosphate buffer which contained 0.2 per cent glucose as the substrate. Placed in the center well was 0.2 ml. of 20 per cent KOH. Total volume was 3.0 ml. Gas phase was air. Temperature was 38° C. Homogenates were kept at -79° C. for 24 hours.

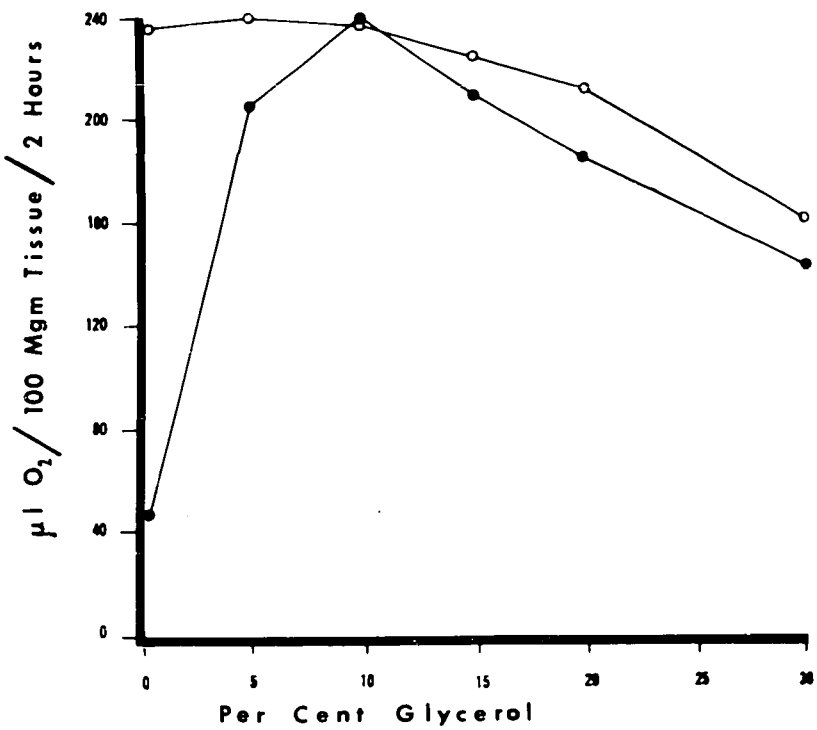

Figure 3. The effect of various concentrations of glycerol on oxygen consumption of fresh and frozen homogenates of rat brain. Each reaction vessel contained 2.8 ml. of 10 per cent homogenate (280 mgm. tissue). The reaction medium was Krebs-Ringer-phosphate buffer which contained 0.2 per cent glucose as the substrate. Glycerol varied as indicated on the absissa. In the center well was placed 0.2 ml. 20 per cent KOH. Total volume was 3.0 ml. Temperature was 38° C. Gas phase was air. Frozen preparations were maintained at -79° C.

- O Fresh homogenates*
- ● Frozen homogenates*

* See Table I for the number of vessels represented by each point.

found that glycerol has no significant effect on fresh homogenates in concentrations up to 15 per cent. With the frozen tissue it was necessary to determine at what concentration glycerol would be the most effective in maintaining the rate of oxygen consumption at a level of fresh tissue and yet not cause an inhibition of oxygen consumption due to the concentration of the glycerol itself. It can be seen that glycerol in the concentration of 10 per cent seems to protect the tissue fully against a temperature of -79° C. The oxygen consumption of frozen homogenates treated with 10 per cent glycerol is approximately that of fresh homogenates treated with glycerol. It appears that as the glycerol concentration of the fresh homogenates increases, the rate of oxygen consumption decreases. A possible explanation of this trend is discussed on page 48. Also to be noted is the fact that at all other concentrations of glycerol (except 10 per cent) the oxygen consumption of the frozen homogenates is relatively lower than that of the fresh homogenates. The mechanism of this occurrence at the higher concentrations of glycerol is not understood.

Experiment VI

Table I and Figure 3 show that when homogenates containing 15 to 30 per cent glycerol (which theoretically should afford a protective action equivalent to that of 10 per cent glycerol) are frozen, oxygen consumption is lower than with 10 per cent glycerol. Since glycerol is viscous, it may alter the oxygen solubility of the homogenates, thus lowering oxygen consumption. It was decided that by freezing concentrated (20 to 40 per cent) homogenates with the higher concentrations of

glycerol and diluting them to 10 per cent homogenates, thus lowering the glycerol concentrations to 10 per cent or less, full protection might be given the preparations without lowering the oxygen consumption. Figure 4 is a comparison of the results obtained from experiments in which there was a dilution of the homogenate and glycerol and the results of experiments in which there was no dilution of the homogenate and glycerol. There is a progressive lowering of oxygen consumption as the degree of dilution of glycerol increases. This is contrary to the results which were expected.

Experiment VII

In view of the experience of Lovelock (1952) and Sloviter (1951a, 1952), it seemed possible that the lowered oxygen consumption was due to the rapid dilution of glycerol. Slow dilution over an interval of 30 minutes was then attempted to determine if the rapid dilution had caused the decrease.

Oxygen consumption measurements were done on 10 per cent homogenates which had 10 per cent glycerol-Krebs-Ringer-phosphate buffer in the reaction medium. Three sets of vessels were prepared. One set of vessels contained 10 per cent homogenate in Krebs-Ringer-phosphate buffer with no glycerol. A second set of vessels contained 10 per cent homogenate prepared in 10 per cent glycerol-Krebs-Ringer-phosphate buffer solution. The third set of vessels contained preparations which were made as 30 per cent homogenates with 30 per cent glycerol in the homogenizing medium. This set was divided into three different series of vessels: (a) The first series was diluted immediately with Krebs-

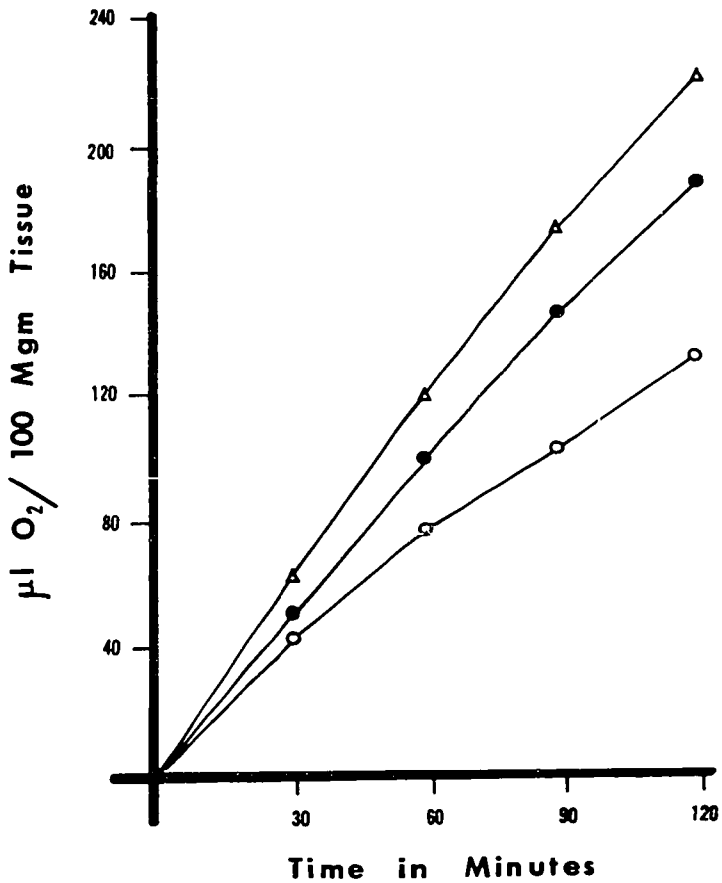

Figure 4. The effect of diluting the glycerol content of fresh rat brain homogenates on the oxygen consumption. The initial glycerol concentration in all vessels was 15 per cent. Each reaction vessel contained 2.8 ml. of 10 per cent homogenate (280 mgm. tissue) in Krebs-Ringer-phosphate buffer containing 0.2 per cent glucose as the substrate and the concentration of glycerol as stated. In the center well was placed 0.2 ml. of 20 per cent KOH. Total volume was 3.0 ml. Temperature was 38° C. Gas phase was air.

△ Final glycerol concentration: 15 per cent (13 vessels)
● Final glycerol concentration: 7.5 per cent (28 vessels)
○ Final glycerol concentration: 3.0 per cent (14 vessels)

Ringer-phosphate buffer to a 10 per cent homogenate containing 10 per cent glycerol; (b) the second series was diluted dropwise to a 10 per cent homogenate containing 10 per cent glycerol over an interval of 30 minutes; and (c) the third series was diluted rapidly to a 10 per cent homogenate containing 10 per cent glycerol at the end of the 30 minute period. All vessels remained in an ice bath from the time of homogenization until they were put on the Warburg manometers which was an interval of 30 minutes duration.

If the assumption that the decreased oxygen consumption was due to the rapidity of glycerol dilution is correct, then the homogenates which were diluted rapidly should consume oxygen at a much lower rate than the other vessels, and the original 30 per cent homogenates with 30 per cent glycerol-Krebs-Ringer-phosphate buffer which were diluted gradually to 10 per cent should respire at the same rate as the original 10 per cent homogenate. However, this was not the case (Figure 5). All the homogenates which contained 30 per cent glycerol originally, consumed oxygen at a lower rate than did the homogenates containing 10 per cent glycerol originally. A possible explanation of this is that the inhibition occurs at the moment glycerol is added rather than when the glycerol is diluted.

Experiment VIII

A group of experiments was also done to determine whether the addition of relatively high concentrations of glycerol to homogenates caused the inhibition of oxygen consumption. Oxygen consumption measurements were made on 10 per cent homogenates which had 30 per cent

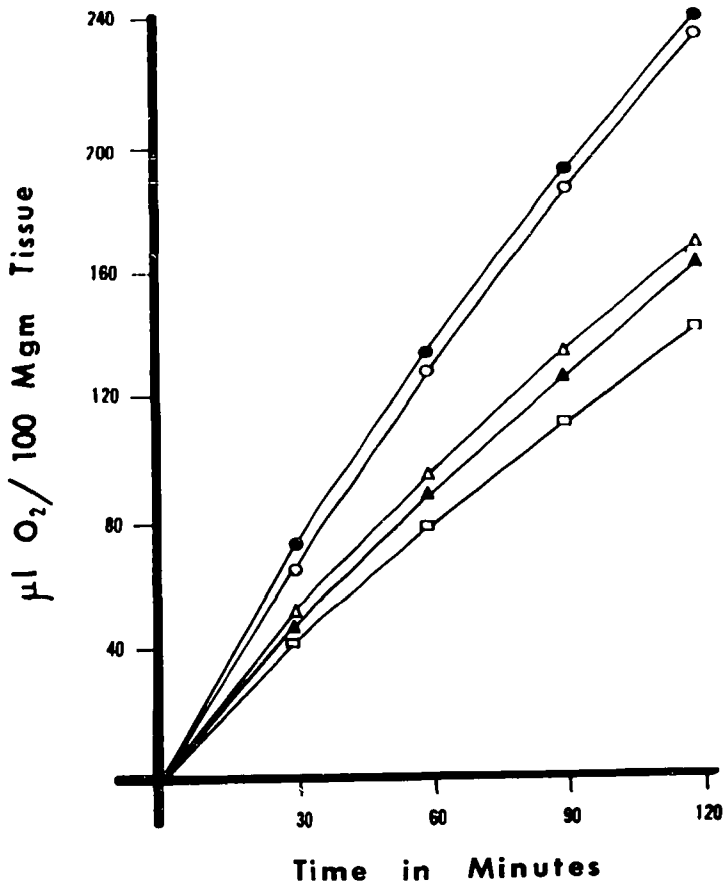

Figure 5. The effect of rapid and gradual dilution on the oxygen consumption of fresh, glycerol-treated rat brain homogenates. There was an interval of 30 minutes for all vessels from homogenization until beginning of oxygen consumption measurements. Each reaction vessel contained 2.8 ml. of 10 per cent homogenate (280 mgm. tissue) in Krebs-Ringer-phosphate buffer containing 0.2 per cent glucose as a substrate. In the center well was placed 0.2 ml. 20 per cent KOH. Total volume was 3.0 ml. Temperature was 38° C. Gas phase was air.

- ● No glycerol (4 vessels)
- ○ 10 per cent glycerol (3 vessels)
- △ 10 per cent glycerol, diluted from 30 per cent glycerol immediately (3 vessels)
- ▲ 10 per cent glycerol, diluted from 30 per cent glycerol dropwise over a 30 minute interval (3 vessels)
- □ 10 per cent glycerol, diluted from 30 per cent glycerol at the end of a 30 minute interval (3 vessels)

glycerol-Krebs-Ringer-phosphate buffer as a reaction medium. Three sets of vessels were prepared. One set of vessels contained a 10 per cent homogenate in Krebs-Ringer-phosphate buffer with no glycerol in the medium. A second set of vessels contained a 10 per cent homogenate in 30 per cent glycerol-Krebs-Ringer-phosphate buffer, which had been homogenized in 30 per cent glycerol-Krebs-Ringer-phosphate buffer. A third set of vessels contained preparations which were made as 30 per cent homogenates with 10 per cent glycerol in the homogenizing medium. They were changed to 10 per cent homogenates in 30 per cent glycerol by the addition of glycerol-buffer. This set was divided into three different series of vessels: (a) The first series had a glycerol-Krebs-Ringer-phosphate buffer solution added to it immediately to make it a 10 per cent homogenate with 30 per cent glycerol in the reaction medium; (b) a second series was prepared as (a) but the glycerol-Krebs-Ringer-phosphate buffer solution was added to it gradually over a period of 30 minutes so that its final glycerol concentration was 30 per cent; and (c) the third series of vessels was also prepared as (a) but the glycerol-Krebs-Ringer-phosphate buffer solution was rapidly added to the homogenate at the end of a 30 minute interval in order to make it a 30 per cent glycerol solution. All preparations remained in an ice bath from the time of homogenization until they were put on the Warburg manometers, which was an interval of 30 minutes duration. If the assumption that the decreased oxygen consumption was due to the rapidity of the addition of glycerol is correct, the series of vessels to which glycerol is added gradually to make a final concentration of 30 per cent should respire more than

any of the others. This, however, did not occur; all the homogenates containing 30 per cent glycerol respired at approximately the same rate (Figure 6).

Experiment IX

To be of practical value, the tissue homogenates must be preserved for lengths of time in excess of 24 hours. Tissue homogenates containing different concentrations of glycerol-Krebs-Ringer-phosphate buffer solutions (5, 10, and 15 per cent) were frozen at -79° C. for periods of 14 and 28 days. At the end of this time oxygen consumption measurements were made. Table III shows that at these concentrations of glycerol, oxygen consumption is approximately the same at the end of 14 days and 28 days as it was at the end of one day at -79° C.

Experiment X

It is possible that even though the frozen homogenates are protected from sub-zero cold by glycerol and do consume oxygen at a normal rate, the process of freezing might injure or destroy some enzymes or cofactors in the metabolic pathway of glucose. Previous workers have discovered that the enzymes of the glycolytic part of the pathway are not injured by freezing (Lynen and Burkhardt, 1944; Novikoff, et al., 1948).

Experiments were conducted to determine the effect of freezing and glycerol on the oxygen consumption of dilute homogenates, using alpha-ketoglutarate and succinate as substrates. It was found that glycerol has an inhibiting action on the oxygen consumption when these two substrates were used (Figure 7 and Figure 8). Glycerol appears to offer

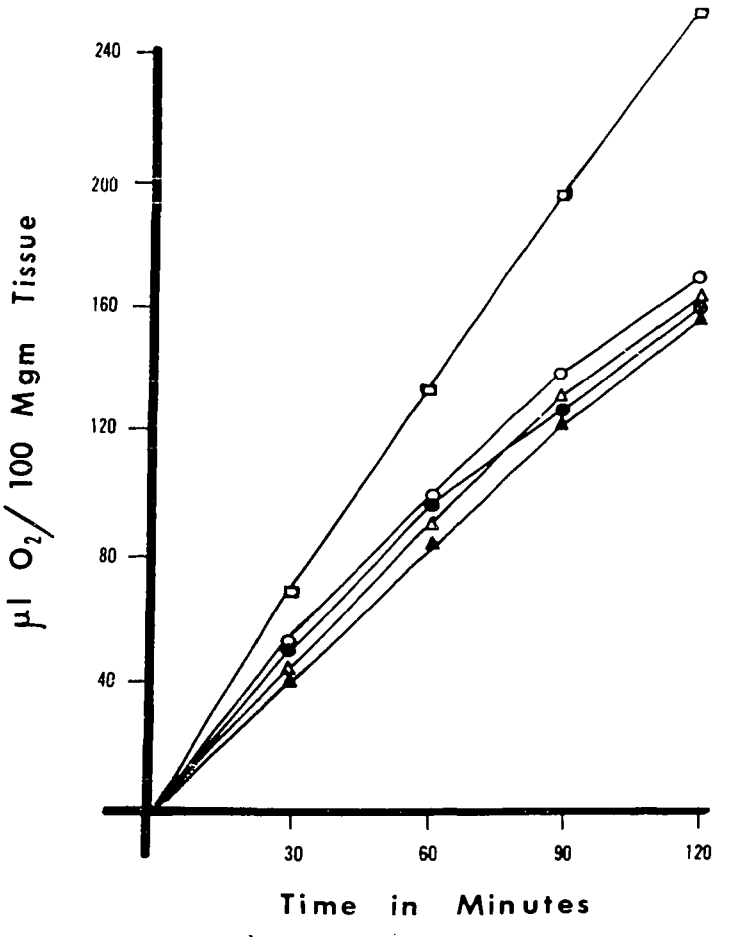

Figure 6. The effect of rapid and gradual addition of glycerol on the oxygen consumption of fresh rat brain homogenates. There was an interval of 30 minutes from homogenization until beginning of oxygen consumption measurements. Each reaction vessel contained 2.8 ml. of 10 per cent homogenate (280 mgm. tissue) in Krebs-Ringer-phosphate buffer containing 0.2 per cent glucose as the substrate and the indicated concentrations of glycerol. In the center well was placed 0.2 ml. 20 per cent KOH. The total volume was 3.0 ml. Temperature was 38° C. Gas phase was air.

- □ No glycerol (4 vessels)
- ○ 30 per cent glycerol (3 vessels)
- ● 30 per cent glycerol, increased from 10 per cent glycerol immediately (3 vessels)
- △ 30 per cent glycerol, increased from 10 per cent glycerol dropwise over a 30 minute interval (3 vessels)
- ▲ 30 per cent glycerol, increased from 10 per cent glycerol at the end of a 30 minute interval (3 vessels)

TABLE III

EFFECT OF PROLONGED FREEZING (-79° C.) ON THE OXYGEN CONSUMPTION OF GLYCEROL-TREATED RAT BRAIN HOMOGENATES

CONCENTRATION OF GLYCEROL IN MEDIA	TOTAL MICROLITERS O_2 / 100 MGM. TISSUE, WET WEIGHT / 2 HOURS							
	UNFROZEN		FROZEN					
	FRESH	NO. OF VESSELS	1 DAY	NO. OF VESSELS	14 DAYS	NO. OF VESSELS	28 DAYS	NO. OF VESSELS
5%	241	6	207	6	206	5	216	6
10%	237	8	240	22	245	5	245	5
15%	225	6	211	10	215	5	219	5

Each reaction vessel contained 2.8 ml. of 10 per cent homogenate (280 mgm. tissue). The reaction medium was glycerol-Krebs-Ringer-phosphate buffer in which the glycerol concentration varied as indicated above. Placed in the center well was 0.2 ml. 20 per cent KOH. Total volume was 3.0 ml. Gas phase was air. Temperature was 38° C.

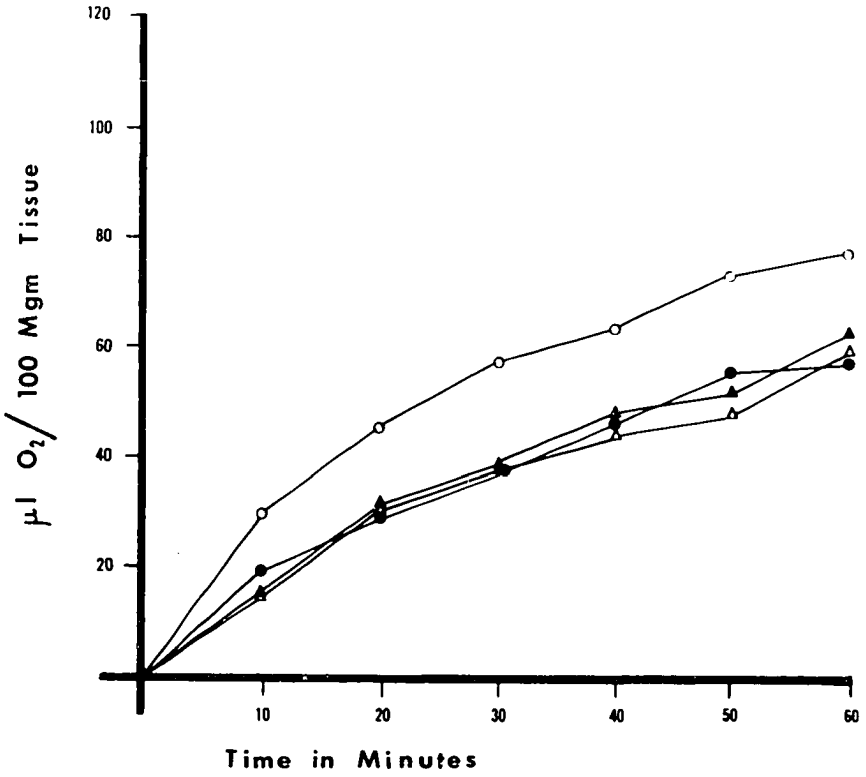

Figure 7. The oxidation of alpha-ketoglutarate to succinate in the presence of malonate in fresh and frozen rat brain homogenates with and without glycerol. The following additions were made to the reaction vessels: 0.5 ml. of 10 per cent rat brain homogenate (50 mgm. tissue), 0.1 ml. of M sodium malonate, 0.5 ml. of distilled H_2O, 0.4 ml. of 0.5 M KCl, 0.1 ml. of 0.1 M $MgCl_2$, 0.1 ml. of 4 X 10^{-4} M cytochrome c, 0.2 ml. of 0.1 M KH_2PO_4 plus KOH (to pH 7.4), 0.3 ml. of 0.01 M ATP, 0.2 ml. of 0.2 per cent DPN, 0.1 ml. of 0.154 KCl and 0.3 ml. of 0.1 M sodium alpha-ketoglutarate. Added to the center well was 0.2 ml. of 20 per cent KOH. Total volume was 3.0 ml. Temperature was 38° C. The gas phase was air.

- O Unfrozen homogenate, without glycerol (4 vessels)
- ● Unfrozen homogenate, with glycerol (4 vessels)
- Δ Frozen homogenate, without glycerol (4 vessels)
- ▲ Frozen homogenate, with glycerol (4 vessels)

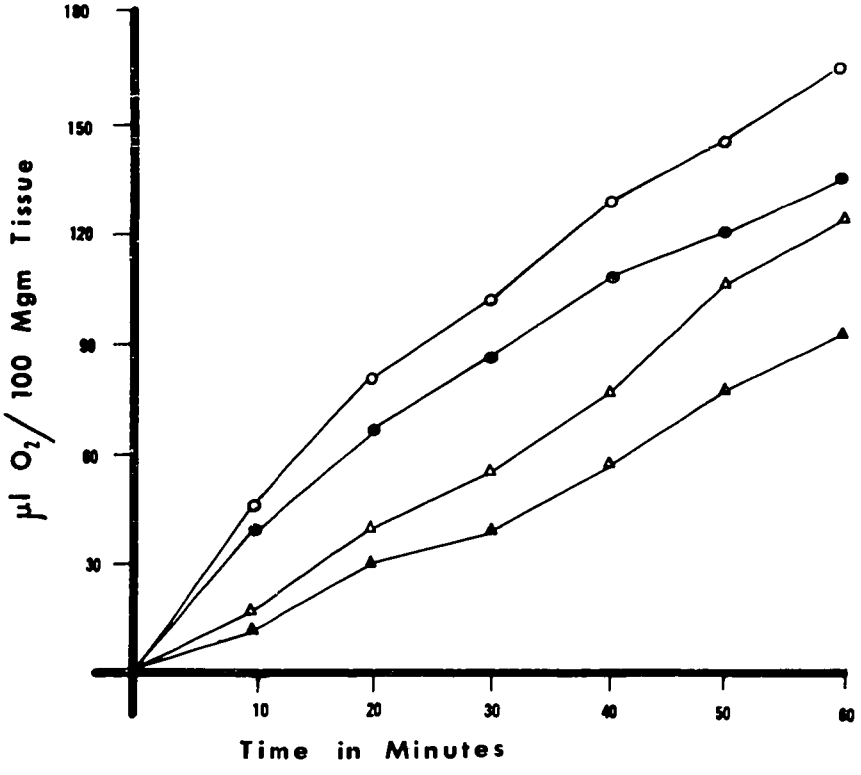

Figure 8. The oxidation of succinate to fumarate in fresh and frozen rat brain homogenates with and without glycerol. The following additions were made to the reaction vessels: 0.5 ml. of 10 per cent rat brain homogenate (50 mgm. tissue), 0.5 ml. of distilled H_2O, 0.4 ml. of 0.5 M.KCl, 0.1 ml. of 0.1 M $MgCl_2$, 0.1 ml. of 4 X 10^{-4} M cytochrome c, 0.2 ml. of 0.1 M KH_2PO_4 plus KOH (to pH 7.4), 0.3 ml. of 0.1 M ATP, 0.2 ml. of 0.2 per cent DPN, 0.2 ml. of 0.154 M KCl. Added to the sidearm was 0.3 ml. of 0.1 M sodium succinate. Added to the center well was 0.2 ml. 20 per cent KOH. The total volume was 3.0 ml. Gas phase was air. Temperature was 38° C. The vessels were allowed to equilibrate for 15 minutes in the Warburg bath, after which the succinate was dumped into the vessel from the sidearm. After an interval of 5 minutes oxygen consumption measurements were begun.

- O Unfrozen homogenate, without glycerol (4 vessels)
- ● Unfrozen homogenate, with glycerol (4 vessels)
- ▲ Frozen homogenate, without glycerol (4 vessels)
- Δ Frozen homogenate, with glycerol (4 vessels)

no protection against the effects of low temperature when alpha-ketoglutarate is used as a substrate. However, it does give some protection when succinate is used as a substrate, the frozen preparations without glycerol respiring at a lower rate.

Experiment XI

The enzymatic activity of frozen "mitochondrial preparations" to which 10 per cent glycerol had been added was tested. Although it has been shown that freezing does not injure succinic dehydrogenase (Nowinski and De Robertis, 1943; Lynen and Burkhardt, 1944), it was decided to test the activity of the succinoxidase system as well as that of alpha-ketoglutaric oxidase. Succinic dehydrogenase does not need DPN as a hydrogen acceptor whereas alpha-ketoglutaric oxidase does need it. Recent investigations have disclosed that adenine nucleotides are in some way destroyed or partially inactivated by freezing (Mondy and Daniel, 1954; Albaum, et al., 1952). Results of experiments in the present study indicate that frozen glycerol-treated "mitochondrial preparations" are capable of consuming oxygen at a higher rate than those which have been frozen but not treated with glycerol, when alpha-ketoglutarate and succinate are used as substrates. This is evidence which substantiates the concept that the enzymes formerly thought to be affected by freezing are protected by treatment with glycerol (Figure 9 and Figure 10).

Usually phosphorylation accompanies oxidation but, due to influences such as the action of drugs, oxidation may be uncoupled from phosphorylation; that is, oxidation may occur without the normally concurrent accumulation of high energy phosphate bonds. It was thought

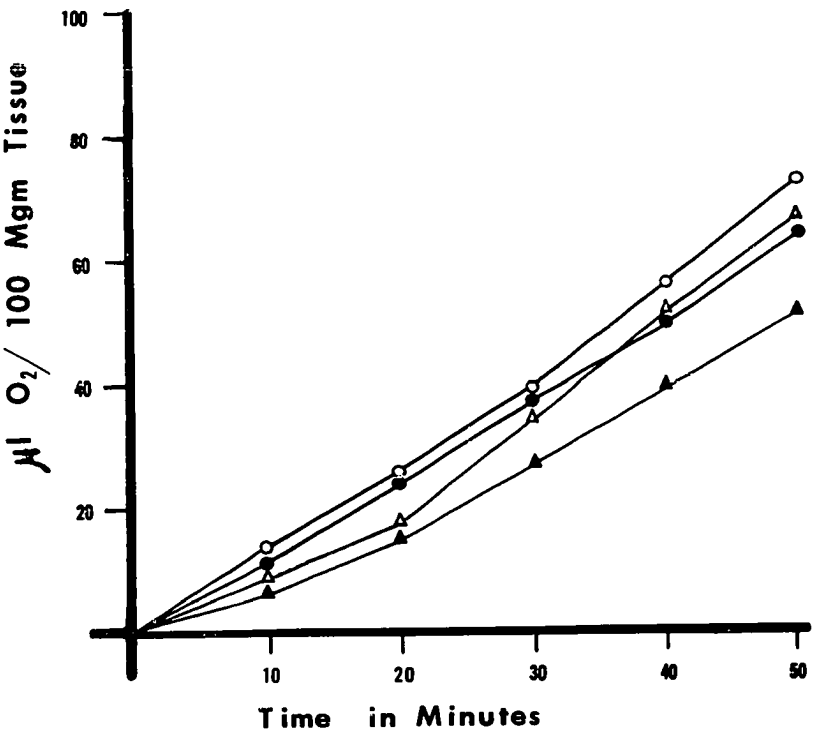

Figure 9. The oxidation of alpha-ketoglutarate to succinate in fresh and frozen "mitochondrial preparations," with and without glycerol. The following additions were made to the reaction vessels: 0.5 ml. washed "mitochondrial preparations" (from 125 mgm. tissue), 0.2 ml. of 0.1 M alpha-ketoglutarate, 0.2 ml. of 0.5 M glucose, 0.05 ml. of 0.05 M ethylenediamine tetra-acetic acid, 0.01 ml. of 4 X 10^{-4} M cytochrome c, 0.4 ml. of 0.1 M. KH_2PO_4, 0.2 ml. of a reaction mixture containing 0.05 M ATP, 0.01 M DPN, 0.1 M $MgSO_4$. 7 H_2O, 0.2 ml. hexokinase, 0.3 ml. 10 per cent trichloroacetic acid. The TCA was added to the sidearm. Placed in the center well was 0.2 ml. 20 per cent KOH. Total volume was 2.26 ml. Gas phase was oxygen. Temperature was 26° C.

- O Unfrozen "mitochondrial preparations", without glycerol (2 vessels)
- ● Unfrozen "mitochondrial preparations", with glycerol (2 vessels)
- ▲ Frozen "mitochondrial preparations", without glycerol (2 vessels)
- Δ Frozen "mitochondrial preparations", with glycerol (2 vessels)

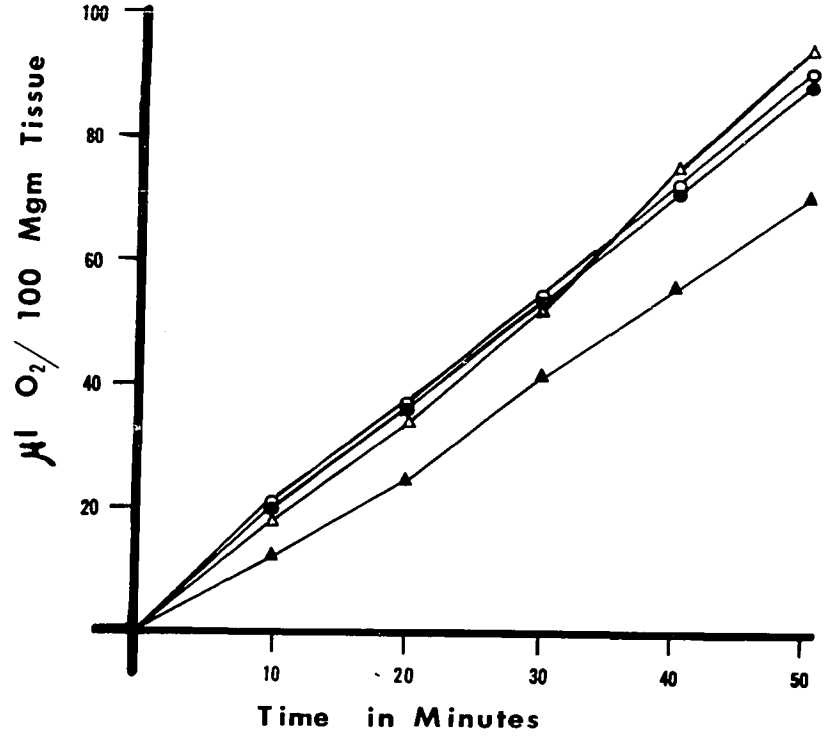

Figure 10. The oxidation of succinate to fumarate in fresh and frozen "mitochondrial preparations," with and without glycerol. The following additions were made to the reaction vessels: 0.5 ml. "mitochondrial preparation" (from 125 mgm. tissue), 0.2 ml. 0.1 M succinate, 0.2 ml. 0.5 M glucose, 0.05 ml. 0.05 M ethylenediamine tetra-acetic acid, 0.01 ml. 4 X 10^{-4} M cytochrome c, 0.4 ml. 0.1 M KH_2PO_4, 0.2 ml. of a reaction mixture containing 0.05 M ATP, 0.01 M DPN, 0.1 M $MgSO_4 \cdot 7H_2O$, 0.2 ml. hexokinase, 0.3 ml. 10 per cent trichloroacetic acid. The TCA was added to the sidearm. Placed in the center well was 0.2 ml. 20 per cent KOH. Total volume was 2.26 ml. Gas phase was oxygen. Temperature was 26° C.

- ● Unfrozen "mitochondrial preparations," without glycerol (2 vessels)
- ○ Unfrozen "mitochondrial preparations," with glycerol (2 vessels)
- ▲ Frozen "mitochondrial preparations," without glycerol (2 vessels)
- △ Frozen "mitochondrial preparations," with glycerol (2 vessels)

that this might happen to tissue when it is frozen. Phosphorylation was measured in the "mitochondrial preparations" and, as can be noted by Table IV, there is no appreciable reduction of the phosphorus-oxygen ratio in the preparations tested. The ratio of the phosphorus used to the oxygen consumed in the frozen untreated "mitochondrial preparations" using succinate as a substrate is unaccountably high. A possible explanation for this will be presented in Chapter 4. It must be realized that these experiments are pilot in nature and are in no manner conclusive.

Experiment XII

As previously noted, there have been reports to the effect that freezing causes a dilution of cofactors rather than, necessarily, an inactivation of the enzymes themselves. With this concept in mind, various cofactors were added, both separately and collectively, to homogenates, and oxygen consumption measured. Table V illustrates that the addition of ATP, DPN, and cytochrome c separately to homogenates which had been frozen for 24 hours at -79° C. has no appreciable effect on the oxygen consumption. Table V also shows that the oxygen consumption of rat brain homogenates is not appreciably altered when the three cofactors are added together.

Experiment XIII

In view of the success obtained with homogenates of rat brain, it was decided to determine whether the protective action of glycerol would apply to brain homogenates of other animals as well. Rabbit brain

TABLE IV

EFFECT OF GLYCEROL ON THE OXYGEN CONSUMPTION AND PHOSPHORUS UTILIZATION OF FROZEN AND FRESH "MITOCHONDRIAL PREPARATIONS" USING SUCCINATE AND ALPHA-KETOGLUTARATE AS SUBSTRATES

SUBSTRATE	"MITOCHONDRIAL PREPARATION"	MICROATOMS OXYGEN	MICROMOLES PHOSPHORUS	PHOSPHORUS OXYGEN
SUCCINATE	Unfrozen, no glycerol	7.86	7.5	0.95
	Unfrozen, with glycerol	8.12	7.2	0.89
	Frozen, no glycerol	6.34	22.2	3.50
	Frozen, with glycerol	8.40	15.4	1.83
ALPHA-KETOGLUTARATE	Unfrozen, no glycerol	6.42	12.2	1.90
	Unfrozen, with glycerol	5.72	8.3	1.45
	Frozen, no glycerol	4.56	11.3	2.48
	Frozen with glycerol	5.90	6.7	1.14

The contents of the reaction vessels and pertinent experimental data are given under Figure 9 and Figure 10.

TABLE V

EFFECT OF ADDED COFACTORS ON OXYGEN CONSUMPTION

OF FROZEN RAT BRAIN HOMOGENATES

ADDED COFACTORS	NUMBER OF VESSELS	TOTAL MICROLITERS O_2/ 100 MGM. TISSUE, WET WEIGHT / 2 HOURS
ATP	4	110
DPN	4	119
CYTOCHROME *c*	4	110
ATP, DPN, CYTOCH. *c*	3	114
NO COFACTORS	6	127

Each reaction vessel contained 2.8 ml. of 10 per cent homogenate (280 mgm. tissue) in Krebs-Ringer-phosphate buffer solution containing 0.2 per cent glucose as the substrate. The homogenate was frozen as a 40 per cent homogenate and diluted after thawing to a 10 per cent homogenate with Krebs-Ringer-phosphate buffer containing the desired additive or additives. Additives were: Adenosine triphosphate (ATP), 5×10^{-4}M; Diphosphopyridine nucleotide (DPN), 0.5 per cent; cytochrome *c*, 1×10^{-5}M. In the center well was placed 0.2 ml. 20 per cent KOH. Total volume was 3.0 ml. Gas phase was air. Temperature was 38° C. Preparations were maintained at -79° C. for 24 hours.

homogenates were made, treated with various concentrations of glycerol, and then their oxygen consumption was measured in the same manner as were the rat brain homogenates. Figure 11 shows the effect of various concentrations of glycerol on fresh homogenates of rabbit brain. Glycerol apparently has no significant effect on fresh rabbit brain homogenates in concentrations up to 15 per cent. Above this concentration of glycerol the oxygen consumption of the homogenates decreases to a slight degree.

The oxygen consumption of homogenates frozen in 7.5 per cent glycerol-Krebs-Ringer-phosphate buffer solution was measured at the end of one, two, and three days in the ice-chest at -79° C. Table VI shows no appreciable decrease in oxygen consumption of the frozen homogenates over the three day period. The fresh and frozen homogenates treated with 7.5 per cent glycerol consume oxygen at approximately the same rates. These, in turn, consume oxygen at the same rate as do the fresh untreated homogenates.

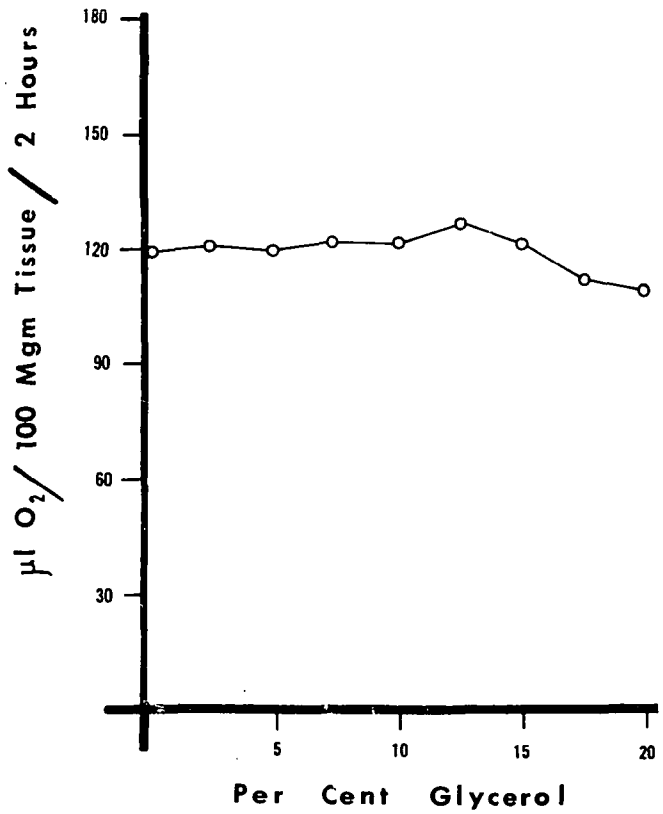

Figure 11. The effect of glycerol on the oxygen consumption of fresh rabbit brain homogenates. In each reaction vessel was placed 2.8 ml. of 10 per cent homogenate (280 mgm. tissue) in Krebs-Ringer-phosphate buffer containing 0.2 per cent glucose as the substrate. The glycerol concentration was varied as indicated on the abcissa. Placed in the center well was 0.2 ml. of 20 per cent KOH. Total volume was 3.0 ml. Gas phase was air. Temperature was 38° C.

O Fresh homogenates, (2 vessels represented by each point).

TABLE VI

EFFECT OF FREEZING (-79° C.) ON THE OXYGEN CONSUMPTION OF GLYCEROL-TREATED RABBIT BRAIN HOMOGENATES

TIME FROZEN IN 7.5 % GLYCEROL	TOTAL MICROLITERS O_2 / 100 MGM. TISSUE, WET WEIGHT / 2 HOURS
UNFROZEN	120
24 HOURS	125
48 HOURS	120
72 HOURS	115

Each reaction vessel contained 2.8 ml. of 10 per cent homogenate (280 mgm. tissue) in Krebs-Ringer-phosphate buffer containing 7.5 per cent glycerol and 0.2 per cent glucose as the substrate. Placed in the center well was 0.2 ml. 20 per cent KOH. Total volume was 3.0 ml. Gas phase was air. Temperature was 38° C. Preparations were maintained at -79° C. for the desired periods of freezing.

CHAPTER IV

DISCUSSION

In the preliminary experiments completed to determine the effects of low temperatures (-79° C.) on the oxygen consumption of rat brain homogenates, it is not surprising to find that frozen untreated homogenates of a higher concentration consume oxygen at a higher rate than do the homogenates of a lower concentration. The explanation for this undoubtedly lies in the presence of a higher concentration of water in the more dilute homogenates. During freezing this water presumably undergoes the process of crystallization resulting in the destruction of intracellular components not only by mechanical but also by chemical means. Microscopic studies of mammalian corneal epithelium, sperm, red blood cells, amoebae, and testicular tissue show that the cells freeze internally on lowering the temperature to -79° C. and disintegrate on thawing (Smith, et al., 1951; Smith and Smiles, 1953-1954). De Robertis and Nowinski (1942) have found that in frozen liver cells, nuclear retraction, mitochondrial destruction, and cytoplasmic vacuolization occur. Lovelock (1954) contends that as a result of ice formation large islands of highly concentrated salts remain which are very likely to cause protein denaturation as well as other chemical changes.

The lack of protection against cold afforded by glycerol to

chunks or pieces of rat brain can be explained. When using the glycerol-freezing method on intact cells an equilibration period is required, presumably to allow permeation of glycerol into the tissue. Evidently, due to the physical size of the tissue chunks, the necessary permeation of the tissue was not effected.

It is well known that the ionic content of the medium is an important factor when measuring oxygen consumption of tissue slices and homogenates. In many studies involving the glycerol-freezing method of preservation, the tonicity of the suspending medium has been considered only in gross terms (Smith, 1952a; Parkes and Smith, 1953; Deanesley, 1954a, 1954b). If, as is commonly accepted, cellular membranes and presumably the "membranes" of the intracellular organized bodies of brain homogenates allow complete permeation by glycerol, the osmotic effect of glycerol should be negligible. The fact that the ionic content and also the osmotic effect of the medium is reduced by the addition of glycerol should be realized together with the possibility that such solutions are hypotonic to intracellular fluid and are undesirable for enzymatic studies. The increased oxygen consumption obtained in "adjusted" glycerol media as compared with "unadjusted" glycerol solutions is argument for the use of "adjusted" glycerol media.

Experiments were completed to determine whether an equilibration time is necessary in order for the glycerol to permeate the active components of the homogenates. The lack of an increase in oxygen consumption after prolonged equilibration periods indicates that the effective permeation of the homogenate by glycerol occurs immediately, whether

the tissue is homogenized in glycerol or whether glycerol is added following homogenization. If any equilibration period is required it is presumably too short to be detected.

The slight decrease in oxygen consumption of tissues in which there is a delay in the time interval between homogenization and oxygen consumption measurements is an expected occurrence. It has been found that there is a gradual loss of enzyme activity with an increase in the time interval. Luyet and Gehenio (1940) find that tissues cannot withstand temperatures which slowly approximate the freezing point of the tissue. At this temperature there is a greater possibility for ice crystals to form and for membranes to be destroyed.

Glycerol appears to protect and preserve brain tissue homogenates fully at a concentration of 10 per cent. The similarity between oxygen uptake of the glycerol-frozen and fresh homogenates is the chief criterion for such a conclusion. In lower concentrations, glycerol appears to protect the homogenates partially, and it seems reasonable to assume that the only reason that it does not protect fully is that it is not present in sufficient quantities to do so. At higher concentrations of glycerol lower oxygen consumption rates are observed. It has been shown that increasing the salt concentration of the homogenization fluid lowers the oxygen solubility, and hence changes the flask constants only by a relatively small amount (Umbreit, et al., 1949). However, glycerol is a very viscous substance and, with an increase in concentration, the oxygen will diffuse more slowly throughout the liquid phase. Glycerol may exert an influence on oxygen solubility and lower the vessel con-

stants, thus altering the apparent oxygen uptake by increasing amounts at the higher concentrations of glycerol.

Complete explanation cannot be given for the decreased oxygen uptake of the frozen homogenates, as compared with that of the fresh homogenates, at concentrations of 15 per cent, 20 per cent, and 30 per cent glycerol. It would seem that the freezing and thawing process renders the tissue preparations more susceptible to the deleterious effects of glycerol under these conditions.

An analysis of results from the experiments on the diluted and undiluted homogenates tends to nullify the hypothesis that the inhibiting effect of higher concentrations of glycerol on oxygen consumption is caused by an increased viscosity of the homogenate. However, it is probable that although glycerol permeates a homogenate completely, the influx of buffer caused by the process of rapid dilution may cause a disturbance in the spatial relationships within the cellular particulate matter. Sloviter (1951a, 1952), Lovelock (1952), and Chaplin and Veall (1953) have noted that red blood cells have a short survival after freezing when treated with glycerol if an attempt is made to remove all or most of the glycerol by one quick process of dilution or dialysis. Thus, the enzymes of the tissue homogenates treated with glycerol might not be destroyed by the large amount of glycerol in them, but might, rather, be affected by the process of rapid dilution. However, in the experiments done to determine whether the process of rapid dilution is deleterious to the homogenate in regard to its ability to consume oxygen, it will be noted that the rapid dilution does not decrease oxygen consump-

tion unless it takes place immediately before oxygen consumption measurements are made. Perhaps a short equilibration period may be needed for the buffer and glycerol to come into equilibrium in the homogenate before oxygen consumption measurements are started.

On the other hand, the experiments done to determine whether the rapid addition of glycerol to the homogenate causes a decrease in oxygen consumption show that all of the 30 per cent homogenates consumed oxygen at the same approximate rate and that in no instance did the gradual increase of glycerol concentration result in higher oxygen consumption measurements. On the basis of these experiments, it is assumed that glycerol in higher concentrations has an inhibiting effect on the oxygen consumption of the homogenates; a possible explanation cannot be presented at this time. It is possible that glycerol has an inherent toxicological action (such as denaturation of proteins) when used in these preparations.

The experiments which were done with dilute homogenates and with "mitochondrial preparations" were designed to determine whether treatment with glycerol serves to protect specific enzyme systems as well as such treatment seems to protect oxygen consumption by the complete aerobic enzyme system when glucose is used as a substrate. The results of these experiments are inconclusive.

The ability of "mitochondrial preparations" to phosphorylate after treatment with glycerol and freezing is further evidence that glycerol does protect these preparations against the effects of low temperatures, in the respect that apparently no uncoupling of oxidation

and phosphorylation occurs. The extremely high P/O ratio obtained in the frozen untreated "mitochondrial preparations" using succinate as a substrate can possibly be explained. Brain homogenates, in general, are known to contain a high concentration of adenosine triphosphatase, an enzyme which is able to split the end phosphate from adenosine triphosphate. It may be that freezing the "mitochondrial preparations" inactivates the adenosine triphosphatase of brain and gives an abnormally high amount of phosphorus used in the experiment.

The failure of adenosine triphosphate, diphosphopyridine, and cytochrome c to cause an increase in the oxygen consumption of the frozen homogenates indicates that in these experiments the dilution or perhaps the destruction of these factors is not a limiting factor in oxygen consumption. These experiments give no indication of an effect of freezing on the enzymes of the homogenates. If freezing does affect the cofactors involved in carbohydrate metabolism, then it appears that glycerol protects these cofactors.

Homogenates of rabbit brain respire at a much slower rate than do the rat brain homogenates. However, the results indicate that these are as completely protected as the rat brain homogenates. Since there is no decrease in oxygen consumption of the frozen rabbit brain homogenates after they have been frozen for three days, it appears that glycerol exerts its protective effect on rabbit brain tissue, as well as on rat brain tissue, over prolonged periods of time.

CHAPTER V

SUMMARY AND CONCLUSIONS

1. Rat brain homogenates, using glucose as a substrate, consume less oxygen (50 to 80 per cent) after being frozen (-79° C.) and thawed.

2. Rat brain homogenates, frozen at -79° C. in 40 per cent concentration, consume oxygen in a medium containing glucose, at a higher rate than do preparations frozen as 10 per cent homogenates.

3. Rat brain chunks which have been treated with various concentrations of glycerol and then frozen, thawed, and homogenized, consume oxygen, using glucose as a substrate, at the same rate as do homogenates frozen without glycerol.

4. Both fresh and frozen glycerol-treated homogenates, using glucose as a substrate, consume oxygen at a more sustained and rapid rate when the glycerol-Krebs-Ringer-phosphate reaction medium was altered to contain the total ionic content of Krebs-Ringer-phosphate buffer.

5. No appreciable period of equilibration is needed to permit glycerol to exert its protective effect on the ability of rat brain homogenates to consume oxygen when glucose is used as a substrate.

6. Glycerol, in concentrations below 15 per cent, has no significant effect on the oxygen consumption of fresh rat brain homogen-

ates when glucose is used as a substrate.

7. The oxygen consumption of homogenates treated with 10 per cent glycerol, and subsequently frozen for periods as long as 28 days, is approximately that of fresh homogenates treated with 10 per cent glycerol when glucose is used as a substrate.

8. There is a decrease of oxygen consumption of rat brain homogenates in a buffer medium containing glucose and 15 per cent glycerol when frozen and then diluted to 7.5 per cent glycerol or to 3.0 per cent glycerol.

9. Neither rapid dilution nor rapid addition of glycerol can be said to be totally responsible for the decrease in oxygen consumption caused in homogenates containing glycerol in high concentrations (to 30 per cent).

10. Rat brain homogenates prepared in glycerol-Krebs-Ringer-phosphate buffer solutions containing 5 per cent glycerol, 10 per cent glycerol, and 15 per cent glycerol, were frozen at -79° C. for one day, 14 days, and 28 days. Oxygen consumption of homogenates frozen in 5 per cent glycerol was approximately the same for all time periods of freezing indicated. Glucose was used as a substrate. The same was true for the other two groups.

11. The addition of adenosine triphosphate, diphosphopyridine nucleotide, or cytochrome c to frozen homogenates of rat brain, either separately or together, fails to increase the oxygen consumption when glucose is used as a substrate.

12. Glycerol appears to inhibit the activity of the alpha-

ketoglutaric oxidase system and the succinoxidase system of dilute homogenates but appears to have no effect on those systems in "mitochondrial preparations."

13. Freezing appears to inhibit the activity of the alpha-ketoglutaric oxidase system and the succinoxidase system of dilute homogenates and "mitochondrial preparations."

14. Glycerol appears to protect partially the alpha-ketoglutaric oxidase system of frozen "mitochondrial preparations" but not that of the frozen dilute homogenates.

15. Glycerol appears to protect partially the succinoxidase system of frozen dilute homogenates and "mitochondrial preparations."

16. Glycerol, in concentrations up to 15 per cent, has no significant effect on the oxygen consumption of fresh rabbit brain homogenates when glucose is need as a substrate.

17. Homogenates of rabbit brain frozen at -79° C. in 7.5 per cent glycerol respire at approximately the same rate as do unfrozen homogenates without glycerol when glucose is used as a substrate.

BIBLIOGRAPHY

Albaum, H. G., H. F. Drury, and T. Cayle. 1952. Some observations on the adenine nucleotide levels of rat tissues stored at low temperatures. Arch. Biochem. and Biophys. 41: 274-277.

Bahrmann, E. 1932. Reizleitung und elekrische Leitfahigkeit des Nerven vei tiefen Temperaturen. Ztschr. f. Biol. 92: 366-372. Cited by Luyet and Gehenio, 1940. p. 60.

Becquerel, P. 1932(a). La vie latente des spores des Mousses aux basses temperatures. Compt. rend. Acad. Sci. (Paris). 194: 1378-1380. Cited by Smith, et al., 1954.

Becquerel, P. 1932(b). L'anhydrobiose des tubercules des Renoncules dans l'azote liquid. Compt. rend. Acad. Sci. (Paris). 194: 1974-1976. Cited by Smith, et al., 1954.

Becquerel, P. 1932(c). La revivescence des plantules dessechees soumises aux actions du vide et des tres basses temperatures. Compt. rend. Acad. Sci. (Paris). 194: 2158-2159. Cited by Smith, et al., 1954.

Becquerel, P. 1950. La suspension de la vie audessous de 1/20° K. absolu par demagnetization adiabetique de l'alun de fer dans le vide le plus Eleve. Compt. rend. Acad. Sci. (Paris). 231: 261-263. Cited by Smith, et al., 1954.

Belehradek, J. 1935. Temperature and Living Matter. Berlin, Germany. Cited by Luyet and Gehenio, 1940. p. 34.

Berger, L., M. W. Slein, S. P. Colowick, and C. F.Cori. 1946. Isolation of hexokinase from Baker's yeast. J. Gen. Physiol. 29: 379-391.

Billingham, R. E. and P. B. Medawar. 1952. The freezing, drying, and storage of mammalian skin. J. Exp. Biol. 29: 454-467.

Brody, T. M. and J. A. Bain. 1952. Mitochondrial preparations from mammalian brain. J. Biol. Chem. 195: 685-696.

Brown, I. W., Jr. and H. F. Hardin. 1953. Recovery and *in vivo* survival of human red cells. A.M.A. Arch. Surg. 66: 267-282.

Bunge, R. G., W. C. Keetel, and J. K. Sherman. 1954. Clinical use of frozen semen. Fertility and Sterility. 5: 520-529.

Chambers, R. and H. P. Hale. 1932. The formation of ice in protoplasm. Proc. Roy. Soc. London, Series B. 110: 336-352. Cited by Luyet and Gehenio, 1940. p. 58-59.

Chang, M. C. 1953. Resumption of heartbeat in the rabbit embryo after exposure to low temperatures. Science. 118: 550-552.

Chaplin, H. and P. L. Mollison. 1953. Improved storage of red cells at -20° C. Lancet. 1: 215-218.

Chaplin, H. and N. Veall. 1953. Removal of glycerol from previously frozen red cells. Lancet. 1: 218-219.

Cross, R. J., J. V. Taggart, G. A. Covo, and D. E. Green. 1949 Studies on the cyclophorase system. VI. The coupling of oxidation and phosphorylation. J. Biol. Chem. 177: 655-678.

Deanesley, R. 1954(a). Immature rat ovaries grafted after freezing and thawing. J. Endocrinology. 11: 197-200.

Deanesley, R. 1954(b). Spermatogenesis and endocrine activity in grafts of frozen and thawed rat testis. J. Endocrinology. 11: 201-206.

De Robertis, E. and W. W. Nowinski. 1942. Action of freezing and drying on the metabolism of tissues. I. The respiration of frozen and dried guinea pig liver. Revista de la Sociedad Argentina de Biologia. 18: 333-345.

Dickens, F. and F. Simer. 1931. The metabolism of normal and tumor tissue. IV. The respiratory quotient in bicarbonate media. Biochem. J. 25: 985-993.

Eastscott, H. H. G., A. G. Cross, A. G. Leigh, and D. F. North. 1954. Preservation of corneal grafts by freezing. Lancet. 1: 237-239.

Elliott, K. A. C. 1948. Metabolism of brain tissue slices and suspensions from various mammals. J. Neurophysiol. 11: 473-484.

Elliott, K. A. C. and Henry M. 1946. Studies on the metabolism of brain suspensions. III. Respiration at low oxygen. J. Biol. Chem. 163: 351-359.

Elliott, K. A. C. and B. Libet. 1942. Studies on the metabolism of brain suspensions. I. Oxygen uptake. J. Biol. Chem. 143: 227-245.

Fiske, C. H. and Y. Subbarow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375-400.

Fulton, J. D. and A. U. Smith. 1953. Preservation of Entamoeba histolytica at -79° C. in the presence of glycerol. Ann. Trop. Med. Parasit. 47: 240-246.

Grand, N. G., J. F. Keane, Jr., W. H. Bauer, and B. J. Luyet. 1952. Transplantation of a mouse adrenocarcinoma frozen in liquid nitrogen after treatment with ethylene glycol. Biodynamica. 7: 133-139. Cited from Chemical Abstracts. 47: 7062 b.

Green, D. E., W. F. Loomis, and V. H. Auerbach. 1948. Studies on the cyclophorase system. I. The complete oxidation of pyruvic acid to carbon dioxide and water. J. Biol. Chem. 172: 389-403.

Hammond, B. 1952. Effect of freezing liver tissue on its thiamine pyrophosphate content. Biochim. and Biophys. Acta. 9: 103.

Hepburn, J. S. 1915. The behaviour of enzymes at low temperatures. J. Amer. Pharm. Assoc. 4: 682-685.

Jahn, T. L. 1933. Studies on the physiology of the euglenoid flagellates. IV. The thermal death time of Euglenia glacilis Klebs. Arch. Protistenk. 79: 248-262. Cited from Luyet and Gehenio, 1940. p. 31.

Kadisch, E. 1931. Beitrage zur Wirkung der Kalte auf pathogene Padenspilze, Hefen und Backterien Ausdehnung dieser Versuche bis in de Nahe des absoluten Nullpunktes (bis -272° C.). Med. Klin. 27: 1074-1078; 1109-1112. Cited from Luyet and Gehenio, 1940. p. 25.

Keeley, R. L. A., A. C. Gomez, I. W. Brown, Jr. 1952. An experimental study of the effects of freezing, partial dehydration, and ultra-rapid cooling on the survival of dog skin grafts. Plastic and Reconstructive Surgery. 9: 330-344.

LePage, G. A. 1948. Analyses for tissue metabolites with in situ freezing techniques. Methods in Medical Research. 1: 337-357. V. R. Potter, editor-in-chief. Year Book Publishers, Inc. Chicago.

Lipman, C. B. 1936. Normal viability of seeds and bacterial spores after exposure to temperatures near the absolute zero. Plant Physiol. 201-205. Cited from Luyet and Gehenio. 1940. p. 22.

Lovelock, J. E. 1952. Resuspension in plasma of human red blood cells frozen in glycerol. Lancet. 1: 1238-1239.

Lovelock, J. E. 1953(a). The hemolysis of human red blood cells by freezing and thawing. Biochim. and Biophys. Acta. 10: 414-426.

Lovelock, J. E. 1953(b). Mechanism of the protective action of glycerol against hemolysis by freezing and thawing. Biochim. and Biophys. Acta. 11: 28-36.

Lovelock, J. E. 1954. Biophysical aspects of the freezing and thawing of living cells. A discussion on the survival of tissues at low temperatures. Proc. Roy. Soc. Med. 47: 57-66.

Luyet, B. J. and P. M. Gehenio. 1940 Life and Death at Low Temperatures. Normandy, Mo. Biodynamica.

Luyet, B. J. and F. Gonzales. 1952. Protective action of glycerol against freezing injury in embryonic tissues of the chick. Biodynamica. 7: 101-106. Cited in Chem. Abstracts. 47: 7061g.

Luyet, B. J. and F. Gonzales. 1953. Growth of nerve tissue in liquid nitrogen. Biodynamica. 7: 141-144. Cited in Chem. Abstracts. 48: 5891.

Luyet, B. J. and E. L. Hodapp. 1938. Revival of frog's spermatozoa vitrified in liquid air. Proc. Soc. Exp. Biol. Med. 39: 433-434.

Luyet, B. J. and J. F. Keane. 1952. Comparative efficiency of ethylene glycol, glucose, and sodium chloride in protecting tissues against freezing injury. Biodynamica. 7: 119-131. Cited in Chem. Abstracts. 47: 7062a.

Luyet, B. J. and G. Thoennes. 1938. The survival of plant cells immersed in liquid air. Science. 88: 284-285.

Lynen, F. and L. Burkhardt. 1944. Experiments with kidney extracts on the metabolism of animal tissue after freezing in liquid air. Z. Physiol. Chem. 281: 83-89.

McLean, A. L. 1918. Nature. 102: 35-39. Cited by Luyet and Gehenio. 1940.

Mondy, N. I. and L. J. Daniel. 1954. Effect of freezing and thawing on choline dehydrogenase. Arch. Biochem. & Biophys. 48: 49: 402-408.

Moran, T. 1925. Effect of low temperature on hen eggs. Proc. Roy. Soc. London, Series B. 98: 436-56.

Murigin, I. I. 1937. Bull. Biol. Med. Exp. 4: 100-102. Cited by Luyet and Gehenio, 1940. p. 94.

Novikoff, A. B., V. R. Potter, and G. A. LePage. 1948. Phosphorylated intermediates in tumor glycolysis. IV. Glycolysis in tumor homogenates. Cancer Research. 8: 203-210.

Nowinski, W. W. and E. De Robertis. 1943. Action of freezing and drying on the metabolism of tissues. II. Changes of succinic dehydrogenase in the liver tissue of the guinea pig. Revista de la Soc. Argentina de Biologia. 19: 527-534.

Parkes, A. S. and A. U. Smith. 1950. Effect of low temperatures on ovarian tissue. J. Endocrinology. 7: xxxix.

Parkes, A. S. and A. U. Smith. 1953. Regeneration of rat ovarian tissue grafted after exposure to low temperatures. Proc. Roy. Soc. London. Series B. 140: 455-470.

Park, W. H., A. W. Williams, and C. Krumwiede. 1924. Pathogenic Micro-organisms. 8th ed., p. 698. New York. Lea and Febiger.

Polge, C., A. U. Smith, and A. S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature. 164: 666.

Polge, C., 1951. Functional survival of fowl spermatozoa after freezing at -79° C. Nature. 167: 949-950.

Potter, V. R., G. A. LePage and H. L. Klug. 1948. The assay of animal tissues for respiratory enzymes. VII. Oxalacetic acid oxidation and the coupled phosphorylations in isotonic homogenates. J. Biol. Chem. 175: 619-634.

Ochoa, S. 1944. Alpha-ketoglutaric dehydrogenase of animal tissues. J. Biol. Chem. 155: 87-100.

Rivers, T. M. 1927. Effect of repeated freezing (-185° C.) and thawing on colon bacilli, virus III, vaccine virus, herpes virus, bacteriophage, complement, and trypsin. J. Exp. Med. 45: 11-21.

Sherman, J. K. 1954. Freezing and freeze-drying of human spermatozoa. Fertility and Sterility. 5: 357-371.

Simonin, C. 1931. Researches sur la surviedes tissues exposes aux basses temperatures. Comp. rendu soc. de biol. 107: 1029-1032.

Smart, H. F. 1935. Growth and survival of micro-organisms at sub-freezing temperatures. Science. 82: 525.