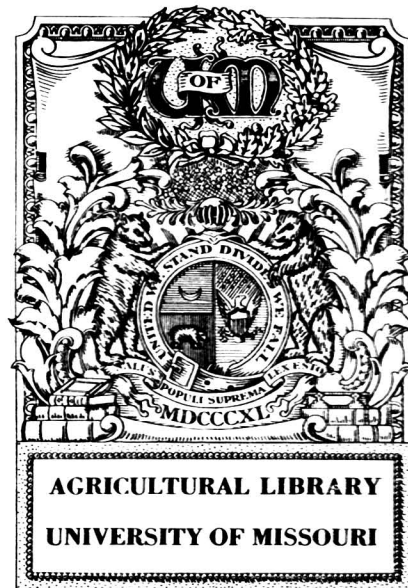


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THE METABOLISM AND LOCALIZATION OF GLYCEROL

BY BOVINE SPERMATOZOA

A Thesis

Submitted to

The Graduate Faculty of the

University of Missouri

in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy



by

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## INTRODUCTION

Since the discovery that glycerol, a trihydroxy alcohol, would provide protection for spermatozoa during the freezing and thawing process, many questions have arisen concerning its metabolism and mode of action. Most of the recent work, with various types of single cells and tissues, has shown that the cells are permeated by glycerol and that entry must occur before full protection is afforded. However, the methods employed in the majority of these studies have not proven successful for bovine spermatozoa, possibly due to its extremely inflexible cellular membrane and/or the slow rate at which glycerol enters the cell.

The majority of the information concerning glycerol entry into bovine spermatozoa has been supplied by metabolic studies. These studies have indicated that glycerol is metabolized by spermatozoa both anaerobically and aerobically in quantities too large to attribute the action to extracellular enzymes alone. However, relatively little information is available concerning the intracellular loci of glycerol action.

Thus, this investigation was undertaken to provide additional information concerning the metabolism and localization of glycerol in the bovine spermatozoa.

## REVIEW OF LITERATURE

### Metabolism

A yeast fermentable sugar, present in the secretions of the seminal vesicles, has been found to be the primary source of nutrients for bovine spermatozoa. In the course of most investigations it had been called the "reducing sugar" or glucose; however, Mann, in 1946 (59), discovered that the reducing carbohydrate of the seminal plasma was not glucose, but rather d(-) fructose. Fructose was purified from seminal plasma and identified by its reducing value, optical activity, preparation of methylphenyl fructosazone, and yeast-fermentation tests.

In the seminal plasma of several species, including the bull, fructose accounts for practically all the yeast fermentable sugar. Fructose varies from about 50 mg. per cent in the boar to as much as one gram per 100 ml. in the bull (60).

Erb, et al. (24) have found that there is an inverse relationship between sperm numbers and fructose concentration, especially after the sperm concentration reaches  $1.2$  to  $1.4 \times 10^9$  spermatozoa per milliliter of semen.

The primary function of fructose in semen is to supply the spermatozoa with readily glycolyzable material. On storage, the fructose falls progressively and lactic acid accumulates. Since fructose is the normal sugar present in semen it is the one normally used, but spermatozoa also have the enzymes necessary to metabolize

glucose and mannose.

Mann (59) has found that the breakdown of fructose, glucose, and mannose by spermatozoa is initiated by a hexokinase interaction with adenosine triphosphate (ATP) to form monophosphohexose. Thus formed, it is further metabolized through diphosphohexose, phosphotriose, phosphoglyceric acid and pyruvic acid to lactic acid. This is apparently true for ram, boar, and stallion spermatozoa, as well as for the bull. Moore (68) has shown that ram, boar, and stallion spermatozoa are able to utilize those hexoses which possess a spatial arrangement of the attached groups on carbon atoms three and four identical to that of glucose. These hexoses are glucose, fructose and mannose.

It has been further shown that ram spermatozoa do not possess the enzyme systems required to cause hydrolysis of alpha- or beta-glucosidic linkages; therefore, they are incapable of utilizing di- or poly-saccharides. This does not agree with Plaut and Lardy's investigation concerning bull spermatozoa (75). These workers feel that bovine spermatozoa have the ability to utilize maltose and that small amounts of seminal plasma increase the ability of spermatozoa to utilize this sugar.

Kampschmidt, Mayer, and Herman (45) found that sucrose or galactose was not metabolized by bovine spermatozoa, but that as long as metabolizable sugar was present in the semen, sucrose or galactose may be used to maintain a high degree of motility during the storage period. They also found that as the ratio of glucose to

buffer is increased in the storage media, there is an increase in storage time of the spermatozoa. It is well known that fructose is utilized both aerobically and anaerobically. Several workers (19, 24, 26, 59) have shown that there is a greater utilization of fructose under anaerobic than under aerobic conditions. Mann (59) has shown that washed sperm can survive anaerobically only in the presence of some glycolyzable carbohydrate. It was further shown, using a calcium free Ringer's solution with 0.5 per cent fructose, that the rate of fructose utilization is greater in nitrogen than in air and that the ratio between lactic acid produced and fructose utilized is greater under nitrogen. If anaerobic fructolysis is allowed to proceed almost to depletion of fructose and at this point fresh Ringer-fructose phosphate is added, the spermatozoa are revived and continue to produce lactic acid. Under oxygen, sperm cells may survive due to the utilization of other compounds, probably through the Krebs cycle.

Walton and Dott (102) studied the activity of undiluted ram and bull spermatozoa by the use of a Rothschild impedance bridge. The semen samples were placed, undiluted, in a constant stream of Krebs-Ringer-bicarbonate saturated with either 5.0 per cent carbon dioxide plus 95.0 per cent oxygen for aerobic experiments, or 5.0 per cent carbon dioxide plus 95.0 per cent nitrogen for anaerobic experiments. The greatest activity was obtained with fructose under aerobic conditions. Less activity was noted when either oxygen or fructose was omitted separately, and in the absence of both fructose

and oxygen, spermatozoan activity quickly ceased.

Ehlers and Erb (19) studied the utilization of fructose with emphasis on the amount of fructose utilized which could be accounted for as lactic acid. In this particular study, pure anaerobic or aerobic conditions were not maintained, but an attempt was made to duplicate field conditions in artificial breeding. Three types of extenders were used: 1) phosphate buffer, 2) heated homogenized milk, 3) egg yolk phosphate. In the presence of phosphate buffer, with incubation for one hour at 37°C. under aerobic conditions, it was found that 40 per cent of the fructose was utilized, as compared to 42 per cent under anaerobic conditions. The average amount of fructose utilization accounted for as lactic acid was 72 and 73 per cent, respectively. When the three types of diluters were compared, lactic acid production was highest in egg yolk, intermediate in milk, and least in the presence of phosphate.

Hopwood, et al. (43) have studied fructose utilization from the standpoint of its relation to fertility. These workers feel that a measure of the rate of fructolysis, as expressed by a "fructolysis index", might be useful in predicting fertility. It was also found that the rate of fructose utilization was essentially a first order reaction. Semen was incubated at 37°C. for three hours and the fructose content determined at 20, 40, 60, 80, 120, and 180 minutes.

It has been shown (60) that under anaerobic conditions the final product of fructose utilization, by bull spermatozoa, is lactic acid, which cannot be oxidized further. However, it has also been shown

(19) that the lactic acid increase does not account for all the sugar utilized. There appear to be several factors which affect this. Samples high in sperm concentration have more of the fructose utilized, accountable as lactic acid. As the dilution rate was increased, there was a marked decline in fructose utilization and an even greater decline in lactic acid formation. It was also noted that young bulls' semen had relatively high fructose utilization on the basis of sperm numbers, but fructose utilization, accounted for as lactic acid, was markedly lower than for other semen. Apparently, geographical location also has an effect on fructose utilization. Erb, et al. (23) found that the utilization rate of fructose by spermatozoa was 11 per cent higher for Colorado as compared to Washington.

Flerchinger, et al. (26) have demonstrated that bovine spermatozoa are capable of utilizing lactic, pyruvic, and acetic acid and that lactic acid is a normal constituent of semen. This is in agreement with Mann (60) for boar semen. He reported 21 mg. per cent lactic acid present in normal, fresh ejaculated boar semen. Humphrey and Mann (44) reported that citric acid is a normal constituent of the semen of man, bull, ram, boar, and stallion, but that it is higher in the bull than in the other species. It was also noted that citric acid was metabolized both aerobically and anaerobically by spermatozoa.

Erb, et al. (24) have found that bull sperm are capable of metabolizing pyruvate with an increase in respiration rate under

aerobic conditions. They further state that pyruvic acid may be metabolized by dismutation under anaerobic conditions in suspensions containing fluoride. They point out that by adding the probable increase in pyruvic acid under anaerobic conditions, 80 and up to 91 per cent recoveries of fructose are possible.

Melrose and Turner (65) studied the metabolism of pyruvate in bull spermatozoa using KRP or KRC (calcium-free) at 37°C. It was found that the respiration of washed bull spermatozoa was increased by the addition of pyruvate and that the pyruvate was, in part, converted to lactate. DPN (2-4 dinitrophenol) abolished the conversion of pyruvate to lactate and inhibited the oxidative disappearance of pyruvate. Fluoride decreased the rate of pyruvate utilization, but lactic acid production was not affected. Glew (32) adopted the above technique of Melrose and Turner in an attempt to relate pyruvate metabolism to fertility. Two hundred and ninety-one samples of semen from 74 dairy bulls were tested and graded according to metabolic response. Four distinct metabolic groups were found with a highly significant relationship between metabolic responses and fertility levels of the bulls.

Flipse and Almquist (28) state that failure to account for fructose utilization by lactic acid formation may indicate the existence of alternate pathways of metabolism or the formation and the breakdown of lactic acid into other compounds. However, in view of results obtained by Erb, et al. (24), part of this breakdown of fructose may be accounted for as pyruvic acid which would not



necessitate either an alternate pathway or further breakdown of lactic acid. Nevertheless, Flipse and Almquist (28) found that during the anaerobic dissimilation of glucose- $C^{14}$ , the major portion of radioactivity appeared as lactic acid although some activity was found in carbon dioxide, volatile acids, and an unidentified compound. They also state that spermatozoa probably metabolize glucose to lactic acid, and that lactic acid may be further broken down to carbon dioxide, acetate and other compounds. Results with immotile spermatozoa indicated that carbon dioxide may be formed without passing through the intermediate stage of lactation.

Flipse (27) studied the uptake of glucose- $C^{14}$  by bovine spermatozoa and found that uptake increased with increased incubation time, the relationship being approximately linear. A more rapid uptake was associated with anaerobic than with aerobic conditions, this being attributed to a more rapid glycolysis under nitrogen, with the liberation of  $C^{14}$  containing metabolites from the cell. A reduced uptake was obtained in the presence of seminal plasma which suggested the presence of some substance in seminal plasma which: 1) is used in preference to glucose, 2) inhibits uptake of radioactive glucose, or 3) stimulates glycolysis and the release of radioactive metabolites. It was noted that a small amount of radioactivity was associated with dead spermatozoa incubated with glucose- $C^{14}$  at  $37^{\circ}C$ . under aerobic or anaerobic conditions.

Work on semen metabolism has indicated that phosphorus-containing enzymes play an important role in sperm glycolysis. Flerchinger and Erb (25) have studied the phosphorus relationship in

an attempt to correlate it with fertility and sperm cell concentration. During incubation for one hour at 37°C., the total phosphorus was found to increase 1.4 mg. per cent. They showed that total phosphorus increased almost directly in proportion to increases in sperm concentration.

In an attempt to test the hypothesis that a mechanism similar to that of muscle is responsible for the energy-requiring processes of bovine spermatozoa, Grunfeld and Merilan (36) have studied the effects of supplementary amino acids and adenosine phosphates on motility and metabolism of bovine spermatozoa. Addition of AMP to whole semen had no apparent effect on the rate of respiration and depressed anaerobic glycolysis. Additions of AMP and ATP to washed spermatozoa stimulated respiration and anaerobic glycolysis. There was a rapid utilization of added ATP, but its utilization in vivo may be different from that seen in vitro. AMP caused a quick temporary increase in motility, while samples to which creatine had been added maintained a higher motility than the controls. It was noted that ATP is quickly utilized by washed spermatozoa, indicating that ATP may be removed during washing or that in the absence of seminal plasma there is an over stimulation of the cells. Walton and Dott (102) state that: "it should be noted that whatever pathway provides the energy, the final link between metabolism and motility is the same, namely, the generation of energy-rich phosphate bonds (A.T.P.) which, acting with some contractile substance, comparable to actomyosin in muscle, sends rhythmical contractions down the

length of the sperm tail".

Mann (58) has shown that ATP is continually broken down and re-synthesized in the course of sperm glycolysis, with the ATP content and anaerobic survival depending upon the maintenance of sperm glycolysis since ATP is readily dephosphorylated.

A rapid uptake of radioactive phosphorus administered as disodium phosphate was demonstrated by Bishop and Weinstock (6) with bull spermatozoa. When the seminal plasma was removed by centrifugation and the sperm resuspended in Tyrode's solution, maximum uptake was attained in 5 to 45 minutes. Uptake was shown to be most rapid in highly motile samples which were presumed to be more metabolically active. It was found that 75 per cent of the  $P^{32}$  which entered the cell was in the form of inorganic phosphate and only about six per cent was combined as phospholipid. Removal of the adhered  $P^{32}$  could be accomplished by double washing. However, washing of spermatozoa produces many and varied effects on spermatozoan metabolism. Mayer (62) has pointed out that washing decreases endogenous respiration and causes a greater increase in respiration when glucose or succinate is added. Washing spermatozoa before cold shock had a more depressing effect than cold shock alone.

In 1953, White (103) investigated the effects of one and two washings on the motility, oxygen uptake, and aerobic glycolysis of ram, bull, and rabbit spermatozoa in a phosphate-fructose diluent at 37°C. He found no significant effect of washing on bull spermatozoa as far as oxygen consumption or motility were concerned. Lactic acid

production was decreased by washing twice, while no effect was noted from a single washing.

In another study, White (104) found that total oxygen uptake of ram and bull spermatozoa, in a three hour period at 37°C., was not affected by washing four times in a sodium phosphate-fructose diluent or by centrifugation four times without washing. However, lactic acid production by the spermatozoa of both species was significantly reduced by washing four times, whereas centrifuging had no effect. When 0.004 M KCL was added to the washing solution, ram and bull spermatozoa produced more lactic acid than in the absence of potassium. From this it was concluded that potassium is lost during washing and dilution and that it is more important in glycolysis than the oxidative mechanisms.

Smith, Mayer and Merilan (97) studied the effect of washing bovine spermatozoa four times with isotonic saline. They found that washing with saline produced a highly significant stimulation of succinic dehydrogenase and aldolase. From their study, it appears that the primary harmful effect of washing may be due to the loss of necessary components of the cellular enzyme systems or the enzymes themselves. Further studies have shown that egg yolk, a common extender for bovine spermatozoa, stimulates succinic and malic dehydrogenases and glyceraldehyde-3-phosphate activity of bovine sperm cells and that the stimulation was not necessarily dependent upon washing (96).

### Glycerol Utilization

In 1949 Polge, et al. (79) discovered that if glycerol was added to the freezing medium a significant number of fowl spermatozoa would survive the freezing and thawing process. This discovery opened new and important fields of study concerning not only the freezing and thawing procedures with glycerol, but also the metabolism of glycerol and related compounds.

Mann (60) observed that glycerol, unlike sugars and fatty acids, was not oxidized by ram or bull spermatozoa. It was believed that glycerol exerted its protective action by preventing denaturation during freezing and thawing.

White, et al. (105) have studied the metabolism of glycerol, in combination with various substrates, and its effects on motility. They found that a combination of glycerol and arabinose greatly improved the survival of bull spermatozoa during slow freezing. In this study four extenders were used: 1) Phosphate-fructose, 2) phosphate-fructose plus 7.5 per cent glycerol, 3) phosphate-fructose plus 1.25 per cent arabinose, 4) phosphate-fructose, 7.5 per cent glycerol and 1.25 per cent arabinose. It was found that lactic acid could be produced by ram and bull spermatozoa from glycerol, but not from arabinose. It was postulated that arabinose might accelerate the entry of glycerol into the spermatozoa, but no explanation was offered concerning where glycerol enters the glycolytic cycle. The observation that glycerol is converted to lactic acid suggests that

it does enter the spermatozoa. The cells were centrifuged twice at 300 times gravity and metabolic studies conducted in a Warburg Respirometer at 37°C. for two to three hours, with a shaking rate of 1114 strokes per minute.

In contrast to Mann's earlier work (60), Mann and White (61) have found that glycerol (0.01 M) was utilized aerobically by washed ram spermatozoa,  $3 \times 10^8$  cells per milliliter. The disappearance of glycerol was accompanied by an increased oxygen uptake, and by an accumulation of lactic acid, which appeared to be an intermediate product in the oxidative breakdown of glycerol. When glycerol was replaced by dihydroxyacetone, the oxygen consumption of spermatozoa was increased and lactic acid increased in about the same amounts as from glycerol. Apparently phosphoglycerol is dephosphorylated by washed ram spermatozoa both anaerobically and aerobically, but under anaerobic conditions glycerol accumulated as the final product of phosphoglycerol breakdown. It was also found that there is little or no disappearance of glycerol under anaerobic conditions and that glycerol has no appreciable effect on the rate of anaerobic fructolysis in sperm suspensions to which fructose has been added.

The inability of spermatozoa to utilize glycerol under anaerobic conditions, as reported by Mann and White (61), does not agree with the observations of O'Dell, Flipse, and Almquist (72) for bovine spermatozoa. They designed an experiment to determine if glycerol-1- $C^{14}$  enters the bull sperm cell and whether or not glycerol-1- $C^{14}$  is metabolized by bull spermatozoa.

Semen was incubated under nitrogen at 5°C. in the presence of: 1) fresh pasteurized skim milk heated to 92°C. for ten minutes, 2) 0.9 per cent sodium chloride in the presence of ten uc. of glycerol, with a final concentration of 6.5 to 12.5 per cent glycerol. When skim milk was used, they obtained only small amounts of radioactivity and concluded that the milk might contain substances which compete with glycerol for entry into the sperm cell. However, in the presence of sodium chloride (18-20 hours equilibration) as an extender, spermatozoa produced rather large amounts of radioactive carbon dioxide. By using cells separated from the seminal plasma, it was shown that oxidation of the glycerol was due almost entirely to the metabolic processes of the spermatozoa. The greater activity of spermatozoa, in the absence of seminal plasma, was thought to be due to removal of metabolizable material such as fructose. It was concluded that glycerol, as measured by anaerobic radioactive carbon dioxide production, entered bull spermatozoa in measurable amounts and that a minimum of four washings was needed to remove all of the absorbed glycerol from the sperm cells.

Glycerol metabolism in Bacillus subtilis has been studied by Wiame, et al. (106) and they concluded that in strain (S1) glycerol is oxidized through the tricarboxylic acid cycle, while in strain (M2) the glycerol is actively oxidized before it reaches the tri-carboxylic acid cycle. Thus, it appears that dihydroxyacetone goes to dihydroxyacetone phosphate to pyruvate and to the Krebs cycle or condenses to hexose derivatives. In strain (M2), which contains no

triokinase, 90 per cent goes to dihydroxyacetone while in strain (S $\bar{1}$ ) fewer reducing substances are formed, primarily a mixture of dihydroxyacetone and acetylmethylcarbinol.

Wood and Werkman (108) have studied the dissimilation of glycerol and utilization of carbon dioxide from glycerol. They found, with several species of *Propionibacterium*, that the total carbon dioxide liberated during fermentation of glycerol, plus that remaining in the form of carbonate, is less than the original carbon dioxide in carbonate form. The decrease results from utilization of carbon dioxide by bacteria during their dissimilation of glycerol. They believed that with these bacteria the formation of succinic acid from glycerol was not necessarily accompanied by an evolution of carbon dioxide, because the carbon dioxide was utilized as it was formed.

Hauge, et al. (39) have found that glycerol is rapidly oxidized to dihydroxyacetone by *Acetobacter suboxydans* but that the conversion proceeds more slowly from dihydroxyacetone. In these bacteria it has been found that formation of dihydroxyacetone phosphate must take place from glycerol alpha-phosphate or from dihydroxyacetone plus ATP. At this time, dihydroxyacetone phosphate is converted by isomerase and aldolase to fructose diphosphate which loses one phosphate radical, resulting in the formation of fructose-6-phosphate. At this point, it appears to be involved with compounds similar to some of those found in the monophosphate shunt (73). Eventual breakdown occurs through the pentose cycle.

It has been pointed out by Thimann (99) that glycerol is fer-



mented to alcohol, lactic acid, formic acid, carbon dioxide, and acetic acid by several species of bacteria. Under anaerobic conditions, fermentation ceases before all the glycerol is used. In animal tissues it appears that the most common mechanism for glycerol breakdown is through the pathways of carbohydrate metabolism, probably by phosphorylation to phosphoglycerol to dihydroxyacetone phosphate to phosphoglyceric aldehyde or phosphoglyceric acid and then to pyruvic and lactic acids (18, 40).

Glycerol apparently has some beneficial effects which may or may not be due to metabolism alone. Ryley (83) found during the study of protozoan metabolism that glycerol, as well as many other compounds, stimulated respiration to some extent. In addition, Flipse and Almquist (29) found that motility of bovine spermatozoa was maintained longer in a milk-glycine diluent containing glycerol than in any other combination tested. In a later investigation (30) it was found that glycine and/or glycerol reduced the decline in fertility of semen used the third and fourth day after collection. It was also noted that oxygen consumption was slightly less when glycerol was omitted from milk-glycine-glycerol or saline-glycine-glycerol extender.

McLean (64) has observed an increase in survival time from 12-14 days for control semen to 22-25 days for semen extended in egg yolk citrate or boiled, homogenized milk plus ten per cent glycerol. The preliminary results indicated that the addition of glycerol to diluted bull semen stored at 2-3°C., increased its use five days beyond

its normal usable period, with not more than a five per cent loss in conception rate.

#### Glycerol Equilibration

Although a large number of workers have studied the relationships between glycerol equilibration time, extending medium, and fertility, there is still disagreement on the proper length of equilibration time and per cent glycerol to use for best results.

In 1952, Polge and Lovelock (77) obtained good survival rates after equilibrating bull semen for 15 to 20 hours in a final dilution of 10 per cent glycerol and 25 per cent egg yolk with 3.92 per cent sodium citrate.

Mixner and Saroff (67) used 7.5 per cent glycerol in egg yolk citrate extender with an equilibration time of 18 hours and found a higher per cent of motile spermatozoa after freezing and thawing than in any other concentration of glycerol ranging from 2.5 to 12.5 per cent. In a later study, Saroff and Mixner (84) demonstrated an interaction between egg yolk and glycerol levels. Six per cent glycerol was superior for 18.4 and 23.0 per cent egg yolk, while 8 per cent glycerol was superior for 27.6 per cent egg yolk. Also, it was noted that the per cent of motile sperm increased with increased equilibration time. In another experiment, using various levels of egg yolk, glycerol, and 18 hours equilibration time, the best results were obtained with 7 per cent glycerol and 20 per cent egg yolk.

Cragle and Myers (14) studied various levels of sodium citrate, glycerol, and the hours of equilibration time necessary for acceptable survival of bovine spermatozoa. Their results indicated acceptable recovery of progressively motile spermatozoa using 2.4 to 3.3 per cent sodium citrate, 4.5 to 8 per cent glycerol, and equilibration time of 6.5 to 16.0 hours.

Later Cragle, et al. (15) employed a three-dimensional central composite experimental design, and maintained the egg yolk constant at 24 per cent in an attempt to determine optimal levels of sodium citrate, glycerol, and equilibration time. It was estimated from their results that 2.9 per cent sodium citrate, 7.6 per cent glycerol, and 14.9 hours equilibration was optimal for freezing bovine spermatozoa in an egg yolk citrate extender. This concentration of glycerol is within the optimal range of six to eight per cent found by Miller and VanDemark (66). However, in this study, six hours equilibration was adequate for optimal survival. Williams and Green (107) also obtained good survival after freezing and thawing using a seven per cent glycerol solution; however, in their study, an 18 hour equilibration time was employed.

In contrast to the longer equilibration times, O'Dell and Hurst (71) showed statistically higher recovery rates when sperm cells were equilibrated in glycerol for 0 hours as compared to 18 hours. In this investigation, both skim milk and egg yolk citrate extenders were used. The egg yolk citrate diluter contained 20 per cent egg yolk and 80 per cent 0.1 M sodium citrate. The glycerol was added

in five steps at six minute intervals to give a final concentration of eight per cent glycerol.

In Israel, Schindler (85) obtained the best freezing results with eight per cent glycerol, which agrees with O'Dell and Hurst (71). However, in this study an equilibration time of five hours proved most satisfactory.

Graham, et al. (33) froze bovine spermatozoa in pasteurized, homogenized, heated whole milk plus 10 per cent glycerol and egg yolk citrate with seven per cent glycerol. Portions of the semen collections were allowed to equilibrate four, eight, and twelve hours prior to freezing. Seventy-five day non-returns showed a conception rate of 63.4 per cent, 65.2 per cent, and 67.8 per cent for four, eight, and twelve hours, respectively. The difference between four and twelve hour equilibration was just significant at the five per cent level.

A group of Australian workers (7, 21, 22) have studied the advantages of 30 minute equilibration as compared to equilibrating overnight. In some of their preliminary studies (21) it was found that non-equilibrated semen frozen in glycerol and arabinose gave a higher three-months non-return rate than the equilibrated controls. Results indicated that 63 per cent of the cows settled with non-equilibrated semen while the equilibrated controls showed a 56 per cent non-return. In more recent publications (7, 22), additional evidence has been presented to substantiate their earlier investigations. Control semen was diluted in egg yolk citrate, buffered with

sodium phosphates and stored at  $4^{\circ}\text{C}$ . with a final concentration of  $3 \times 10^7$  sperm cells. A second portion of the original sample was diluted to  $3 \times 10^7$  cells with 7.5 per cent glycerol and 1.25 per cent arabinose. One-half of this sample was frozen after 30-50 minutes (non-equilibrated), while the remaining portion was allowed to equilibrate overnight. Fertility tests on 1,014 cows showed no significant differences in the fertility of the control semen held at  $4^{\circ}\text{C}$ . and the semen frozen to  $-79^{\circ}\text{C}$ . with or without equilibration. Thirty-one day non-returns showed a 73, 78, and 72 per cent conception rate, respectively. However, semen frozen under both conditions showed fewer live sperm than the controls. In addition, it was observed that the non-equilibrated semen showed fewer live sperm. Thus it appears that semen equilibrated for 18 hours gives a better revival rate without any increase in fertility. This supports the findings that arabinose might accelerate the entry of glycerol into the sperm cell (105).

Hafs and Elliott (38) found that 25 per cent egg yolk and 1.0 per cent fructose gave higher revival rates than either glucose or xylose in the same concentrations. Equilibration time was held constant at 18 hours.

Although egg yolk citrate is still the most common extender for bovine spermatozoa, Rakes and Stallcup (80) have studied the possibility of using glycine as a replacement for citrate. In this study a dilution of 1:20 and an equilibration time of 18 hours was used. It was noted that glycine does not buffer the semen as well as sodium

citrate, but after six days storage at  $-78^{\circ}\text{C}$ . the glycine-yolk extenders were reported to give better results than yolk citrate mixtures.

#### Protective Action of Glycerol

Smith, et al. (95) conducted one of the first investigations on the nature of glycerol action. In their study it was noted that the shape of the ice crystals was modified in the presence of glycerol and this suggested that the mechanical stress of freezing might be reduced when glycerol is employed in the freezing media.

In 1950, Smith (92) studied the prevention of hemolysis of red blood cells during freezing and thawing. It was observed that, without any pretreatment, red blood cells of the rabbit could be stored in vitro for four weeks if the temperatures were maintained at  $+2^{\circ}\text{C}$ . to  $+8^{\circ}\text{C}$ . However, if 10 to 15 per cent glycerol was used in Ringers' solution with 0.85 per cent sodium chloride and the cells frozen, hemolysis could be prevented for extended periods of time. Later Sloviter (91) found in the freezing of human red blood cells in the presence of glycerol that the glycerol must be removed before the cells could be placed in a solution containing no glycerol. Red blood cells containing glycerol, regardless of whether or not they had been frozen, would hemolyze rapidly when placed in plasma or other isotonic media. This was believed to occur as a result of the increased osmotic tension of the cellular contents. The hypertonic cell would draw in water, swell, and rupture. It was further noted

that equilibration time was one of the many factors to consider when freezing tissue.

Lucke and Parpart (52) used a photoelectric method to study the permeability of cancer cells and mouse red blood cells to various compounds. A beam of light, of constant intensity, was passed through a chamber containing the cellular suspension. The light impinged upon a photronic cell, and the current from the cell was picked up by a Kipp torsion string galvanometer. It was found that the amount of light passing through the suspension decreased as the cells shrank. In turn, the galvanometer current decreased and a downward deflection was recorded. The reverse occurred when the cells swelled.

The rate of entry into tumor cells was found to be fastest for ethylene glycol and slowest for erythritol with diethylene glycol, triethylene glycol, and glycerol intermediate, in that order. The same order was found to be true for mouse red blood cells, but the relative rates of entry were observed to be much faster than for tumor cells. It was further noted that both were relatively impermeable to sodium chloride. The time required for tumor cells in glycerol to reach minimum volume was 15 seconds as compared to 1 second for red blood cells. The time required for the cells to reach one-half equilibrium volume in glycerol was 210 and 7 seconds for tumor cells and red blood cells, respectively.

Lovelock (48) used  $10^{-5}$  M copper sulfate solution in combination with glycerol to prove the entry of glycerol into red blood cells. The entry of glycerol was prevented by washing the cells in 0.9 per

cent sodium chloride solution containing  $10^{-5}$  M copper sulfate. This concentration of copper sulfate was also used in the suspending media for freezing the cells. The sample was divided and one-half frozen immediately and the remaining portion kept at  $40^{\circ}\text{C}$ . for 20 minutes. This allowed the glycerol to permeate the cell in spite of the copper ions. In these experiments it was found that ten minutes at  $40^{\circ}\text{C}$ . was sufficient time for glycerol to enter the cell even in the presence of copper. At lower temperatures, when copper was present, a considerably longer equilibration time was necessary to prevent hemolysis. At temperatures below  $0^{\circ}\text{C}$ ., it was found that in the presence of copper the cells are impermeable to glycerol. From these results, it was suggested that glycerol would protect the cell only when allowed to enter the cell and that cells impermeable to glycerol would not survive the freezing and thawing process.

From this and other work (50), Lovelock postulates that the protective action afforded by glycerol is one of "salt buffering". It is well known that when a solution freezes, ice separates as a pure substance, thus allowing the dissolved and solid substances to concentrate in channels of fluid between the ice crystals. Therefore, Lovelock states that the salts are concentrated around the cells, in some cases nearly ten times as high as normal, and the cell would be killed by excess salinity before ice crystals could be formed in sufficient number to cause death. In view of this evidence, he states, "living cells can enjoy the protection offered by neutral solutes only when permeated by them. There is no benefit in preventing the salt



concentration of the medium outside unless the medium inside is also checked." This suggests that the internal KCl concentration is as important as the external NaCl in causing damage.

In red blood cells, the greatest amount of the destructive action occurs between  $-3^{\circ}\text{C}$ . and  $-40^{\circ}\text{C}$ ., which has been shown to correspond to the region in which the cell is exposed to the greatest salt concentration, and that a 2.5 M concentration of glycerol will eliminate this critical area (48).

In 1940, Luyet and Geheio (53) published a treatise concerning injury and death by low temperature. At that time, some nine years before the protective action of glycerol was discovered, no mention was made of increased salt concentration. In a more recent publication (54), chick embryo heart slices, frozen in the presence of glycerol, were found to be viable after freezing and thawing. Actual observations made while freezing was proceeding showed that ice crystal formation was retarded greatly as compared to the untreated controls. From these results, the protective action of glycerol was thought to be exerted by draining water from the tissues or by impregnating them, increasing the solute concentration and exerting a binding action on the water, thus preventing crystal formation. Easy penetration into the tissue to be frozen was listed as one of the properties of glycerol necessary to protect the cells.

In another study (55), using the same type of tissue, 100, 60, and 30 per cent glycerol were all found to be effective in protecting the chick embryo heart slices. It was found that an equilibration

time of 15 seconds was as effective as four minutes.

Taylor (98) has frozen rat and mouse skin in the presence of glycerol and found that protective treatments reduced the amount of ice formed in the frozen tissue. In this work, phase microscope studies suggested that glycerol may enter the cells and its action to be one of dehydration.

Lovelock (49) has devised a method of calculating the amount of solute necessary to protect cells during freezing and thawing. Of the compounds used, it was found that the theoretical and actual figures were closer for glycerol than for any of the other substances studied. Several substances were tested, including methanol, formamide, ethanol, acetamide, ethylene glycol, propylene glycol, glycerol, diethylene glycol, erythritol, monoacetin, D-xylose, triethylene glycol, glucose, sucrose, and polyethylene glycol. It was found that for compounds which readily penetrated the cell, ten minutes at 20°C. was a sufficient equilibration time. However, solutes which crossed the cell membrane more slowly required one hour at 37°C.

The poorly penetrating substances were erythritol, xylose, and glucose, and it was observed that sucrose and polyethylene glycol did not enter the cells. Only three of the compounds used gave complete protection at all temperatures. They were glycerol, ethylene glycol, and diethylene glycol. It was concluded that weight concentration of material required to protect the cell was in proportion to the molecular weight of the compound being tested.

Smith (93) investigated suspension fluids for freezing rabbit

ovarian granulosa cells. It was found that cells frozen slowly in 15 per cent glycerol and rabbit serum showed the best survival. It was thought that 15 per cent glycerol might protect the cells by partial dehydration due to osmotic withdrawal of the water.

Luyet (56), in studying the protective action of glucose and sodium chloride, concluded that their protective action was due to dehydration. The concentrations which afforded maximum protection during freezing and thawing were 1.5 M glucose and 1.0 M sodium chloride. At these concentrations, both exert the same osmotic pressure.

Although more attention has been given to the freezing of bull spermatozoa and red blood cells, many types of tissues have been frozen. Smith, in a review (94), has pointed out various tissues which have been frozen and thawed with some success: rabbit eggs, testicular tissue, adrenal cortex, anterior pituitary and ovarian tissue.

It has been pointed out by Smith (94) that bull spermatozoa must be cooled slowly when being frozen because they are sensitive to cold shock between  $+37^{\circ}\text{C}$ . and  $0^{\circ}\text{C}$ . and to thermal shock between  $0^{\circ}\text{C}$ . and  $-79^{\circ}\text{C}$ . The most critical stage has been found to be between  $0^{\circ}\text{C}$ . and  $-15^{\circ}\text{C}$ .

When Polge, et al. (79) discovered that glycerol would protect fowl spermatozoa during freezing and thawing, they also found that the glycerol must be removed before fertilization could occur. In a later study (78) with bull spermatozoa it was found that glycerol did not

interfere with fertilization. In fact, some of the evidence suggested that fertilizing power might be enhanced by the addition of glycerol.

Polge (76), in a discussion of low temperature preservation of spermatozoa, indicated that some mechanism similar to "salt buffering" of red blood cells may account for the protective action of glycerol in bull spermatozoa. He further states that "it is evident, also, that glycerol must enter the cell in order to exert its protective action during freezing, as is shown by the necessity of equilibrating bull semen with the glycerol diluent. In this respect, the species variation that we have found in the resistance of spermatozoa in glycerol-containing media, might reflect some differences in their permeability." The variation in spermatozoa may indicate fundamental differences in their permeability to glycerol, resistance to increasing salt concentration, osmotic shock, and temperature shock. It has been pointed out by Lovelock (51) that there is a rather pronounced difference in entry rate of glycerol in red blood cells of different species.

Hendrikse, et al. (41), in their investigation with bull spermatozoa, found that glycerol exerts a considerable osmotic effect and lowers the freezing point. It was suggested that the osmotic activities of glycerol might provide an explanation for the protective and injurious activities found when freezing semen.

When glycerol solutions are added to the mixture to be frozen, heat is released due to orientation of the unpolarized, bipolar water molecules around the glycerol. This orientation might explain the

need for equilibration. It was further noted that equilibration time could be shortened to about four hours by using a stirring apparatus to facilitate the mixing of glycerol in this solution.

Bunge and Sherman (13) have found that ten per cent glycerol was optimal for freezing human spermatozoa and that no other addition was necessary. These workers (88) have also observed that glycerol, or the freezing and thawing process, had no adverse effects on the spermatozoal proteins stained with seven oxidation-reduction potential dyes at pH four through ten.

Sherman (87), working with human spermatozoa, found that a pre-treatment of five minutes was just as effective as a 30 minute equilibration time. He concluded that no more than 25 seconds exposure of human spermatozoa to glycerol was necessary to protect the cells from death during freezing and thawing, regardless of the site or mode of glycerol action.

#### Autoradiography

Autoradiography is a method for detecting radioisotopes, based on their ability to affect silver bromide crystals of photographic emulsions. These crystals act as micro-detectors of radiation and are useful in localizing a radioactive substance in a body or tissue, and in some cases, single cells where a Geiger counter would fail. Essentially, an autoradiogram is obtained by placing a radioactive tissue in contact with a photographic emulsion, allowing sufficient time for exposure, and then developing as in ordinary photography. The re-

sulting picture (autoradiogram) consists of accumulations of black silver halide granules overlying the areas in the tissue specimen which contains the radioactive material (9). As with other methods involving the use of radioisotopes, it must be assumed that the chemical behavior of a labeled substance is identical to that of the same non-radioactive substance. Thus the amount of radioactivity used must be small enough not to cause any reactions that the unlabeled substance would not also produce (35).

Photographic emulsions, in general, consist of silver bromide crystals or "grains" embedded in gelatin, the bromide concentration and mean diameter varying with the different types of emulsions. The photographic mechanism, in the case of radioactive emissions, is similar to that for radiant energy. White light causes an excitation and release of electrons from some of the bromide ions. These electrons are trapped within the crystals and silver ions are attracted to these points, resulting in the deposition of photolytic silver. The developer, a reducing substance, converts silver ions to metallic silver, which then precipitates, making the crystals visible as black granules. When a radioactive particle passes through a grain it will, by direct collisions, release orbital electrons which migrate to the sensitivity specks. The efficiency of the release of these electrons will depend upon the energy of the particle (35). A certain number of grains is always developed due to chemical imperfections, cosmic radiation, exposure to light during manufacture, and handling of the emulsion in the dark room (17).

In 1946, Belanger and Leblond (4) were the first to develop a method for obtaining autoradiograms of histological sections by spreading the photographic emulsion over them. Bayley (3), in 1947, pointed out that the procedure was hardly applicable for single cells, until better emulsions were made available. In 1947, Mullins (69) was one of the first to apply the procedure to the study of single cells. In 1951, Eidinoff, et al. (20) used the method to study tritium in yeast. Also in 1951 King, et al. (46) used track autoradiography to localize  $P^{32}$  in Paramecium caudatum.

Gross, et al. (35) pointed out that long-lived isotopes, such as the 5500 year half-life  $C^{14}$ , must contain many more atoms of isotope per millicurie than the short-lived elements, and therefore cannot be prepared carrier free. As a result, these radioelements are often less suitable for tracer experiments. However, in the past few years, more and more labeled compounds have become available, which tends to render them very useful in autoradiographic studies, especially  $C^{14}$ . The maximum emulsion range for this isotope is but a few microns and thus the possibility of diffusion is reduced.

Bloom, et al. (8) injected 75-150 uc. of  $C^{14}$  as  $BaC^{14}O_3$  and  $NaHC^{14}O_3$  to study carbon 14 deposition in bone and obtained good autoradiograms.

MacDonald, et al. (57), in 1948, were perhaps the first to use  $C^{14}$  for autoradiograms in which the method allowed the experimenter independence in staining and developing. Liver slices from a rabbit, starved for 48 hours, were incubated two hours in vitro with

$\text{NaHC}^{14}\text{O}_3$ , in presence of a pyruvate substrate. They used Type M stripping film, a 10 u. emulsion with a 7 u. base. The tissue section was mounted on a glass slide and the stripping film cemented, base side down, onto the tissue. The emulsion was covered with a guard slide and the sandwich wrapped with Scotch tape. After 25 days exposure, the slides were developed. The film and tissue slice were then removed together and the emulsion side cemented down. This allowed the section to be exposed for staining.

In 1948, Armstrong, et al. (2) attempted to determine the localization of  $\text{C}^{14}$  in rats after intraperitoneal injections of  $\text{NaHC}^{14}\text{O}_3$  or peritoneal cavity implantation of  $\text{CaC}^{14}\text{O}_3$ . For preparation of the autoradiographs, the material to be studied was dried and ground on a fine grain carborundum stone. Then it was placed in direct contact with Eastman No-Screen X-ray Film and exposed in the dark for two weeks. It was shown that a significant amount of  $\text{C}^{14}$  was found in glycerol as well as in many other compounds and structures.

Boyd, et al. (11) made autoradiograms of  $\text{C}^{14}$  incorporated into individual blood cells. These workers injected glycine- $\text{C}^{14}$  into rats and after 25 hours withdrew blood from the tail vein. The blood was diluted with serum prepared from dog blood, then smeared directly on Eastman NTB emulsion. The smears were dried in air and fixed in methyl alcohol. The blood smears were diluted to insure well defined autoradiograms. The NTB plates were exposed 67 days, then developed in Eastman Kodak D-19 developer. It was found that excessive developing time results in a loss of cellular detail.



Boyd and Levi (10) have studied the localization of glycine- $C^{14}$  in liver sections of the rat. In these experiments, NTB emulsion, 25 and 100 u. thickness on glass plates, was employed. This particular emulsion is rather sensitive and excellent for recording beta tracks such as those given off by  $C^{14}$ . These workers used water to float the tissue sections onto the plates. Partial drying was carried out prior to storage of the plates in black plastic slide boxes and several grams of  $CaCl_2$  were placed in each box to maintain a low humidity. The plates were exposed for 10 days, developed in Eastman D-19 developer for 20 minutes, and then fixed in 30 per cent hypo at  $20^{\circ}C$ .

In 1950, Boyd, et al. (12) employed a technique for studying the incorporation of glycine- $C^{14}$  into individual red blood cells using Eastman Kodak NTB Experimental Plates with a 6 u. emulsion. These workers diluted oxalated blood with fresh, clear serum to obtain the desired cellular concentration. In the dark room, a drop of the cell suspension was placed directly on the emulsion and smeared by pushing forward a clean glass slide. By holding the smearing slide at an angle of  $20^{\circ}$ , the cell suspension was pulled along behind it. The smears were dried in air, fixed by flooding the plates with methyl alcohol for two minutes and then the plates were allowed to air dry again. After storage in a light-tight box, in the presence of a desiccant for 67 days, the slides were developed in Eastman Kodak D-19 for two minutes at  $20^{\circ}C$ ., rinsed in tap water one-half minute, then fixed in 10 per cent sodium thiosulfate until clear. Following washing for 20-30 minutes, the slides were again air dried.

These workers found the photographic-fixing step to be the most critical in the technique. It was further noted that NTB plates with a 6 u. emulsion appeared to be the most sensitive to beta particles for its grain size.

Skipper, et al. (90) studied the hazard involved in the use of  $C^{14}$  by autoradiographing bones of mice having received intraperitoneal injections of 18 uc. of  $NaHC^{14}O_3$  (2.5 mg. per mouse). The animals were injected, then sacrificed at various times to determine  $C^{14}$  retention. The prepared bones were clamped to Eastman No-Screen X-ray Film and exposed from 30 days to 5 months. The autoradiographic assessments were made gravimetrically and the approximate total  $C^{14}$  content of the various organs, tissues, and bones determined. These data, when totaled, provided information with regard to the total radioactivity in the animal and the per cent of the total injected dose retained after a specific time.

Greulich and Leblond (34), in a similar experiment, injected 20-40 uc. of  $C^{14}$  labeled bicarbonate into 12-hour old rats. Sites of deposition of the  $C^{14}$  were determined by autoradiography. These workers employed the "coating" method (9), with two types of melted photographic emulsion: 1) Ansco "Radiographic Emulsion A", and 2) Eastman Kodak NTB3 Emulsion. The Ansco emulsion is very sensitive, but coarse grained, and must be used immediately upon receipt; whereas the Eastman NTB3 Emulsion is less sensitive, but fine grained, and is obtained in pellicles. This makes it necessary to soak the emulsion in a one per cent aqueous solution of "dupanol" (ethyl laurate) for

24 hours before melting at  $37^{\circ}\text{C}$ . The coated autoradiographs were exposed in the dark at  $-17^{\circ}\text{C}$ . for eight to ten months. Following exposure, routine developing techniques were used. The tissues were then stained with an acid hematoxylin solution and counter-stained with eosin. Autoradiograms were made of cerebrum, salivary glands, eye, skin, spleen, urinary bladder, pancreas, various regions of the stomach, liver, lung, duodenum, thyroid, kidney, heart, testis, thymus, uterus and adrenals. Examination of these autoradiograms showed that all organs and tissues of the body studied contained substances formed from the labeled bicarbonate. Thus they concluded that carbon dioxide may be used for organic synthesis by animal tissues. Some of the carbon dioxide produced must be reused in formation of products of the Krebs Cycle, which may enter into the synthesis of carbohydrates and proteins.

In 1954, Levi (47) obtained beta track autoradiographs of yeast and algae. The labeled cells were washed in inactive nutrient solution of the same composition in which they had been grown. Ilford G-5 emulsion was used. It was obtained as a gel and was melted by heating in a water bath at  $43^{\circ}\text{C}$ . for ten minutes. When the emulsion was liquefied, the cell suspension was added with a micropipette. The emulsion containing the cells was poured on the prepared slides. The slides were kept at  $40^{\circ}\text{C}$ . on a level table which was heated by a stream of warm water. This allowed the emulsion to spread evenly to a uniform thickness. The temperature of the table was changed to  $15^{\circ}\text{C}$ . by replacing the warm water with cold, thus allowing the emulsion

to harden. By using this method, Levi found that the cells were well preserved after embedding and processing. Autoradiograms of Scenedesmus algae, labeled with  $C^{14}$  and viewed at 900x, showed many well-defined tracks. It was concluded that this method was very good for studying low energy beta track emissions. One disadvantage of this emulsion is that it remains sensitive to low energy particles for only about two to three weeks.

After autoradiograms have been prepared, the problem of evaluation arises. The method used for evaluation will depend upon autoradiographic techniques, type of emulsion, and energy of the isotope. There are three quantitative methods for determination of radioisotopes in an autoradiogram: 1) densitometric evaluation of random grain autoradiograms, 2) grain counting, 3) track counting (47).

Skipper, et al. (89) injected 4.0 and 100 uc. per mouse, of  $C^{14}$  labeled sodium formate. Blood was collected from the heart and the blood cells were washed twice with inactive heparinized mouse plasma to remove active sera. The cells were then diluted (5:1) in inactive mouse plasma and spread directly on Eastman NTB plates, emulsion thickness 10 u. It was found that after an exposure of six weeks very faint autoradiograms were obtained from cells of mice injected with 4.0 uc. However, the cells from mice injected with 100 uc. produced good autoradiograms in one week and two weeks exposure was found to be too long. The developed autoradiograms were examined by a technique which entailed counting the silver grains immediately surrounding the individual cell and comparing with the background. An eye-

piece reticle which divided the field into 10 u. squares at a magnification of 970x was placed in the microscope. The grains in these squares were counted and an average taken. A count was taken from an area where no cells were seen and subtracted from a count obtained from an area including cells to determine the amount around each individual cell.

Mazia, et al. (63) employed an autoradiographic technique which gave high resolution autoradiograms of Amoeba proteus labeled with adenine- $C^{14}$ . A stripping film technique, using Kodalith Ortho Film and processed with Kodalith Fine Line Developer, which allowed quantitative densitometric measurements of the autoradiograms, was used to determine relative amounts of activity in the different areas. Andresen, et al. (1) have also studied the metabolism of  $C^{14}$  in Amoebae by a stripping film method. Their method allowed quantitative counting of the number of developed photographic grains per unit area in a strip through the length of the section.

In addition to the above mentioned techniques, Schoolar, et al. (86) have devised a method for quantitative determinations of radioactivity in connection with autoradiograms. Carbon  $14$  labeled Isoniazid was given intraperitoneally to adult cats in an attempt to localize the compound and/or its metabolites in brain tissues. Direct counting of various parts of the brain gave disintegrations per minute of dry weight tissue. The autoradiograms were made on Kodak No-Screen X-ray Film and exposed 30 days at  $-26^{\circ}C$ . By using the two methods in combination, these authors were able to localize  $C^{14}$  in the various

structures of the brain.

Walters and Thaine (101) have used Ilford G-5 nuclear track emulsion to obtain high resolution autoradiograms with  $C^{14}$ . By shielding out cosmic radiation with a four inch lead shield, they were able to pick up extremely small concentrations of  $C^{14}$ , which would allow quantitative beta track counting.

Risley (82) has obtained autoradiograms of hamster spermatozoa using intact animals injected with 60-120 uc. of  $H_3P^{32}O_4$ . Good results were obtained with epididymal spermatozoa by suspending them in Tyrode's solution and pipetting the cells directly on Eastman medium lantern slide plates. It was found that 56 to 60 days were required to obtain good results under the conditions of this study.

Bishop (5) studied localization of  $P^{32}$  in the bull sperm cell using an autoradiographic technique. In this study the cells were placed in contact with Eastman NTB plates for the desired exposure time. It was found that the posterior portion of the head and the mid-piece appeared to be the areas of the cell which accumulated the greatest proportion of the isotope.

## MATERIALS AND METHODS

### Semen Collection

Bull semen was collected by means of an artificial vagina from healthy bulls maintained as part of the University of Missouri Agricultural Experiment Station Dairy Herd. The semen collection tube was placed immediately in a thermos bottle containing water at a temperature of 15°C. for transport to the laboratory, where motility ratings were made using a 0-5 scale, with five as excellent and zero as immotile (42). No semen samples rating less than a four motility were used. In some cases a (+) or (-) was used to indicate minor differences between samples.

### Manometry

#### Procedure for Washing Spermatozoa:

The spermatozoa were washed, to remove the seminal plasma, by the addition of either calcium-free Krebs-Ringer phosphate (KRP) or calcium-free Krebs-Ringer bicarbonate (KRC), depending upon which was used as a suspension medium during the experiment. The whole semen was suspended in 167.00 per cent of its original volume, mixed thoroughly and centrifuged for ten minutes at 2000 rpm (100 rpm=30x gravity). The same volume of material that had been added was removed. Extreme care was taken not to disturb the loosely packed cells. Following the removal of the desired quantity of supernatant, the cells were resuspended by gently drawing them up and down in a

small bore pipette. Sperm cell concentration for each washed suspension was determined by the hemocytometer method (42). When the semen was not used immediately after motility ratings were made, the test tube was placed in a 500 ml. beaker three-fourths full of water, and stored in the refrigerator. Thus the water cooled slowly, preventing cold shock to the spermatozoa. Immediately after centrifugation and washing, 0.5 ml. of the semen was pipetted into the reaction vessels with a long-tip pipette.

#### Manometric Technique:

The manometric measurements were made at 38°C. in a rectangular Constant Volume Warburg Respirometer accomodating 14 reaction flasks per experimental run and adjusted to a shaking speed of 110 strokes per minute. The flasks of 15-18 ml. capacity were mercury calibrated by the technique suggested by Umbreit et al. (100).

Each series of experiments were anaerobic. Gassing was accomplished by using a water vacuum pump and manifold gassing arrangement. The reaction flasks and manometers were alternately filled with the gas and evacuated, a minimum of ten times, to assure replacement of the air. The last six experiments were gassed by the technique outlined by Hawk, Oser, and Summerson for anaerobic experiments (40). After attaching the vessels to the manometers, a stream of nitrogen gas (water pumped) was passed through the vessels for 30 minutes. Every five minutes the manometer fluid (Brodie's solution) was run up and down to displace any air that might be in the manometer capillary.



The types of gases used in the course of the experiments were a 95.0 per cent nitrogen and 5.0 per cent carbon dioxide mixture and a 100.00 per cent nitrogen gas.

The stock solutions for KRP and KRC used in the experiments were prepared as follows:

NaCl 4.50 g.

KCl 5.75 g.

$\text{KH}_2\text{PO}_4$  10.55 g.

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  19.10 g.

Phosphate Buffer

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  17.80 g.

HCl 1 N 20.00 ml.

Distilled water to one liter

Bicarbonate Buffer

$\text{NaHCO}_3$  1.30 g. per 100 ml.

Gassed for one hour with carbon dioxide

The first four compounds were placed in separate 100 ml. flasks and water (redistilled over glass) was added to make 100 ml. Made up in these concentrations, they could be stored for several weeks in the refrigerator.

Prior to each experiment, 100 parts of NaCl stock solution, four parts KCl, one part  $\text{KH}_2\text{PO}_4$  and one part  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  were pipetted into a 500 ml. flask. To this mixture was added 218.00 ml. of distilled water. If KRP were desired, 25 ml. of the phosphate buffer were added

to 225 ml. of the foregoing mixture. If KRC were desired, 40 ml. of the carbonate buffer was added to 210 ml. of the mixture.

When the final solution was prepared, it was gassed for ten minutes with the appropriate gas for that experiment.

Radioactive fructose used in the experiments was obtained as D (-) fructose-C<sup>14</sup> (uniformly labeled) from Volk Radio-Chemical Company. Total activity per shipment was 50 uc. contained in 0.785 mg. Dilutions for the experiment were made by adding enough non-radioactive fructose to give a final weight of 0.3 grams. This was dissolved in sufficient distilled water to make a final volume of two ml. Two-tenths ml. of this solution was used per flask, thus giving a final concentration of one per cent fructose containing 2.5 uc. of radioactivity.

The rate of anaerobic glycolysis was measured by carbon dioxide production from the substrate with KRP as the buffer.

The various levels of glycerol to be studied were dissolved in the appropriate buffer (KRP or KRC) and placed in the reaction vessel side-arms after the semen had been added to the buffer in the flask proper.

In the experiments where it was desired to trap the carbon dioxide, 0.2 ml. of 20 per cent potassium hydroxide was added to the center well of the Warburg flask. A folded filter paper strip was placed in each well to increase surface area. In the flasks which served as controls, a 0.2 ml. solution of buffer was added to the center well, thus giving an equal volume in all flasks.

After placing the flasks in the 38°C. bath they were allowed to equilibrate for five minutes. Following equilibration, readings were taken every 5 minutes for 30 minutes to establish a base. The side-arms were then tipped and readings taken at 5 minute intervals for 75 minutes.

At the end of the experimental run, motility was observed on each flask in Series II and III. Immediately after motility ratings were made, per cent live spermatozoa was determined by the technique recommended by Herman and Madden (42).

In the remaining series, pH determinations were made with a Beckman pH meter (Model G) and motility was checked on four random samples to determine if there were live cells for the duration of the experiment.

Samples for radioactivity assay were pipetted, using a long-tip pipette and a ten ml. syringe, into 2.5 cm. diameter cupped stainless steel planchets. These samples were dried by infra-red heat lamps positioned at a sufficient distance to prevent formation of uneven surfaces or charring of the samples. After removal of the filter paper from the center well, the reaction vessel was rinsed out with distilled water, which was also added to the planchet for drying.

The potassium hydroxide filter paper was washed with five ml. of saturated barium hydroxide. The wash was pipetted into a planchet for drying.

The dried samples were assayed with a Model K-3 N. Wood gas flow counter coaxially connected to a Tracerlab 64 Scaler (Fig. 1). The



Figure 1. Tracerlab 64 Scaler and K-3 N. Wood Gas flow counter.

gas used in the counter consisted of 99.05 per cent helium and 0.95 per cent isobutane.

#### Autoradiography

##### Cleaning and Preparation of Slides:

One by three inch frosted-end glass slides were washed for two hours in potassium dichromate cleaning solution. The slides were then rinsed in distilled water several times and given a final rinse in water redistilled over glass and the slides allowed to air dry.

Further preparation of the slides included the addition of some type of adhesive that would prevent the dried emulsion from washing off during the developing and washing process.

It can be seen from Table I that several types of adhesive were used, but the most effective was that used in experiments VI and VII. The composition of this adhesive is given below:

5.0 grams photographic gelatin

0.5 grams chrome alum

distilled water to 1000 ml. final volume

The gelatin was dissolved in 200 ml. of warm water and added to 800 ml. of chrome alum solution. The slides were held by the frosted end and dipped quickly into and out of this solution. If the slides are dipped slowly or the solution is agitated, bubbles tend to adhere to the slide, giving an uneven coating. After dipping, the slides were placed on cheesecloth and allowed to air dry for two hours.

When egg albumin or Mayer's egg albumin was used as an adhesive, a small drop was placed in the center of the slide and spread with the finger. As much of the solution was wiped off as possible in the process, in an attempt to get a very thin film of adhesive. After the adhesive was spread, the slides were allowed to dry in air for 30 minutes to 1 hour.

#### Semen Preparation:

All solutions to be used were prepared in advance of the semen collection. Immediately after motility ratings were made, the semen was extended in the desired ratio (Table I) and allowed to equilibrate with the radioactive glycerol. The length of equilibration time depended upon the type of extender used in each experiment. During equilibration the semen was maintained at 5°C. in a refrigerator. After equilibration, the extended semen was centrifuged at 1800 to 3000 rpm (approximately 540-900x gravity) for ten minutes. As much as possible of the supernatant was drawn off without disturbing the packed cells. This was then measured and an equivalent amount of extender added containing non-radioactive glycerol. The spermatozoa were then resuspended.

The radioactive glycerol used in these experiments was obtained from Nuclear Instrument and Chemical Corporation, Chicago, Illinois, as glycerol-1-C<sup>14</sup>. The total activity of each shipment was 100 uc. Upon receipt, the entire shipment was diluted to one ml. The desired number of microcuries was measured for each experiment with a syringe

and micropipette and added directly to the extended semm. The final concentration of glycerol, by volume, was supplied from a non-radioactive source and added immediately following the addition of radioactive glycerol. The tubes were then stoppered tightly and inverted several times to assure proper mixing.

#### Preparation and Addition of Emulsion to Slides:

##### A. Stripping film and dental plate procedure.

Sixteen slides were divided into two groups and a very thin film of egg albumin was spread on the slides in group one and allowed to dry. Group two was used without any pretreatment. One drop of extended semen was placed on the slide near the frosted end. One end of a clean glass slide, held at an angle of about  $20^{\circ}$  between the two slides, was pushed back until it came into contact with the drop of semen. The slide was then pulled forward, spreading the cells (9). This procedure for spreading the cells was used for all experiments except VI and VII, in which case the cells were mixed with the emulsion before spreading. The slides were then allowed to air dry for approximately one hour. The slides were taken to the dark room and groups one and two were again divided into equal groups. One-half of each group was covered with Kodak Autoradiographic Permeable Base Stripping Film (Experimental) and the other one-half covered with Kodak Dental X-ray Film (DF-58). The film was held to the slides by placing another glass slide on top of the film and binding the glass slides together with masking tape.

B. Handling and preparation of slide and liquid emulsion.

The box containing the emulsion was stored in a refrigerator at 5°C. and 50 per cent humidity. Two hours before the emulsion was to be used, it was removed from the refrigerator and allowed to come to room temperature.

When the other preparations were complete, the package containing the emulsion was opened in the dark room under safe light conditions. The jars containing the emulsion were placed in a water bath for 45-60 minutes maintained at 37°C.

While the emulsion was being warmed, the slides to be coated were placed on a level hot plate (24" X 4½") and held at 37°C. to warm the slides prior to painting.

When the emulsion had reached 37°C. it was mixed by inverting the jar several times. After thorough mixing, 20 to 25 ml. of the emulsion was poured into a glass receptacle which was sitting in a water bath (37°C.). The emulsion was taken from this receptacle with an eye dropper and two drops of emulsion were placed on each slide. The emulsion was spread very gently, but quickly, with a camel's hair brush, which had been warmed to 37°C. in a test tube in the water bath.

After painting, the slides were returned to the level hot plate and allowed to remain for one to two minutes, at which time they were removed to another level table and allowed to dry for 30-60 minutes.

The only deviation from this technique was in experiments VI and VII. In these experiments, 12-16 ml. of the warmed emulsion was



poured into the test tube containing the extended spermatozoa. The spermatozoa and the emulsion mixture were then transferred to the glass receptacle in the 37°C. water bath prior to spreading on the slides.

#### Storage during Exposure:

When the emulsion-covered slides had dried the desired length of time, the slides were stored in a light-tight, 100 slide capacity black plastic slide box. A small box ( $1\frac{1}{4}$ " X  $5\frac{1}{4}$ " X  $3/4$ " ) was filled with calcium chloride and taped inside the slide box containing the slides, to keep the humidity constant. The slide box was then sealed with black Scotch tape and wrapped in aluminum foil to prevent entry of light and moisture. Exposure was carried out in a refrigerator at 0-2°C. When the desired exposure time was reached, the slide box was removed and allowed to reach room temperature before being transported to the dark room for development (9).

#### Developing Procedure:

In experiment I, when Kodak Liquid X-ray Developer and Replenisher and Kodak Fixer Solution A plus Kodak Hardener Solution B were used, the slides were developed in solutions previously prepared and used for X-ray developing. In all other experiments, two liter stainless steel buckets were used for developing and fixing. In no case were any solutions used more than once. The Kodak D-19 was prepared one gallon at a time and used as needed. Fresh developer was not prepared until needed or until a color change or sediment occurred in the

developer. The fixer was prepared one liter at a time and a fresh solution was prepared for each group of slides.

The washing tank (9 3/4" X 15" X 18") was maintained at a temperature of 20°C. and the water changed ten times per hour. The buckets containing the developer and fixer were allowed to float in the washing tank until they had reached a temperature of 20°C. The buckets were removed and the slides developed for the prescribed length of time in a 20 slide capacity slide holder (slides were vertical during developing and fixing), washed for ten minutes in the water bath, and fixed for twice the time required for clearing. After fixing, the slides were washed the desired length of time in the washing tank. Following washing, they were spread on a table to dry. Prior to microscopic examination, the slides were stored in a light-tight calcium chloride desiccator.

#### Staining Procedure:

It was found that with the three stains used both the emulsion and spermatozoa stained, thus a staining procedure was used only in three experiments. The procedures were as follows:

##### 1. Experiment II.

##### Toluidine Blue Method (9)

- a. Stained one minute in one per cent aqueous solution of toluidine blue.
- b. Destained in 70 per cent ethyl alcohol.
- c. Dehydrated rapidly in two changes of normal butyl alcohol.

This staining procedure was employed after the slides had been developed and dried.

2. Experiment IV.

After the cells had been spread on the slides and before the emulsion was added, the slides were stained as follows:

- a. Stained three minutes with three per cent solution of eosin B.
- b. Washed five minutes in running distilled water

3. Experiment V.

In this case, the staining was done before the emulsion was painted on the slides.

- a. Stained three minutes in a three per cent solution of eosin Y.
- b. Washed rapidly by dipping several times in distilled water.

Table I. Conditions of Autoradiographic Experiments

: Exp. No.	: of Slides	: Type of Emulsion	: Semen Dilution Ratio	: Semen	: Extender - Per Cent	: EY:Fructose:NaCl:Na Cit.:Gly.:	: C <sup>14</sup> : uc.:	: Equilibration Time Hrs.	: Adhesive	: Centrifugation X-G:Min.		
I	4	Stripping film	1:21	20		3	8.00	1.25	21	1/2 egg albumin	900	10
	4	Stripping film	1:21		0.90		8.00	1.25	5	1/2 egg albumin	900	10
	4	Dental X-ray	1:21	20		3	8.00	1.25	21	1/2 egg albumin	900	10
	4	Dental X-ray	1:21		0.90		8.00	1.25	5	1/2 egg albumin	900	10
II	40	NTB	1:22		1.00	0.90	8.00	5.00	3	Mayer's egg alb.	900	10
III	20	NTB2	1:22	20		3	8.00	2.50	4	Mayer's egg alb.	690	10
	20	NTB3	1:22	20		3	8.00	2.50	4	Mayer's egg alb.	690	10
IV	40	NTB2	1:22	20		3	8.00	5.00	6	Mayer's egg alb.	900	10
V	40	NTB	1:22		0.90		8.00	5.00	9	Mayer's egg alb.	900	10
VI	40	NTB	1:10		0.70	0.70	1.64	5.00	6	Chrome alum.	690	10
VII	20	NTB2	1:10		0.35	0.35	2.28	5.00	6	Chrome alum.	540	10
	20	NTB3	1:10		0.35	0.35	2.28	5.00	6	Chrome alum.	540	10

Table I. Conditions of Autoradiographic Experiments (continued)

Exp. No.	Stain Before	Stain After	Developer Type	Developer Time (Min.)	Fixer Type	Fixer Time (Min.)	Washing Time (Min.)
I	None	None	Kodak liquid X-ray developer and replenisher	2	Kodak Fixer Solution A + Kodak Hardener Solution B	4	30
	None	None	" " "	2	" " "	4	30
	None	None	" " "	2	" " "	4	30
	None	None	" " "	2	" " "	4	30
II	None	Toluidine blue	D-19	2	Kodak Fixer Solution A + Kodak Hardener Solution B	4	15
III	None	None	D-19	2	Kodak Fixer Solution A + Kodak Hardener Solution B	4	30
	None	None	D-19	2	Kodak Fixer Solution A + Kodak Hardener Solution B	4	30
IV	3% eosin	None	D-19	2	Kodak Fixer Solution A + Kodak Hardener Solution B	4	30
V	3% eosin	None	D-19	2	Kodak Fixer Solution A + Kodak Hardener Solution B	4	30
VI	None	None	D-19	2	Kodak Acid Fixer	3	60
VII	None	None	D-19	2	Kodak Acid Fixer	3	60
	None	None	D-19	2	Kodak Acid Fixer	3	60

## OBSERVATIONS AND RESULTS

### Manometric:

The results of the manometric studies using calcium-free Krebs-Ringer bicarbonate as a buffer are summarized in Figure 2. As compared to the controls, sperm cells incubated in a gas phase of 95.0 per cent nitrogen and 5.0 per cent carbon dioxide showed a depression of anaerobic glycolysis in the presence of 5.5 and 8.3 per cent glycerol.

Under these conditions, an average of 16.0 and 22.0 ul. more carbon dioxide was released in the control flasks than in the flasks containing 5.5 and 8.3 per cent glycerol, respectively.

The data presented in Figure 3 were obtained using the same gassing procedure as that in Figure 2. However, in this case, calcium-free KRP was used as a buffer instead of KRC and carbon dioxide production was measured. These results indicate that gas production by bovine spermatozoa is inhibited by 1.0, 3.3, 5.5, 8.3 and 10.0 per cent glycerol when compared to the controls. The inhibition was not directly proportional to the final concentration of glycerol present in the flasks. In the flasks containing 8.3 and 10.0 per cent glycerol, most of the inhibition occurred between 30 and 60 minutes; whereas, at lower glycerol concentrations less inhibition occurred in the same time interval.

The addition of 1.0 per cent fructose to the flasks containing 1.0, 3.3, or 10.0 per cent glycerol caused an immediate and marked

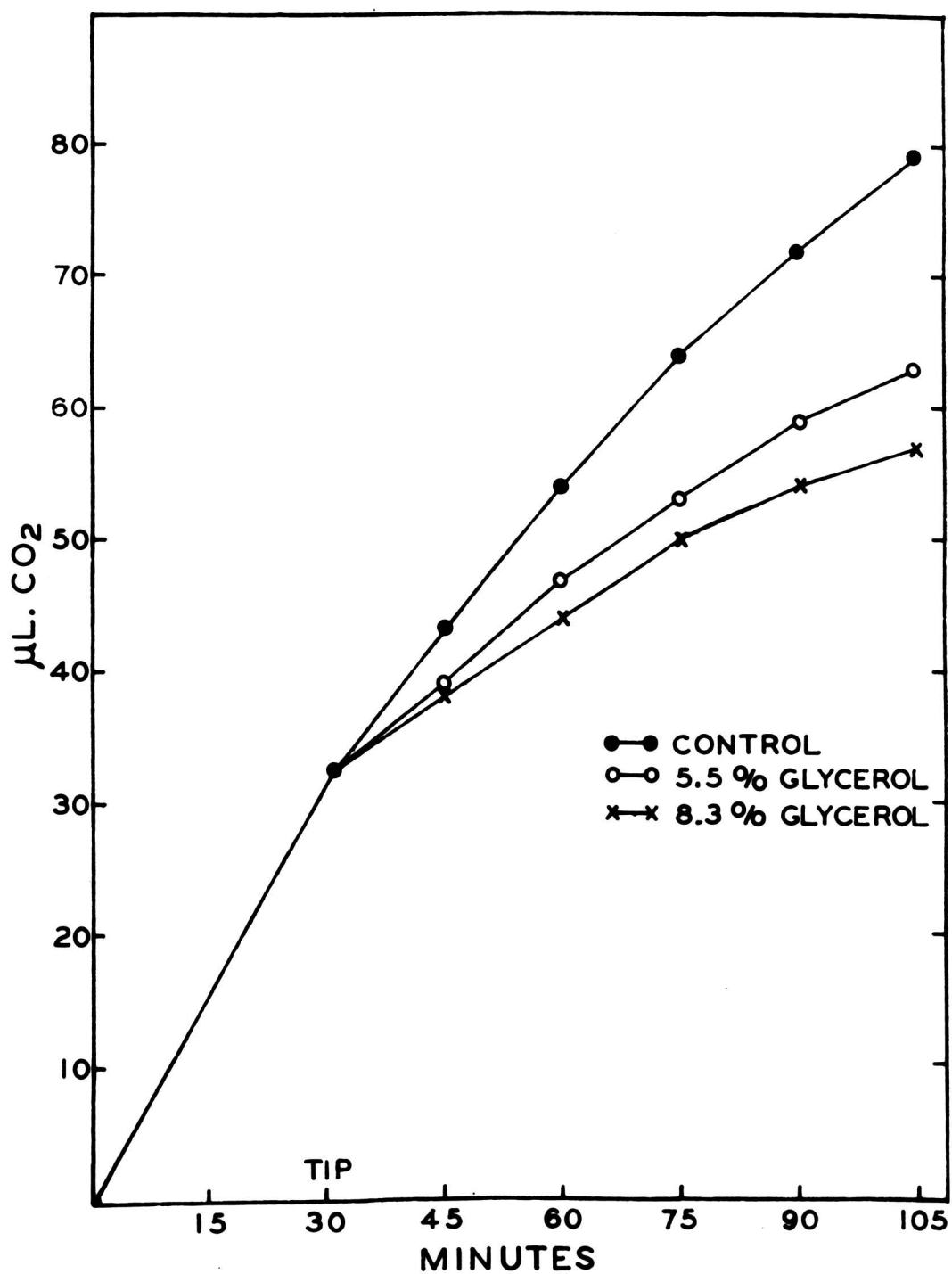


Figure 2. Effect of glycerol on the anaerobic glycolysis of bovine spermatozoa ( $1.13 \times 10^7$  cells/ml.) suspended in calcium-free Krebs-Ringer bicarbonate.

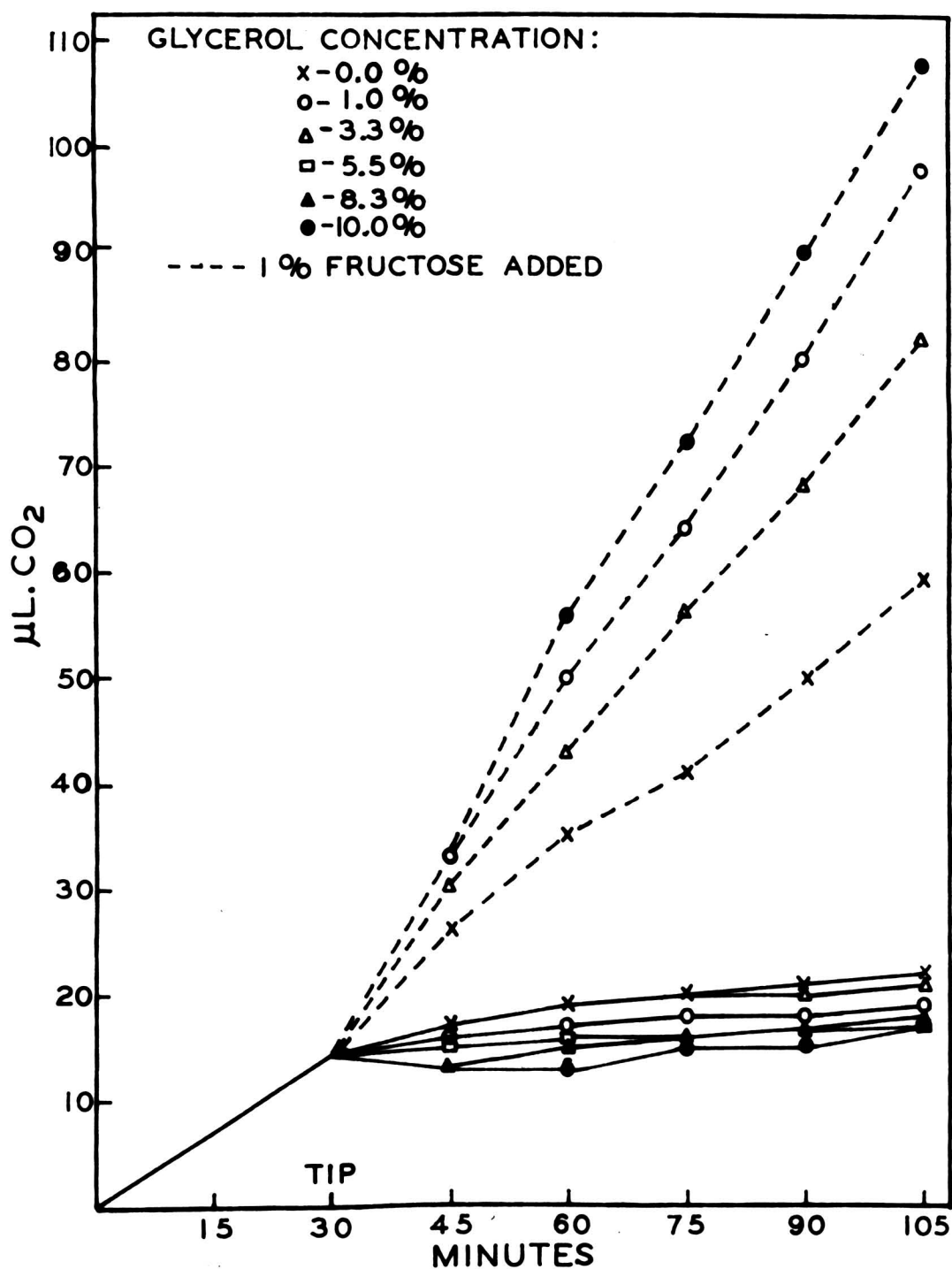


Figure 3. Effect of glycerol, fructose, and glycerol-fructose concentrations on anaerobic metabolism of bovine spermatozoa ( $1.57 \times 10^7$  cells/ml.) suspended in calcium-free Krebs-Ringer phosphate.



stimulation of gas production. When compared with control flasks containing either glycerol or fructose, the greatest degree of stimulation was shown by flasks containing 1.0 per cent fructose plus 10.0 per cent glycerol followed by those containing fructose plus 1.0 per cent and then 3.3 per cent glycerol.

After 105 minutes incubation, motility and per cent alive determinations were made on each flask represented in Figure 3. The data taken from this study are presented in Figure 4. The results of the motility studies show that in all flasks containing glycerol without fructose, motility was lower than in the corresponding glycerol-fructose flasks. The most desirable motility rating was obtained from the samples containing one per cent glycerol and one per cent fructose. Although there was a decline in motility from 1.0 per cent glycerol plus fructose to 3.3 per cent glycerol plus fructose, both maintained a higher level of motility than the flasks containing fructose alone.

In the flasks containing only glycerol no difference in motility was noted between 1.0 per cent and 3.3 per cent glycerol, both of which maintained motility better than the higher glycerol concentrations.

When per cent alive was compared to motility ratings the general trend was much the same with the most notable exception being that ten per cent glycerol maintained slightly greater livability of the cells than 10 per cent glycerol plus fructose, while in motility rating there was little difference between the substrates.

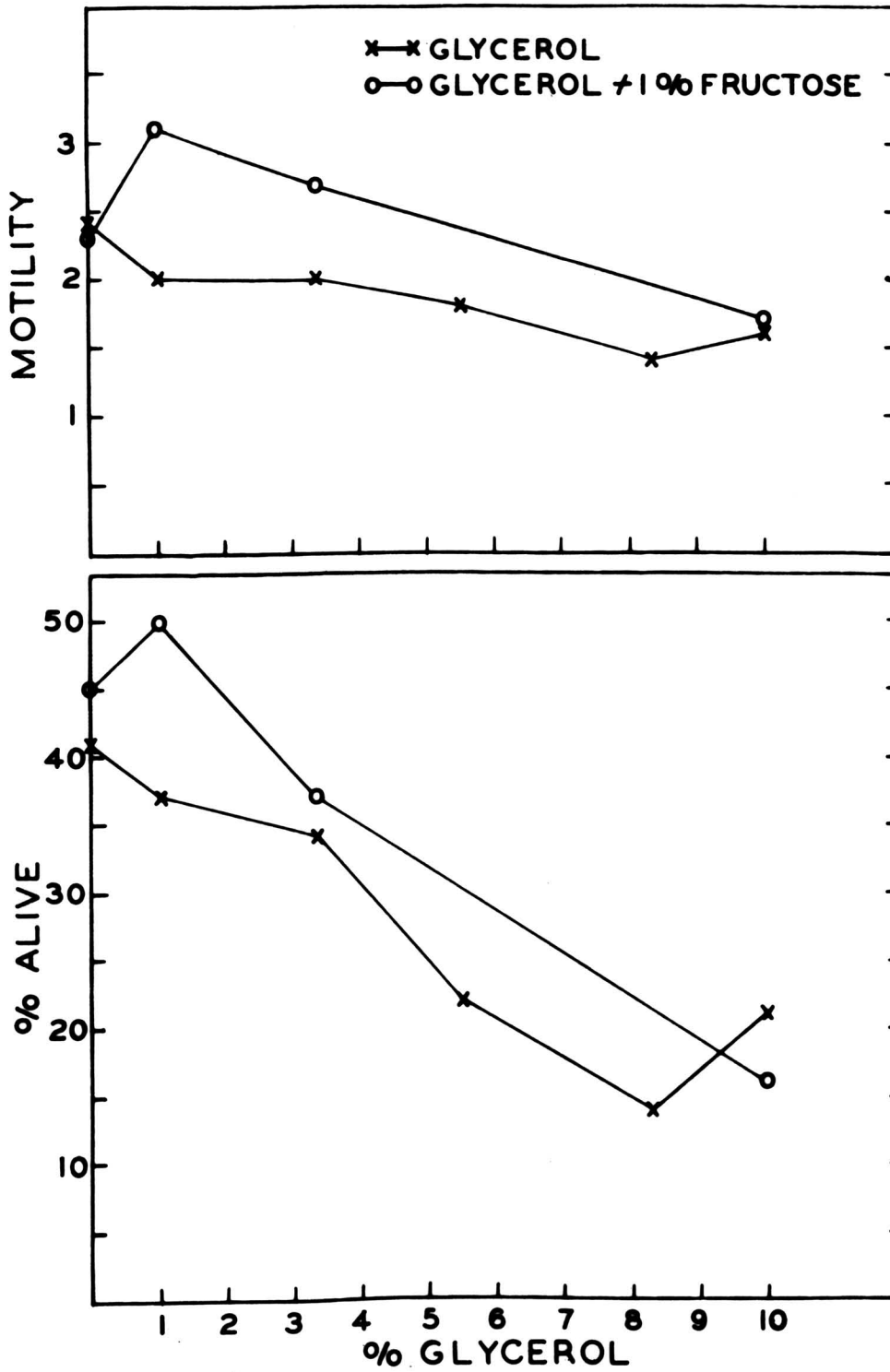


Figure 4. Effect of various concentrations of glycerol and fructose (+ glycerol) on spermatozoan motility and survival after 105 minutes incubation at 38°C.

Although there was no difference in the motility ratings between 1.0 and 3.3 per cent glycerol without fructose, there was a slight difference in the per cent alive count.

The results of pH determinations on the Warburg flask contents are presented in Table II. These data indicate that spermatozoa in the presence of fructose and fructose plus 1.0, 3.3, and 10.0 per cent glycerol produce larger amounts of acid than either the controls or flasks containing only glycerol as the substrate. It was observed that the greatest pH change occurred in the flasks containing 3.3 per cent glycerol plus fructose, followed by 1.0 per cent glycerol plus fructose, fructose, and 10.0 per cent glycerol plus fructose, in that order.

Table II

Decrease in pH Produced by Bovine Spermatozoa ( $1.18 \times 10^7$  Cells/ML.)  
in Various Concentrations of Glycerol, Fructose, and Glycerol Plus  
Fructose after 105 Minutes Incubation at 38°C.

Glycerol Per Cent	Glycerol Initial pH - 7.44	Glycerol + Fructose Initial pH - 7.43
0.00	-0.60	-0.79
1.00	-0.60	-0.81
3.33	-0.64	-0.87
10.00	-0.60	-0.73

When the same type of study was carried out using radioactive glycerol and radioactive fructose, the flasks containing glycerol alone produced a smaller decrease in pH than the flasks containing radioactive fructose and non-radioactive glycerol. The results of this study are summarized in Figure 5. The greatest decrease in pH was observed with 3.3 per cent glycerol plus fructose followed by 10.0 per cent glycerol plus fructose with 1.0 per cent glycerol and fructose showing the least change.

From the summary of  $C^{14}O_2$  production data as shown in Figure 5, it can be seen that the flask containing one per cent glycerol produced more radioactive carbon dioxide than any other concentration, with or without non-radioactive fructose. The flask containing 3.3 per cent glycerol alone produced slightly more  $C^{14}O_2$  than the remaining combinations. Ten per cent glycerol, in the absence of fructose, produced the least amount of gas, while 1.0, 3.3, and 10.0 per cent glycerol with fructose showed essentially no differences.

#### Autoradiographic:

Figures 6a and b illustrate two typical photomicrographs of bull spermatozoa taken from a slide in which the cells had been covered with a thin layer of Eastman Kodak NTB Nuclear Track liquid emulsion. In this trial the cells were spread on the slide before the emulsion was added. The tracks were produced by  $C^{14}$  contained in glycerol- $l$ - $C^{14}$  equilibrated with the spermatozoa three hours before being placed in contact with the emulsion (Exp. II, Table I).

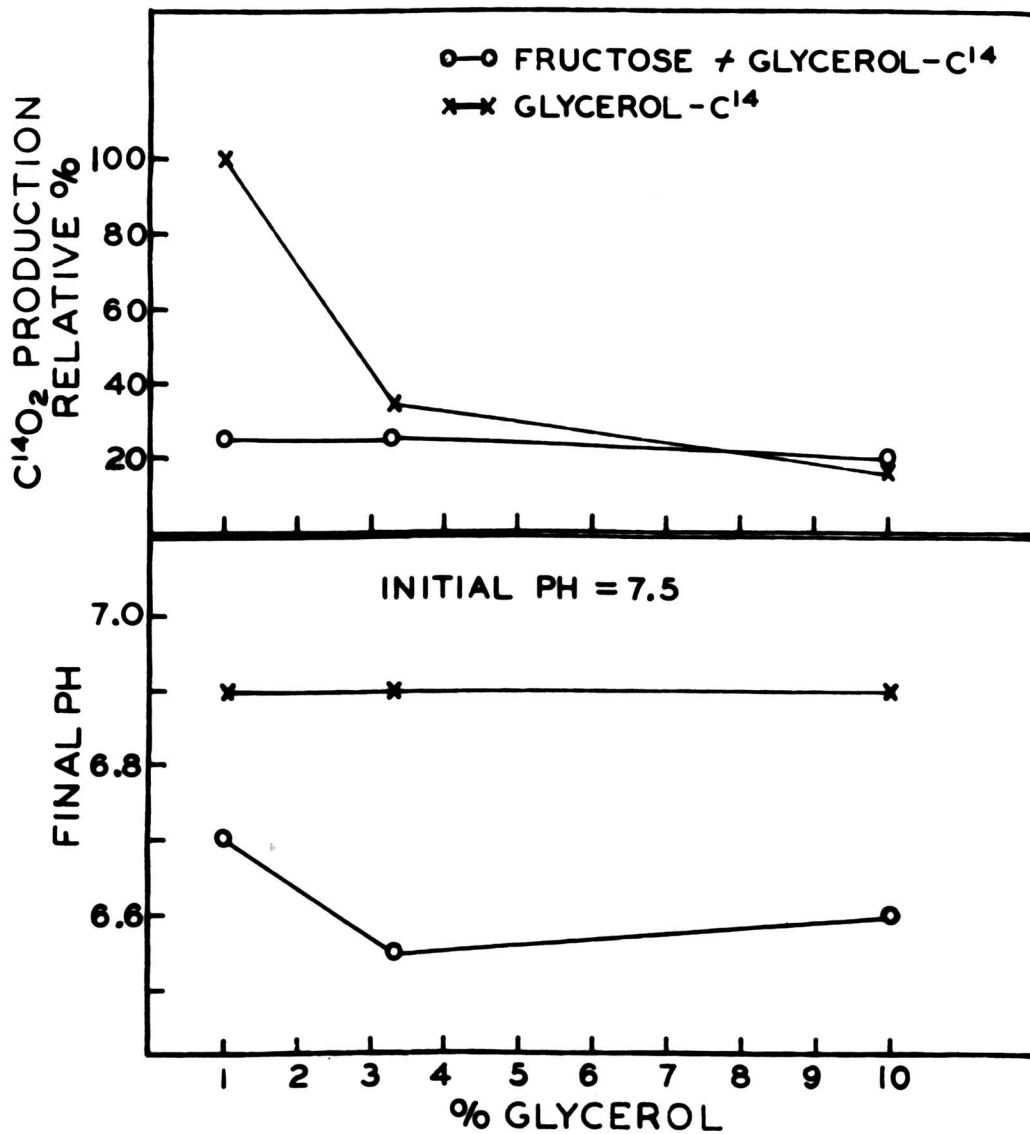


Figure 5. Effect of various concentrations of glycerol-1-C<sup>14</sup> (+ fructose) on C<sup>14</sup>O<sub>2</sub> production and pH changes by bovine spermatozoa ( $1.54 \times 10^7$  cells/ml.) incubated for 105 minutes at 38°C. in calcium-free Krebs-Ringer phosphate under a 100.00 per cent nitrogen atmosphere.

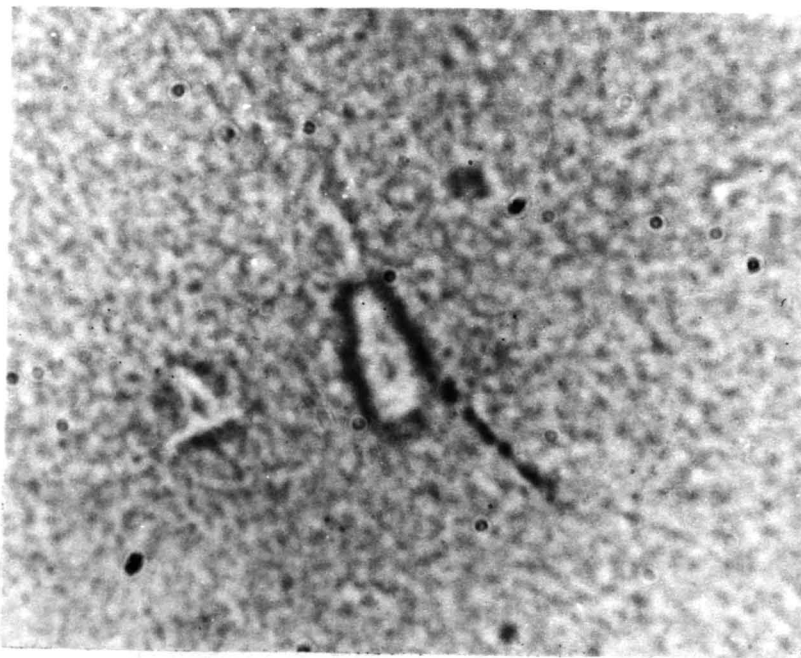


Figure 6a. Bovine spermatozoa showing a  $C^{14}$  beta track in NTB emulsion after 17 days exposure. Magnification 1488 X (Exp. II, Table I).

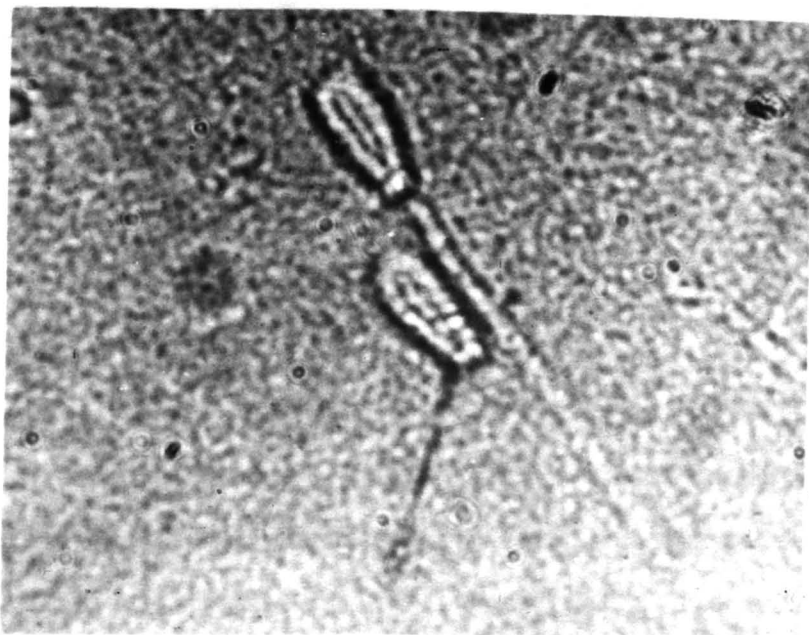


Figure 6b. Bovine spermatozoa showing a  $C^{14}$  beta track in NTB emulsion after 17 days exposure. Magnification 1488 X (Exp. II, Table I).

The slide was stained in toluidine blue after the autoradiograms were developed and processed. Dehydration of the emulsion, after staining, allowed the pictures to be taken under oil immersion.

In both instances (Fig. 6a and b), the track appeared to have originated from the lower portion of the head. However, in Figure 6a the track emerged at the uppermost part of the nuclear area and was recorded along the side of the cell wall. In Figure 6b the track entered the emulsion from the lower part of the nuclear area. In both photomicrographs the track appears to get more diffuse the further it is removed from the cell. Since the cells were below the emulsion, the track was recorded up into the emulsion, thus causing the portion of the track furthest from the cell to be out of the focal plane when the cell is in focus.

The track in Figure 6b is coming from a tailless cell. It was not uncommon that during the resuspending of the cells after the radioactive supernatant is withdrawn some of the tails were broken off. Under the conditions of this experiment, tails also may have separated from the heads due to shrinking and swelling of the emulsion during processing.

Figures 7a and b represent four photomicrographs taken from slides in which the spermatozoa were mixed with NTB emulsion before being spread on the slides. Figure 7b shows two sperm cells with tracks arising from different parts of the cell. The track on the left is coming from the nuclear area of the cell while the one on the right is coming from the mid-piece. The black spots in the two

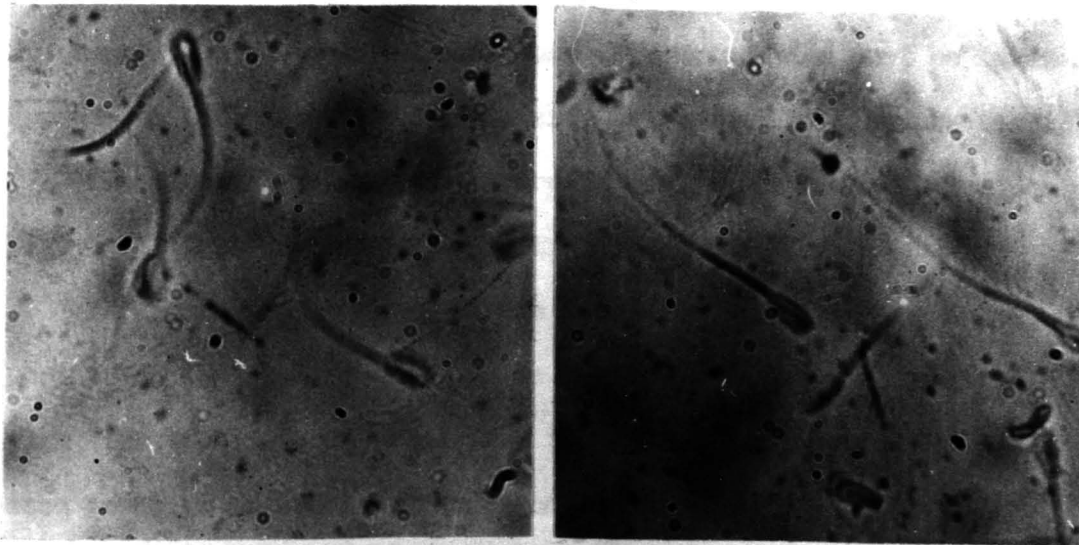


Figure 7a. Bovine spermatozoa showing  $C^{14}$  beta tracks in NTB emulsion after 28 days exposure. Magnification 645 X (Exp. VI, Table I).

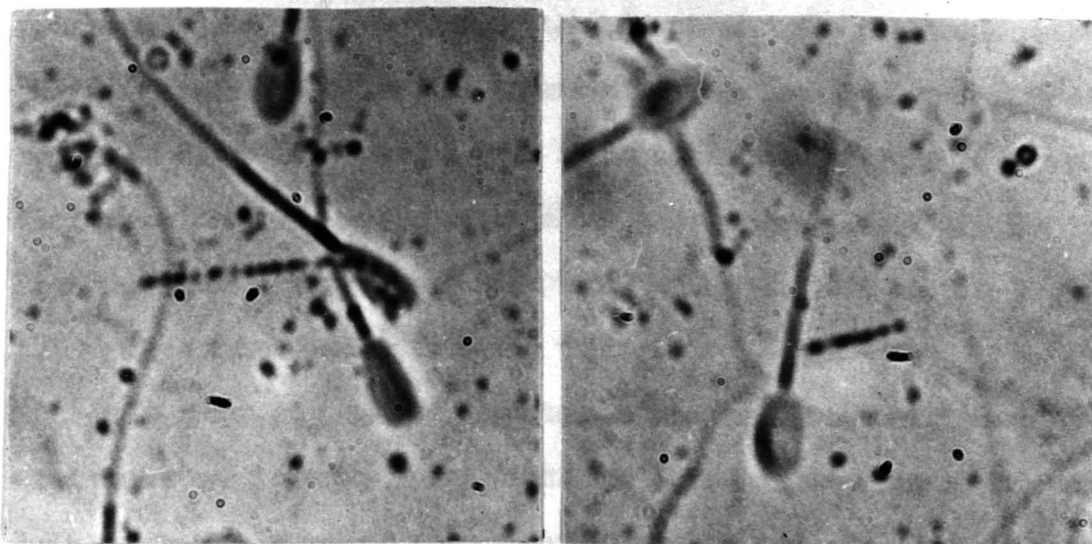


Figure 7b. Bovine spermatozoa showing  $C^{14}$  beta tracks in NTB emulsion after 46 days exposure. Magnification 1000 X (Exp. VI, Table I).



pictures were caused by air bubbles in the emulsion. When the slides are being painted with the emulsion, extreme caution must be taken to spread the emulsion slowly and evenly to prevent bubble formation.

The photomicrographs in Figure 7a are from the same experiment as Figure 7b (Exp. VI) but at a different exposure time.

In Figure 7a the track on the left is coming from the anterior portion of the nuclear area, while in the photomicrograph on the right the track was recorded from the mid-piece in approximately the same position as the track in Figure 7b.

The results of 52 and 116 days exposure time of bovine spermatozoa in NTB emulsion are shown in Figures 8a and b. The  $C^{14}$  beta tracks from the sperm cells in Figure 8a appear to originate from approximately the same position of the mid-piece. The electrons probably contained about the same amount of energy because the length of tracks is almost identical. This is in contrast to Figure 7b where the photomicrograph on the right shows a very short track originating from the upper region of the mid-piece, while the left picture shows a long beta track coming from the tail.

This group of pictures depicts two problems frequently encountered in autoradiography. The light and dark streaks seen in the upper left are probably due to uneven development of the slide. This can be prevented, in some cases, by agitation and separation of the slides during the developing process. Another problem is seen in the lower right photomicrograph, in which the black outlined rough patch resulted from peeling of the emulsion. It was probably loosened during

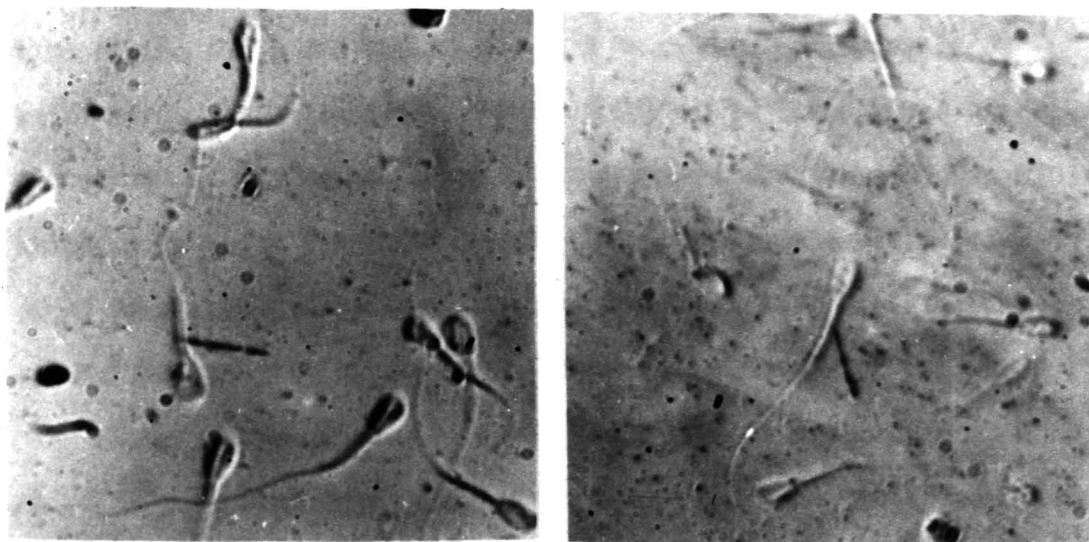


Figure 8a. Bovine spermatozoa showing a  $C^{14}$  beta track in NTB emulsion after 52 days exposure. Magnification 645 X (Exp. VI, Table I).

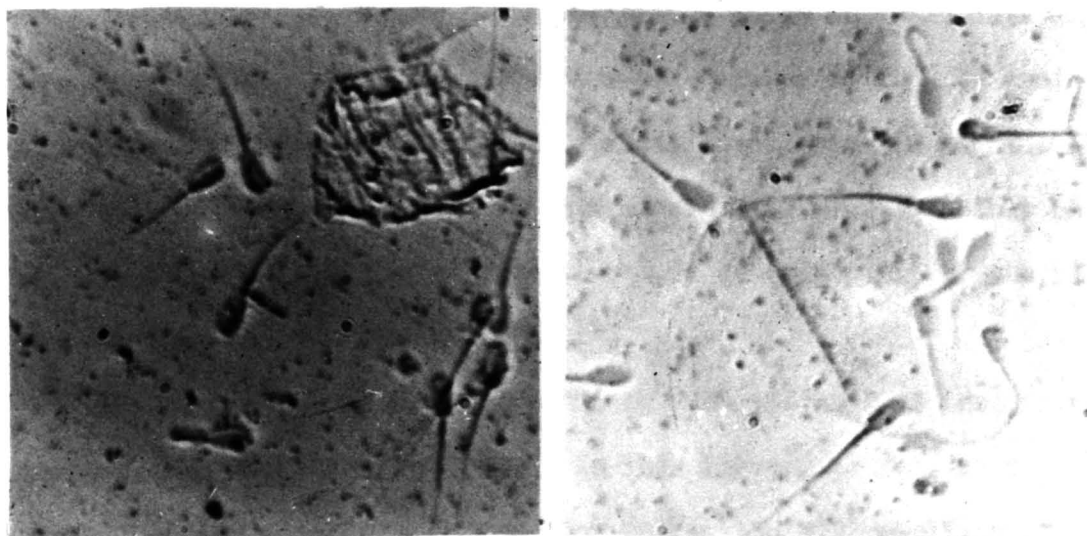


Figure 8b. Bovine spermatozoa showing a  $C^{14}$  beta track in NTB emulsion after 116 days exposure. Magnification 645 X (Exp. VI, Table I).

processing, or from excessive drying of the emulsion during storage.

In the previous figures, NTB emulsion, which is the least sensitive of the three NTB types used, has been shown registering  $C^{14}$  beta tracks up to 116 days exposure. Figure 9a illustrates a photomicrograph of an autoradiogram of bull spermatozoa embedded in NTB3, which is the most sensitive of the three, after an exposure time of eight days. It can be seen that the track has been registered from a point slightly below the mid-piece-tail junction.

Figure 9b represents a picture of sperm cells embedded in NTB2, which is intermediate in sensitivity. In this case the track appears as a black line over the tail. This effect was produced by focusing the microscope to show the sperm cell head more clearly than the tail and beta track.

Figures 10a and b show photomicrographs taken after 14 days exposure. The track shown in Figure 10a was registered in NTB3 emulsion. In some cases there will be a short blank space in an otherwise uniform track as is seen in this case. This might possibly be explained by failure of the track to record at this point or the silver grain not being fully developed.

The beta track in Figure 10b arises from the tail of the spermatozoa suspended in NTB2 emulsion. In this figure, as well as in Figure 9b, the tracks are not as long as for the NTB3 emulsion at the same exposure time.

The photomicrographs in Figure 10a and b were taken from a slide where emulsion was apparently not more than 5  $\mu$ . thick. All of the

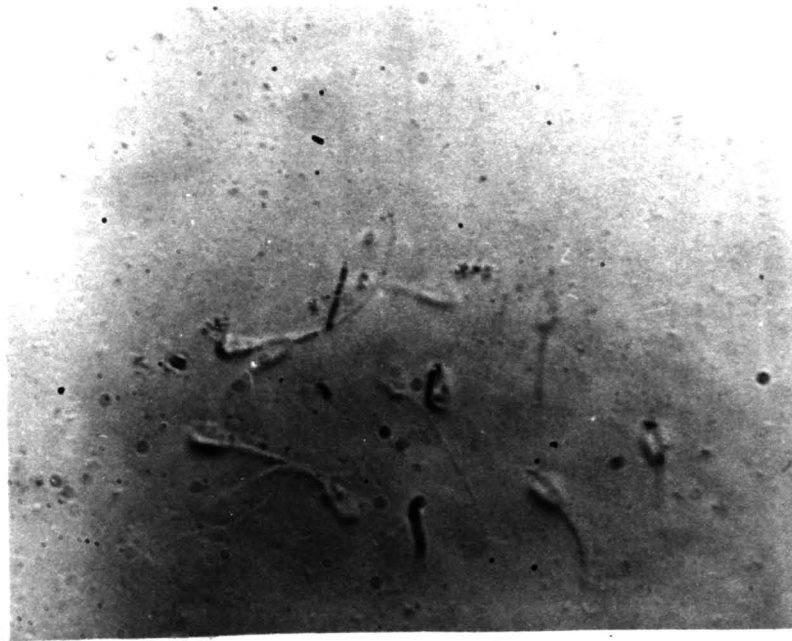


Figure 9a. Bovine spermatozoa showing a  $C^{14}$  beta track in NTB3 emulsion after 8 days exposure. Magnification 645 X (Exp. VII, Table I).

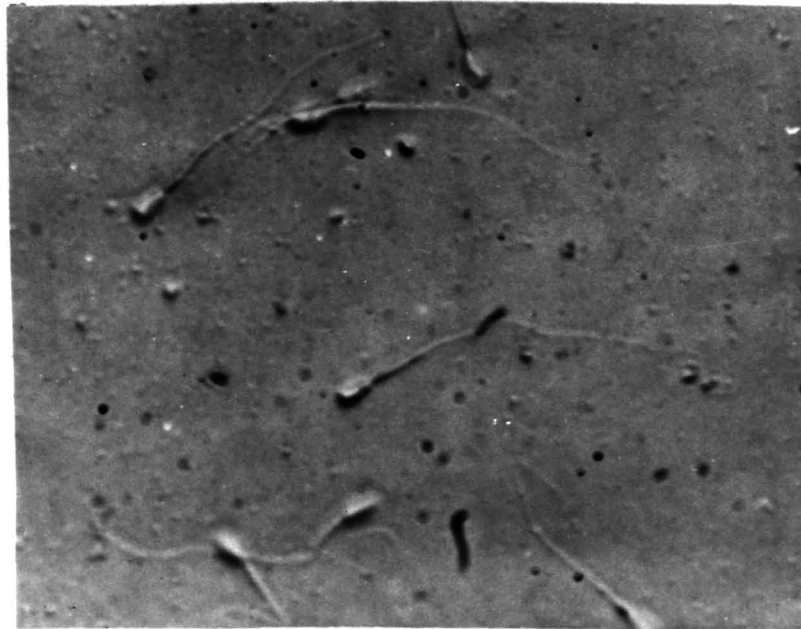


Figure 9b. Bovine spermatozoa showing a  $C^{14}$  beta track in NTB2 emulsion after 8 days exposure. Magnification 645 X (Exp. VII, Table I).



Figure 10a. Bovine spermatozoa showing a  $C^{14}$  beta track in NTB3 emulsion after 14 days exposure. Magnification 645 X (Exp. VII, Table I).

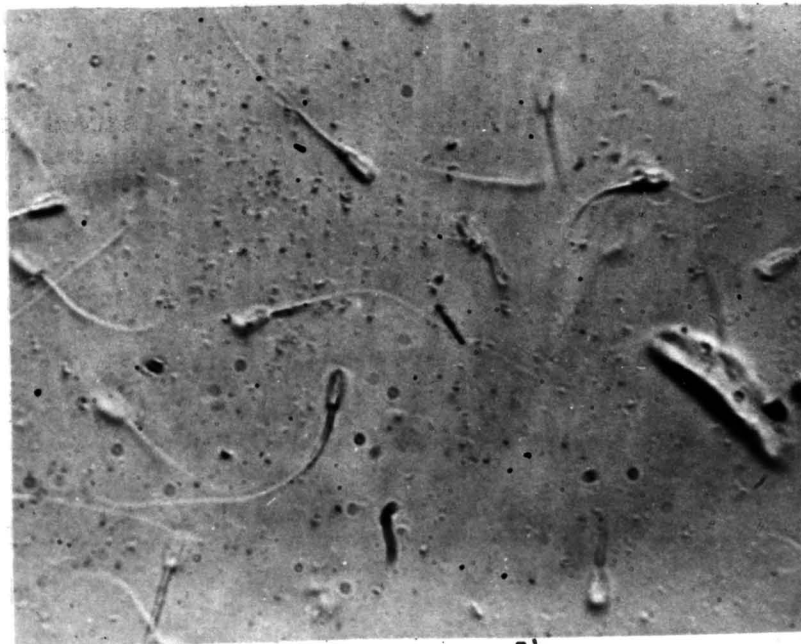


Table 10b. Bovine spermatozoa showing a  $C^{14}$  beta track in NTB2 emulsion after 14 days exposure. Magnification 645 X (Exp. VII, Table I).

cells as well as tracks are in good focus. This is a particular advantage of a thin emulsion layer. However, when the emulsion is spread thin, the tracks may be shorter and not as many recorded if parts of the cell are protruding from the emulsion surface.

The effects of an extended exposure time, with the more sensitive emulsions, are shown in Figures 11a and b. Much more background fog is in evidence in both the NTB3 (upper figure) and NTB2 (lower figure).

From the results presented in the various figures, it is obvious that tracks originate from the head, mid-piece, and tail. As would be expected, exposure time or type of emulsion has little if any effect on their origin. Table III shows the relative percentages of beta tracks arising from various regions of glycerolated bovine spermatozoa.

Table III

Per Cent of  $C^{14}$  Beta Tracks Associated with the Head, Mid-piece, and Tail of Glycerolated Bovine Spermatozoa after 52 Days Exposure with NTB Emulsion

Site of First Developed "Grain"	Head	Mid-piece	Tail
At cell wall	14.9%	13.1%	8.9%
2-3 "grains" from cell wall	14.3%	20.8%	28.0%

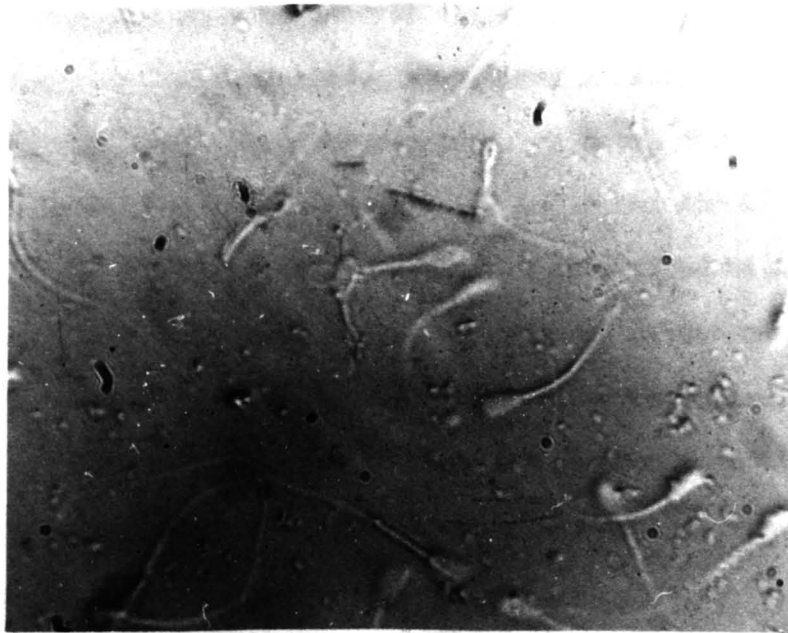


Figure 11a. Bovine spermatozoa showing a  $C^{14}$  beta track in NTB3 emulsion after 52 days exposure. Magnification 645 X (Exp. VII, Table I).

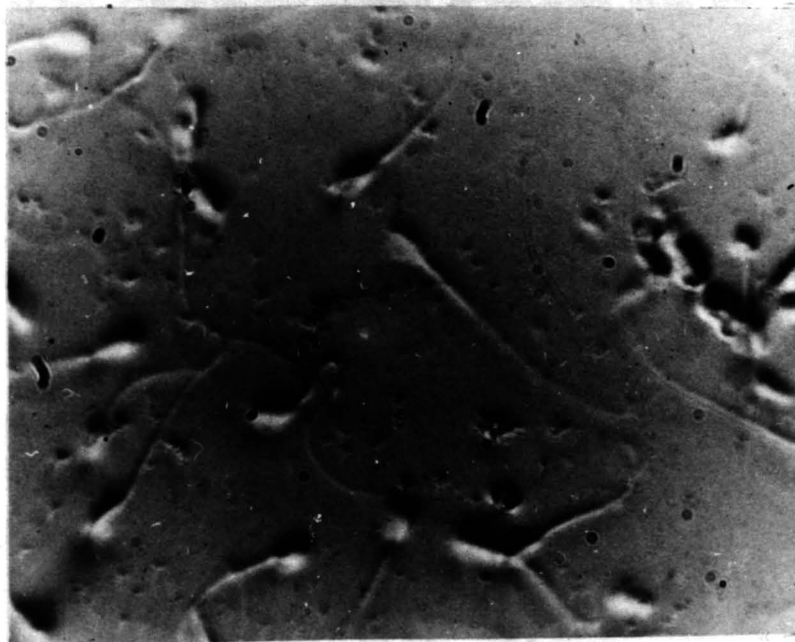


Figure 11b. Bovine spermatozoa showing a  $C^{14}$  beta track in NTB2 emulsion after 52 days exposure. Magnification 645 X (Exp. VII, Table I).

Nine slides were examined microscopically by making eight traverses lengthwise of each slide, at 430 magnification and the point of origin of each track was recorded. A total of 168 beta tracks were observed to originate near the cells and for 62 of the tracks, the first developed silver grain was associated with the cell wall. In the remaining tracks the first recorded evidence of beta track was separated from the cell wall by a distance equivalent to two to three developed silver grains. These latter tracks and their percentages for possible point of origin are given in the lower part of Table III.

These percentage figures indicate that it is difficult to accurately ascertain the point of origin of beta tracks when the first developed silver grain is not closely associated with the cell wall.



## DISCUSSION

### Manometric:

Bovine spermatozoa incubated anaerobically under a gas phase of 95.0 per cent nitrogen and 5.0 per cent carbon dioxide showed, in the presence of 5.5 and 8.3 per cent glycerol, a depression of glycolysis as measured by carbon dioxide released from calcium-free KRC (Figure 2). The control flasks released 16.0 and 22.0 ul. more carbon dioxide than flasks containing 5.5 and 8.3 per cent glycerol, respectively. Glycerol in these concentrations may have inhibited the anaerobic utilization of residual fructose since the washing procedure used in these studies was not designed to remove all the fructose present in the seminal plasma, but merely to dilute it while keeping the spermatozoan concentration high. Therefore, the controls in this study probably utilized the fructose present, while in the flasks containing 5.5 and 8.3 per cent glycerol the glycerol in some way interfered with the fructose utilization.

In a similar study (Figure 3), when calcium-free KRC was replaced with calcium-free KRP, glycerol also inhibited anaerobic glycolysis, but the inhibition was not directly proportional to the concentration of glycerol present. When 8.3 and 10.0 per cent glycerol was added to the flasks, a more pronounced inhibition occurred during the first 15 to 30 minutes.

The addition of 1.0 per cent fructose to the flasks, at the same time 1.0, 3.3, and 10.0 per cent glycerol was added, caused an im-

mediate and marked stimulation of gas production. The greatest degree of stimulation occurred in the flasks containing fructose plus 10.0 per cent glycerol.

These results indicate that the increased carbon dioxide production is the result of the utilization of fructose or glycerol or both. In view of some recent investigations, it appears probable that carbon dioxide is being produced from both glycerol and fructose. Mann (59) and Moore (68) have shown that glucose and fructose appear to be utilized through essentially identical pathways, and Flipse and Almquist (28) have shown that lactic acid produced from glucose-C<sup>14</sup> by bovine spermatozoa was, in part, broken down to carbon dioxide. O'Dell, Flipse, and Almquist (72) found that glycerol-1-C<sup>14</sup> was utilized by bovine spermatozoa under anaerobic conditions and converted in appreciable amounts to radioactive carbon dioxide. An alternate possibility is that fructose might accelerate the entry of glycerol into the cell, as has been postulated by White, *et al.* (105) for arabinose, a non-glycolyzable pentose.

The results of the motility and per cent alive studies show that in all flasks containing glycerol without fructose, motility and per cent alive was lower than the corresponding glycerol-fructose flasks. The only exception was seen in the per cent alive studies with ten per cent glycerol. Since more gas was produced in the 10.0 per cent glycerol-fructose flasks, motility may have been depressed due to a build-up of metabolites toxic to the cells instead of a direct toxic effect produced by this high concentration of glycerol. However, all

flasks containing glycerol alone showed a lower motility, indicating a depression which probably was not associated with a build-up of toxic substances since less metabolic activity was observed in these flasks than in the controls, which exhibited a higher motility. This difference in motility between glycerol and glycerol-fructose might be explained by the report of Kampschmidt, et al. (45) which indicates that as glucose is used to replace the buffer solution, viability of bovine spermatozoa is increased. This effect was attributed to: 1) more glycolyzable sugar present, or 2) decreased electrolyte content of the storage medium. Further support of the electrolyte view has been presented by Hendrikse, et al. (41). They found that glycerol exerted a considerable apparent osmotic effect with a lowering of the freezing point when freezing bovine spermatozoa for use in artificial insemination.

One per cent glycerol in the presence of one per cent fructose appeared to be more beneficial to the sperm cells, from both a motility and per cent alive standpoint, than any other combination studied. Since a rather large amount of metabolic activity was observed in these flasks without the decrease in per cent alive and motility, as observed in the ten per cent glycerol-fructose flasks, perhaps low concentrations of glycerol, in the presence of fructose, can be converted largely to carbon dioxide without the accompanying increase in toxic metabolic substances. Thus, a beneficial rather than a depressing effect was exerted on motility and viability of the cells.

The results of pH determinations, taken after 105 minutes incubation under 100.00 per cent nitrogen, show a rather pronounced difference between glycerol and glycerol plus fructose samples. It was found that the greatest pH change occurred in the flasks containing 3.3 per cent glycerol plus fructose, followed by 1.0 per cent glycerol plus fructose, fructose, and 10.0 per cent glycerol plus fructose, in that order. Lactic acid probably accounts for the largest amount of the pH decrease; however, in view of the investigation of Erb, et al. (24), a considerable amount of this acid may have been pyruvic acid.

In the flasks containing glycerol alone there was a considerable change in pH, but there was little difference between the results for different glycerol concentrations.

When the same type of gassing procedure was used in a study of radioactive carbon dioxide production by bovine spermatozoa from glycerol-1-C<sup>14</sup>, it was found that flasks containing one per cent glycerol, in the absence of fructose, showed the largest amount of radioactive carbon dioxide. However, the pH in this case was identical to that found in flasks containing 3.3 and 10.0 per cent glycerol.

Results presented in Figure 3 with five per cent carbon dioxide present would lead one to expect more radioactive carbon dioxide to be produced when 100.00 per cent nitrogen is used as the gas than actually was produced. Although some radioactive carbon dioxide was obtained (Fig. 5), the amount produced in relation to the activity present in the substrate was very small.

It seems possible that the observations of Greulich and Leblond

(34) might account for the small amount of carbon dioxide produced under these conditions. They found that carbon dioxide may be used for organic synthesis by animal tissues and postulated that some of the carbon dioxide produced may be reused in formation of products of the Krebs Cycle. In support of this view, Wood and Werkman (108) found, in a study with bacteria, that the total carbon dioxide liberated during fermentation of glycerol, plus that remaining in the form of carbonate, was less than the original carbon dioxide in carbonate form. It was suggested that the decrease resulted from the utilization of carbon dioxide by bacteria during their dissimilation of glycerol.

From the results of this study with bovine spermatozoa, it appears that more carbon dioxide is produced by the cells when five per cent carbon dioxide is included in the gassing mixture than when 100.00 per cent nitrogen is used. However, pure nitrogen does not appear to decrease the amount of acid compounds formed, and may increase their formation.

When 100.00 per cent nitrogen is used, if only small amounts of carbon dioxide are produced, it may be reused by the cells themselves; whereas, when carbon dioxide is present initially to stimulate the production, much more may be produced than can be reused by the tissue present. Such might be the case with one per cent glycerol, which was shown to be less toxic to the cells as far as motility and per cent alive were concerned. Therefore, carbon dioxide produc-

tion may be more easily initiated by the cell in the presence of one per cent than ten per cent glycerol when carbon dioxide is included in the gassing mixture.

More  $C^{14}O_2$  was produced from 1.0 per cent glycerol followed by 3.3 per cent and the least amount from 10.0 per cent glycerol. The latter glycerol concentration, when used alone, was found to be more inhibitory to the cells in all phases of the study.

The fact that very little radioactive carbon dioxide was produced when one per cent fructose was added to the three concentrations of glycerol- $1-C^{14}$  might be explained on the basis that no carbon dioxide was initially present to stimulate  $C^{14}O_2$  production and sufficient quantities of fructose were present to supply energy to the cells without glycerol. Thus, more acid and less  $C^{14}O_2$  was the end product, as can be seen in Figure 5.

#### Autoradiographic:

Results of the autoradiographic studies indicate that glycerol- $1-C^{14}$  probably enters the bovine spermatozoa and that the largest amount is present in the posterior portion of the head, as has been pointed out previously by Pickett and Merilan (74). However, from the results it is impossible to say that entry and localization are definite, because the emulsion will not record the  $C^{14}$  decay until the beta particle comes into contact with the emulsion surrounding the cells. Thus, an argument against entry could be presented by pointing out that the glycerol may be only associated with or adhered to the

cell wall in these areas. However, manometric data for spermatozoa presented in this study, as well as in others (61, 72, 105), would oppose the hypothesis that glycerol is merely adhered to the cell or in the cell wall. Levi (47) has pointed out that one disadvantage of this technique is that the origin of the activity cannot be definitely ascertained since the blackened grains first appear at the edge of the cell.

From the photomicrographs, it is difficult to point out the origin of the tracks because the cell and the entire length of the track may not be in the same focal plane. Boyd and Levi (10) have pointed out that localization of a disintegrated atom can be approximated in the cell by changing the focal plane of the microscope, thus tracing the track to its origin. This has been done with all the autoradiograms from which the photomicrographs were made and only those where the origin of the tracks was in close proximity with the cell wall are shown.

It is evident from the photomicrographs that there are sperm cells which do not have beta tracks associated with them and although only cells with single tracks are shown, several were seen on the original autoradiograms which possessed more than one and in a few cases as many as four. When a thin emulsion is used, tracks coming directly up from the cell probably will not be seen. The same could apply to tracks originating on the side of the cell away from the microscope.

A rather large number of the tracks on all autoradiograms were

found some distance from the cell wall. This was also observed by Levi (47), who suggested that failure of the track to register at the beginning or that distortion of the emulsion might account for the distances seen.

The cells that did not show any tracks may have been dead, or in a particular physiological state not favoring uptake of glycerol, or may have contained radioactive glycerol in which  $C^{14}$  decay did not take place during the relatively short exposure times. An additional factor may have been the removal of glycerol by the washing procedure. When bovine spermatozoa are centrifuged at speeds used in this study, after being equilibrated in fructose and sodium chloride, they pack very tightly in the bottom of the centrifuge tube. This was an advantage in one respect since it allowed removal of most of the radioactive substrate without disturbing the cells. However, this washing probably removed considerable amounts of glycerol from the cells.

Boyd and Levi (10) observed tracks starting in the emulsion and running in all directions. This was attributed to random cosmic events, to radioactive substances naturally occurring in the tissue or gelatin, and photoelectrons from X-rays generated by the beta particles themselves. In this study all the radioactive supernatant was not removed, thus producing some tracks not associated with the cells.

Guidotti and Setti (37) criticized  $C^{14}$  as an isotope for use in autoradiography because under the conditions of their experiment, the tracks were "curly". This is not uncommon as has been shown by others (9, 10, 47). However, in this study no such problem was encountered,



although curved tracks and an occasional "curly" track were seen.

Before the importance of localization of any compound in the cell can be fully realized the localization must be associated or correlated with some structure, compound, or group of compounds in the cell.

The association of structural and functional characteristics of spermatozoan morphology have been studied by means of the electron microscope (81, 109) as well as by the use of  $P^{32}$  autoradiograms (5). In the latter study, Bishop found that bovine spermatozoa accumulated  $P^{32}$  in the posterior portion of the head and in the mid-piece region. Risley (82) also employed  $P^{32}$  in a study of hamster spermatozoa. A reasonably close correlation was found between percentage of motile spermatozoa and those producing good autoradiograms. It was further noted that phosphate activity is localized primarily in the mid-piece region of these cells. It was also pointed out that the mid-piece region appears to be the site of the oxidative processes of the cell. DiStefano and Mazia (16) obtained results indicating the uptake of  $P^{32}$  in the ribonucleic acid constituents of the mid-piece of sea urchin spermatozoa.

Nelson (70) found that the majority, and in some cases all, of the succinic dehydrogenase activity of bull spermatozoa was localized in the mid-piece and tails of the cells. In addition, the cytochrome-oxidizing systems were also found in this area.

Friedlander and Frazer (31) have found phosphate activity primarily in the mid-piece of ram spermatozoa, particularly toward the mid-piece-tail junction. This is interesting since a rather large

number of the  $C^{14}$  beta tracks in the present study were found to be coming from this same region.

In the cytochemical studies of these workers (31) with ram spermatozoa, calcium, magnesium, DNA, potassium, and lipids were found both in the mid-piece and nuclear area. Sulfur appeared to be associated with all areas of the cell but little information was obtained on the tail because of its small size. Zittle and O'Dell (110) analyzed various fractions of bull spermatozoa and found that the tails, heads, and mid-pieces contained 23.0, 6.0, and 7.0 per cent lipid, respectively, and that phospholipid was present, especially in the tail. Since phospholipids and other phosphorus containing compounds were found generally throughout the cell, it is possible that glycerol is closely associated with these substances. This would help explain the origin of tracks from all regions of the cell, especially since more appeared to come from the head, followed by the mid-piece, and tail, in that order, which was also approximately the order of phosphorus accumulation.

## SUMMARY AND CONCLUSIONS

Data have been presented on the effect of various concentrations of glycerol on the anaerobic metabolism of bovine spermatozoa, under varying conditions of gassing, plus or minus fructose, radioactive and non-radioactive glycerol, in both calcium-free Krebs-Ringer carbonate and Krebs-Ringer phosphate buffers.

In addition, autoradiographic studies have been conducted in an attempt to determine localization of glycerol-1-C<sup>14</sup> in bovine spermatozoa. Supplementing this is a detailed discussion of the techniques employed in this study and an attempt has been made to correlate the results with other known facts about the cell. The following conclusions were drawn from these data:

1. Glycerol in concentrations varying from 1.0 to 10.0 per cent inhibited anaerobic glycolysis of bovine spermatozoa during 105 minutes incubation at 38°C. with calcium-free KRC or KRP as the buffer. The greatest inhibition was shown by the highest glycerol concentration.
2. The inclusion of one per cent fructose with 1.0, 3.3 or 10.0 per cent glycerol caused a stimulation of carbon dioxide production by the spermatozoa.
3. Glycerol plus fructose maintained a greater degree of motility and livability of bovine spermatozoa at the end of 105 minutes incubation at 38°C. than did either fructose or glycerol alone. One per cent glycerol plus one per cent fructose was

the apparent optimum in maintenance of spermatozoan motility and livability.

4. Bovine spermatozoa, incubated for 105 minutes at 38°C. under a pure nitrogen gas phase, produced more acid when fructose was present in the substrate with 1.0 and 3.3 per cent glycerol than when glycerol or fructose alone was used.
5. More radioactive carbon dioxide was produced by bovine spermatozoa from one per cent glycerol-1-C<sup>14</sup> than from one per cent glycerol-1-C<sup>14</sup> plus fructose. However, more acid was produced in the latter flask.
6. Under the conditions of the autoradiographic studies, embedding the cells in liquid emulsions gave better results than liquid emulsions painted over the cells. However, either of these techniques was superior to the use of stripping film or dental X-ray plates for studying glycerol localization in the bovine sperm cell.
7. Glycerol-1-C<sup>14</sup> apparently enters bovine spermatozoa and the greatest amount seems to accumulate in the nuclear area, followed by the mid-piece and tail, in that order.

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## BIOGRAPHICAL SKETCH

Bill Wayne Pickett was born on December 14, 1930, at Cyril, Oklahoma. He graduated from Central High School, Muskogee, Oklahoma, in 1948 and attended Northeastern Oklahoma A. & M. Jr. College from June 1948 to January 1950. At this time he transferred to Oklahoma A. & M. College, Stillwater, Oklahoma, graduating from that institution in May 1952 after having completed the requirements for the Bachelor of Science degree in January 1952. From January 1952 he was employed by the Oklahoma Extension Service until entering the U. S. Army in July of that year.

After completion of two years duty with the Army, Mr. Pickett was enrolled as a graduate student in the University of Missouri, receiving the degree of Master of Science in August of 1955.

On December 17, 1955, Mr. Pickett was married to Miss Joan Marvin in Columbia, Missouri.

While a graduate student at the University of Missouri, he was elected to the following honorary fraternities: Gamma Alpha and Gamma Sigma Delta.

The undersigned, appointed by the Dean of the Graduate Faculty, have  
examined a thesis entitled

**The Metabolism and Localization of Glycerol**  
**By Bovine Spermatozoa**

presented by **Bill Wayne Pickett**

a candidate for the degree of **Doctor of Philosophy**

and hereby certify that in their opinion it is worthy of acceptance.

C. P. Meilan Major Advisor

G. T. Raydale Departmental Representative

Hennis Drayer







