RESEARCH BULLETIN 592 SEPTEMBER, 1955

UNIVERSITY OF MISSOURI COLLEGE OF AGRICULTURE

AGRICULTURAL EXPERIMENT STATION

J. H. LONGWELL, *Director*

# Effect of Supplementary Amino Acids and Adenosine Phosphates on Motility and Metabolism of Bovine Spermatozoa

F. J. GRUNFELD and C. P. MERILAN

(Publication authorized September 15, 1955)

COLUMBIA, MISSOURI

## TABLE OF CONTENTS



## ABSTRACT


The effects of various phosphocreatine constituents, AMP, and ATP on motility and metabolism of bovine spermatozoa were studied by means of 4° C storage and manometric techniques, respectively.

Of the phosphocreatine constituents added to whole semen, the combination of arginine and glycine favored the maintenance of a higher level of spermatozoan motility while arginine and methionine were the most effective in stimulating respiration and anaerobic glycolysis. Addition of AMP to whole semen had no apparent effect on the rate of respiration and depressed anaerobic glycolysis. Addition of AMP and ATP to washed spermatozoa stimulated respiration and anaerobic glycolysis. Rapid utilization of supplementary ATP and inhibition of its stimulation by EDTA indicated differences in utilization of added ATP and "in vivo" ATP. Decreasing spermatozoan motility was found to be proportional to decreases in ATP-ase activity.



This bulletin reports on Department of Dairy Husbandry Research Project 54, Artificial Insemination

# Effect of Supplementary Amino Acids and Adenosine Phosphates on Motility and Metabolism of Bovine Spermatozoa

F. J. GRUNFELD AND C. P. MERILAN

## INTRODUCTION

The hypothesis that a mechanism similar to that of muscle is responsible for the energy-requiring processes of viable and motile spermatozoa has been presented by several investigators. Their work is summarized in Anderson's reveiw.[1] From this review, it can be seen that the initial approach was based on showing the ability of tissue extracts from the testis to proceed with the reaction between phospho-pyruvic acid and creatine to form phospho-creatine and pyruvic acid in the presence of adenyl pyrophosphate. When various tissue extracts were compared, it was found that next to muscle, testis showed the greatest activity with respect to the above reaction. Since then a great deal of experimentation has been carried on to demonstrate more conclusively that spermatozoan metabolism is essentially of glycolytic character. Most of this work was concerned with investigations of optimal and inhibiting conditions of glycolysis and the utilization of various substrates.

In 1943 Lardy and Phillips,[11] while studying the effect of several metabolic inhibitors on spermatozoan glycolysis, found appreciable quantities of an acid labile ester which appeared to be similar to ATP. This finding was repeatedly demonstrated by these workers[13] in a series of chemical studies on phosphorus partition of bull spermatozoa which indicated the presence of ATP or a chemically similar compound, as well as the corresponding enzyme which breaks down ATP to adenylic acid and inorganic phosphorus. In an additional study on the metabolism of epididymal spermatozoa,[14] these investigators further substantiated their earlier work with similar results, namely, the esterification of inorganic phosphorus to produce an ester which appeared to be ATP. They then expressed their belief that the function of ATP in bovine spermatozoa might be related to the utilization of the energy-rich phosphate esters for the maintenance of motility. This idea of the relationship between the motor function of sperm and the transformations of ATP in sperm was shared by Engelhardt.[5] However, these studies did not fully establish the fact that this substance was adenosine triphosphate and it remained for Mann[18, 20] to separate the readily hydrolyzable phosphorus fraction as a Ba-salt and identify it as adenosine-triphosphate and thus eliminate the possibility of this fraction being a different adenyl-poly-

phosphate. Ivanov and co-workers[8] further reported on a study of the biological effects of ATP isolated from sheep spermatozoa, on actomyosin threads. From their observations they concluded that ATP from ram spermatozoa does not differ from muscle ATP in its ability to react with actomyosin.

The metabolism of sperm has been the subject of numerous studies in which it has been established that fructose[21] is the readily utilizable store of energy for whole semen, whereas washed spermatozoa can utilize glucose or mannose as well. Mann[19] has shown that this ability is due to the fact that all three sugars enter glycolysis under the same enzymatic reaction with ATP. He also presents experimental evidence for the metabolic pathway involved, which is initiated by the esterification of half the readily hydrolyzable phosphate of ATP under the influence of sperm hexokinase to form a hexose-6-phosphate from either glucose, fructose or mannose. ATP then serves as the phosphate group donor and thus is being continuously split by its participation in the initial reaction of glycolysis.

On the other hand, along with this continous breakdown, there is a continuous reconstitution of ATP which Mann has shown to be accomplished by two reactions; (a) the reaction between adenylic acid and the liberated phosphate group from phospho-pyruvate and (b) the reaction between inorganic phosphorus and adenylic acid.

Since the two pyrophosphate linkages of ATP are high-energy linkages,[9] the dephosphorylation of ATP could supply the large amounts of energy needed for spermatozoan motility. However, in muscles another energy-storing device is known, namely, the one involving "phosphagen," proceeding by the way of the Lohmann reaction.[16]

Creatine phosphate + ADP $\rightleftharpoons$ ATP + Creatine

Creatine has been shown to exist in spermatozoa;[1] phosphocreatine also is known to be present in appreciable amounts.[2] Thus there exists the possibility that when needed, this system can aid the resynthesis of ATP broken down during glycolytic activity and also be regarded as a reserve of phosphate-bond energy which becomes available through ADP in the Lohmann reaction.

The purpose of this study was to investigate the possibility of a "phosphagen" energy reserve in spermatozoa by adding to bovine semen various substances which are part of phosphocreatine synthesis and measure their effects by motility ratings, longevity, and the use of manometric techniques.

In addition, observations were made on metabolic activity as affected by the addition of AMP and ATP to egg-yolk citrate diluted semen, undiluted whole semen, and washed spermatozoa, as well as the relationship between ATP-ase activity and motility loss upon storage.

# METHODS AND PROCEDURES

## Collection and Handling of Semen Samples:

Bull semen was collected in an artificial vagina. Samples were used from 2 to 5 hours after collection unless otherwise indicated. Immediately upon collection, the samples were immersed in a beaker of water at approximately 20° C and then placed in a refrigerator to cool to about 4° C. Samples were kept at this temperature until the time of the respective experiments. Semen samples were used singly in these experiments and not as pooled ejaculates.

The egg-yolk citrate diluter (E.Y.C.) used in experiments consisted of fresh egg-yolk with citrate at a ratio of 1:4. Dilution rates are specified in each experiment. Counts for sperm concentrations were determined by the hemocytometer method.

Motility ratings were made using a 0 to 5 scale,[7] full motility rated as 5 and complete immotility as 0. In checking motility, an attempt was made to have at least two persons rate the samples. In some experiments it seemed advisable to employ + and – symbols to point out minor differences in the relative motility observed.

## Procedure for Washing Spermatozoa:

Seminal fluid was removed according to the procedure recommended by White,[31] utilizing two centrifugations at 1500 rpm (approximately 450 X gravity) for 10 minutes each. Depending on whether the experiment was to be performed in an aerobic or anaerobic atomsphere, the suspension medium employed was Krebs-Ringer phosphate (KRP) or Krebs-Ringer bicarbonate (KRC), respectively, as described by Umbreit.[30] The only modification in preparing these solutions was that calcium was excluded and that 0.1 percent glucose was added. Unless otherwise specified, this procedure of washing was followed throughout this study.

## Manometric Technique and Measurements:

These measurements were made with the Warburg Constant Volume Respirometer at 37° C and consisted of both aerobic and anaerobic experiments, which were introduced to measure either the rate of respiration (aerobic) or the rate of glycolysis (anaerobic).

The respiratory rate of spermatozoa was determined as microliters of oxygen taken up by $1x10^8$ sperm cells. All the aerobic measurements in the course of this study were carried out in an atmosphere of air and 0.2 ml. of 20 percent KOH was added to the center well of the Warburg flasks to absorb the carbon dioxide.

Rate of anaerobic glycolysis of spermatozoa was measured as microliters of carbon dioxide released from the Krebs-Ringer bicarbonate buffer by

$1x10^8$ sperm cells in an atmosphere of 95 percent nitrogen and 5 percent carbon dioxide.

The first recording of metabolic activity was preceded by a 15-minute equilibration time. Substrate was added to reaction vessels 15 minutes after the end of equilibration. In all calculations of manometric data, the average of the control samples during first 15 minutes prior to the addition from the side arm was taken as the standard and all other samples were factored by this standard, thus plotting only one line from 0 to 15 minutes.

### Adenosine-Triphosphatase (ATP-ase) Assay:

The ATP-ase assay used throughout this study was the one described by DuBois and Potter.[4] Phosphorus analysis was carried out according to the method of Lowry and Lopez.[17] The unit for ATP-ase activity is expressed as one $\mu$ gm. of inorganic P liberated from ATP by $1 \times 10^8$ spermatozoa incubated for 15 minutes at 37° C and measured with the Coleman Universal Spectrophotometer at 750 m$\mu$. Simultaneous readings of two standards of inorganic phosphate were made with each assay. The authors of this method described a condition in certain tissue extracts in which full color development was inhibited until a certain length of time had elapsed. Ryan[27] encountered this inhibitory effect in bovine spermatozoa and concluded that if sperm concentrations were not greater than $1x10^8$ sperm cells, color development would tend to plateau at 20 minutes. In this study it was found that at least 25 minutes had to elapse before the color development would remain constant, so all readings were made at 25 minutes after the addition of ascorbic acid and molybdate.

## RESULTS

### Effects of Glycine, Arginine, Methionine, Creatine and Adenylic Acid on Spermatozoan Motility.

The relationship of spermatozoan motility to available supplies of compounds involved in the biological synthesis of creatine was studied by storing semen sample aliquots in diluters containing 200 mg. percent of glycine, arginine, methionine, creatine or AMP.

The first of these trials was carried out with samples stored either at room temperature or at 4° C after the additions were made. Each semen sample was initially diluted with E.Y.C. at a ratio of 1:10 and then divided into aliquots for storage at the different temperatures. No apparent difference in motility appeared during 24 hours of storage at 4° C but beyond that and until motility was minimal in the control samples, those samples to which either glycine or arginine was added, exhibited a higher motility rating. AMP additions caused a temporary increase in motility, lasting for three hours at about five to seven hours prior to complete loss of motility.

Among the samples stored at room temperature, it was noted that in two trials, samples containing the creatine maintained a motility rating of two for ten hours beyond the complete cessation of motility in all other samples.

In four subsequent trials the motility determinations were made on samples which had been divided into two fractions, one used as whole semen and the other washed free of seminal fluid. All samples were then diluted with E.Y.C. at a ratio of 1:100 and stored at 4° C.

As indicated by the average values given in Table 1, the motility of both whole semen and washed spermatozoa was maintained at a more uniform level by the diluter containing methionine. However, at least part of this apparent benefit may be due to the initial inhibitory effect of methionine on spermatozoan motility. It was observed that the greatest variation in response of individual samples to the addition of various amino acids occurred with glycine and arginine.

TABLE 1 -- THE EFFECT OF VARIOUS AMINO ACIDS ON THE MOTILITY OF SPERMATOZOA STORED AT 4° C.

| Hours of Storage | | 24 hrs. | 48 hrs. | 72 hrs. | 90 hrs. |
|---|---|---|---|---|---|
| CONTROL | Whole semen | 3 | 3 | 2 | 1 |
| | Washed sperm | 2 | 2 | 2 | 1 |
| ARGININE 0.01 M | Whole semen | 3 | 3 | 2 | 1 |
| | Washed sperm | 2+ | 2+ | 2+ | 1+ |
| GLYCINE 0.01 M | Whole semen | 3+ | 3 | 2 | 1 |
| | Washed sperm | 2+ | 2 | 2 | 1 |
| ARGININE 0.01 M & GLYCINE 0.01 M | Whole semen | 3 | 3 | 2+ | 2 |
| | Washed sperm | 2+ | 2+ | 2 | 1 |
| METHIONINE 0.01 M | Whole semen | 2+ | 2+ | 2+ | 2 |
| | Washed sperm | 2 | 2 | 2 | 2- |

## Effects of Glycine, Arginine and Methionine on Respiration and Anaerobic Glycolysis of Bovine Spermatozoa.

The three amino acids were added to whole semen suspended in Krebs-Ringer phosphate buffer for the aerobic experiment and in Krebs-Ringer bicarbonate for the anaerobic determinations. Results are reported in graphic form (Figs. 1 and 2) rather than in a table showing $-Z_{O_2}$ or $+ Z_G^N$ values because at the end of one hour the differences in metabolic rate were quite small, becoming more pronounced when the manometric runs were continued over a period of time longer than one hour.

Although the increased rate of oxygen consumption and apparent carbon dioxide release was relatively small, the results exhibit a pattern similar

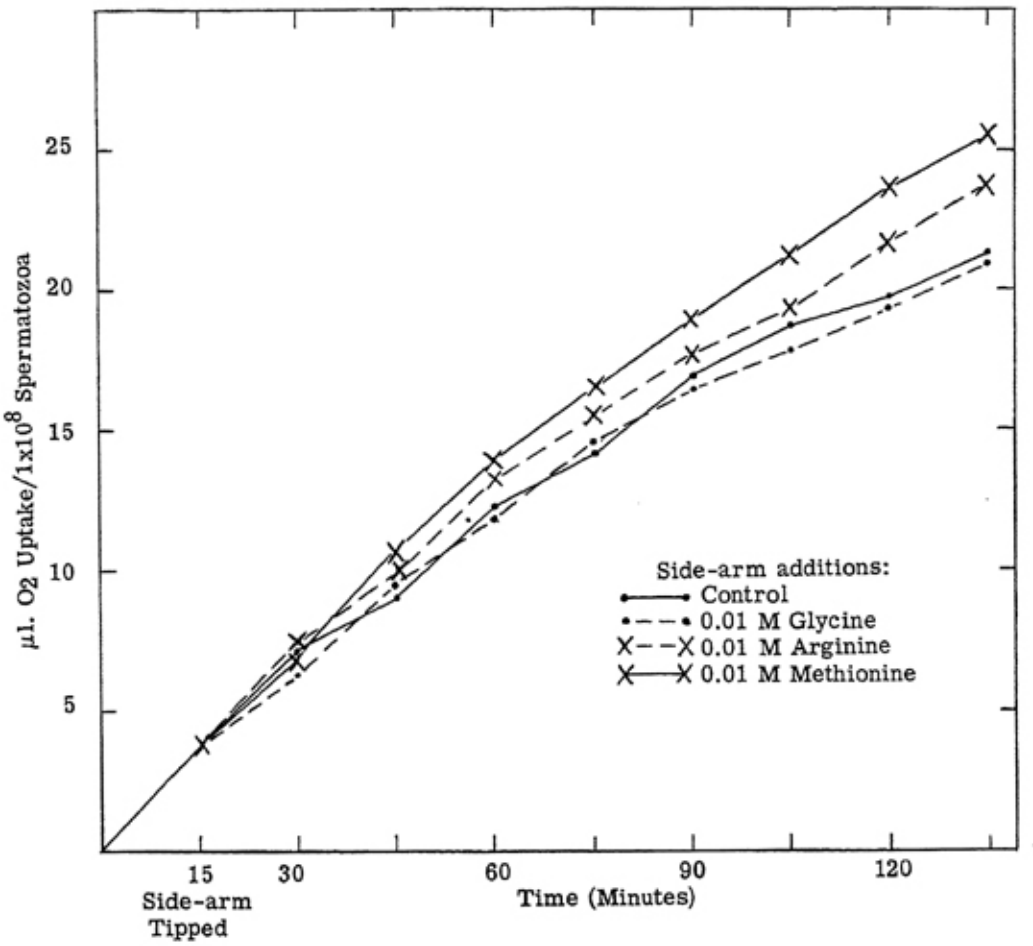


Fig. 1—The effect of various amino acids on the respiration of bovine semen.

to that observed previously, when the effect of arginine, glycine or methionine on stored whole semen was studied. In all of these experiments, the beneficial effect was more in the nature of increased longevity, as shown by the fact that motility was not increased to any significant extent but maintained at a higher level for a longer time.

## Effects of AMP on Respiration and Anaerobic Glycolysis of Whole Semen and Washed Spermatozoa.

The observation that added AMP caused a temporary increase in motility five to seven hours prior to complete cessation of motility suggested the possibility that shortly before glycolytic activity ceases, AMP might be utilized to furnish additional energy to the system by replacing the AMP which had been dephosphorylated to adenosine or deaminized to inosinic acid. It was thus of interest to study the metabolic behavior of whole and washed spermatozoa with the addition of AMP.

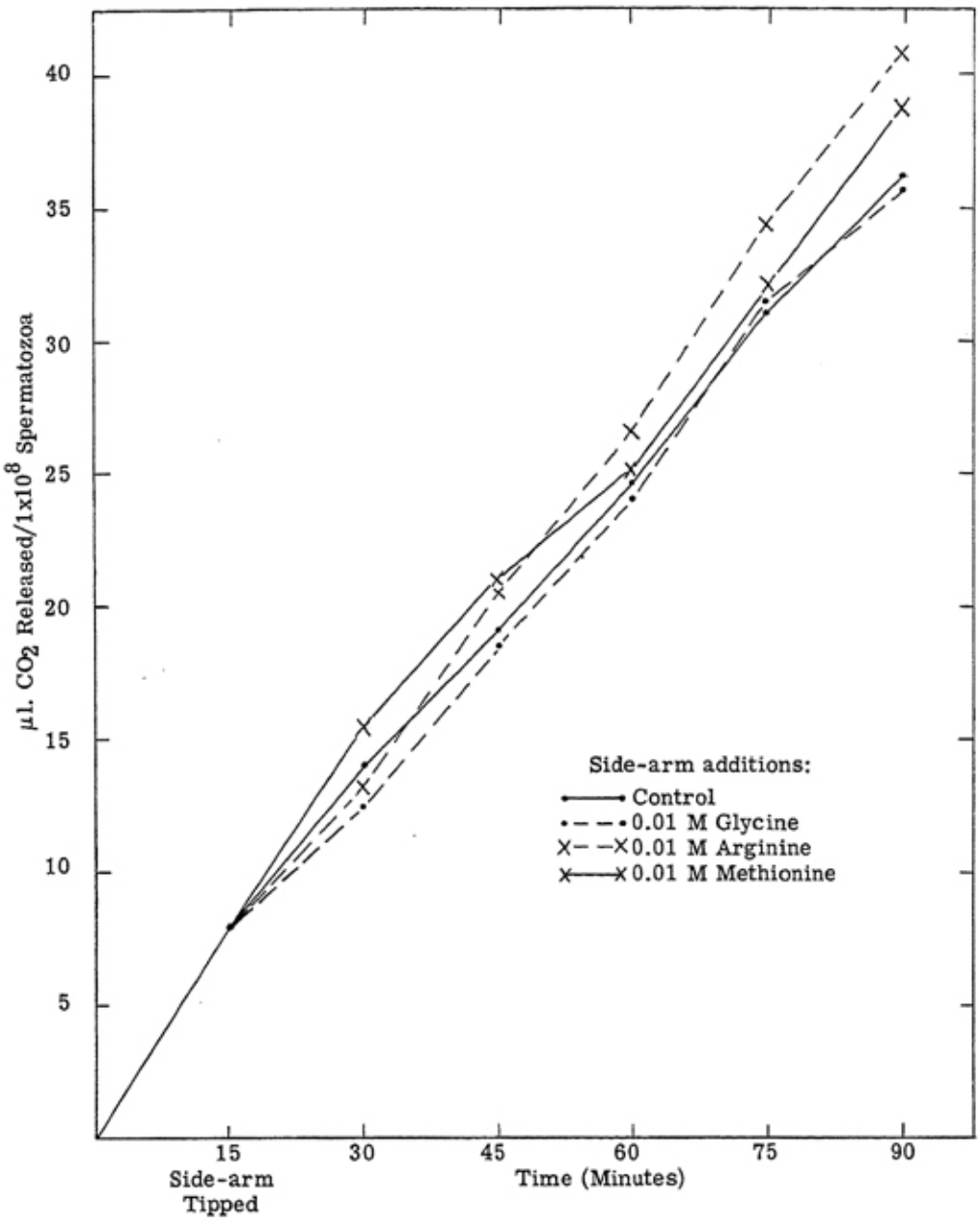


Fig. 2—The effect of various amino acids on anaerobic glycolysis of bovine semen.

In all trials the values obtained for respiration and anaerobic glycolysis were within the range of $-Z_{O_2}$ and $+ Z_G^N$ values reported by other workers.[12, 15, 23, 26] It was interesting to note that in the trials where anaerobic glycolysis of whole semen was studied (Fig. 3), the addition of AMP inhibited glycolysis. The reason for this unexpected behavior in view of the earlier experiment in which AMP added to whole semen diluted with E.Y.C. was

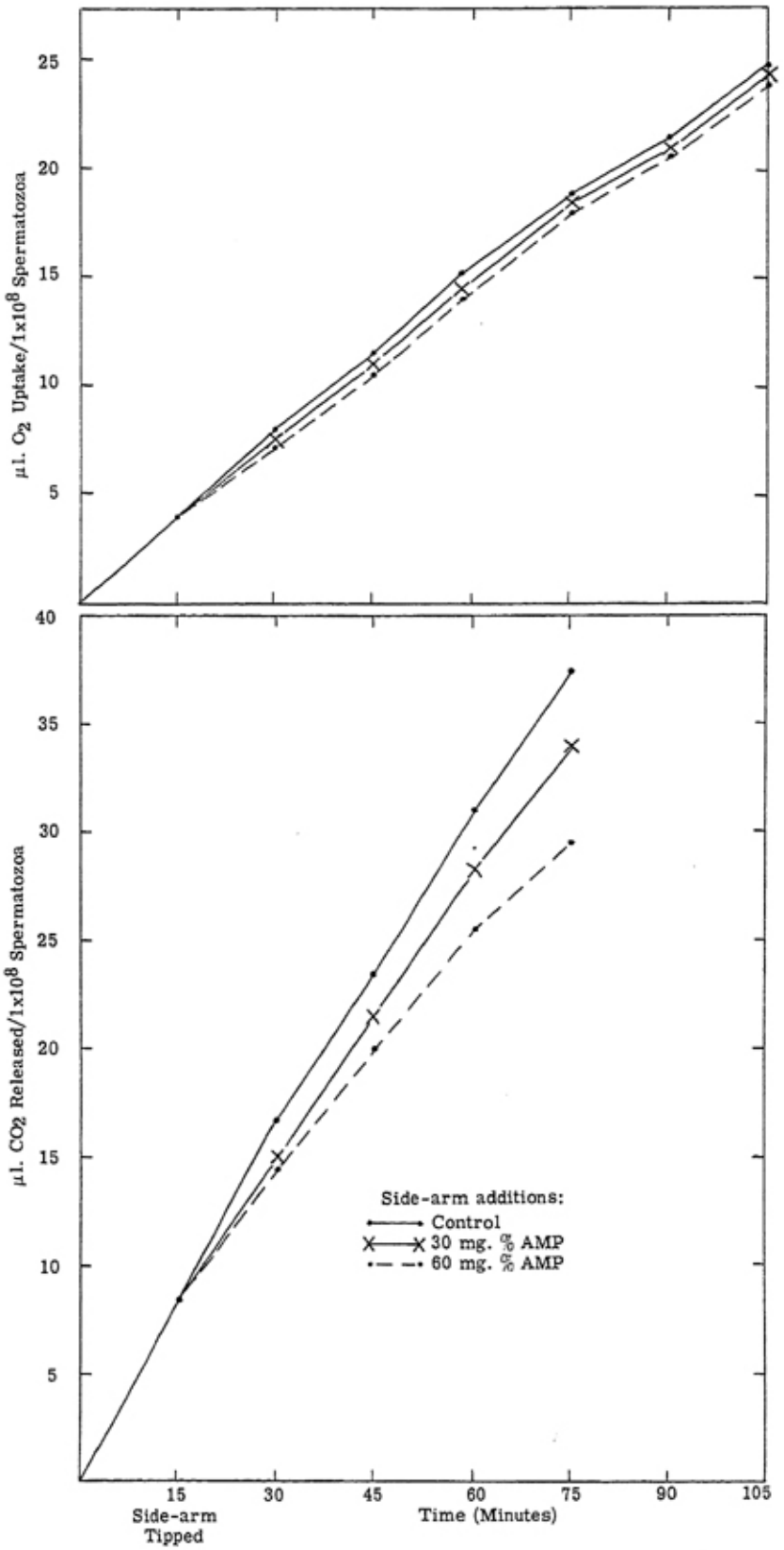


Fig 3.—The effect of AMP on the metabolic activity of bovine semen.

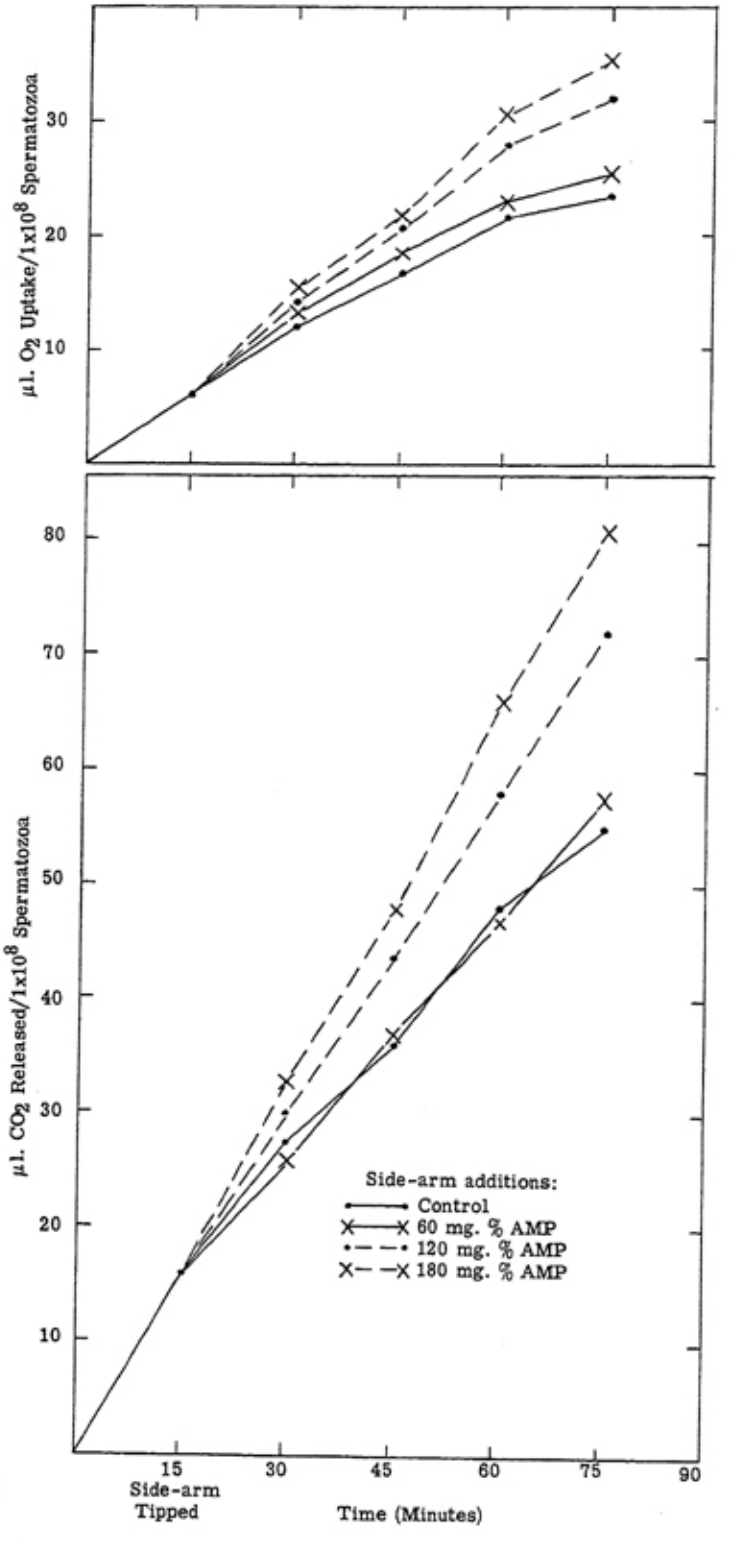


Fig. 4—The effect of AMP on the metabolic activity of washed spermatozoa.

shown to increase motility, is uncertain. Although the enzyme 5-nucleotidase known to be present in bull seminal fluid[19] may have dephosphorylated the added adenylic acid; this would not necessarily account for the actual inhibition of glycolysis. Possibly the increased motility in E.Y.C. diluted whole semen is related to the aerobic utilization of a phospholipid substrate by spermatozoa.[18] Thus, egg yolk containing a relatively large amount of phospholipids might contribute to the phospholipid stores of the sperm. The phospholipid then might be oxidized, resulting in an uptake of inorganic phosphorus, making possible the eventual formation of ATP if additional AMP is present.

The addition of AMP to spermatozoa washed free of seminal fluid resulted in a definite increase in both the aerobic and anaerobic metabolism as measured by oxygen consumption and carbon dioxide release, respectively (Fig. 4). After a few preliminary experiments the concentration of AMP was increased to a minimum of 60 mg. percent and a maximum of 180 mg. percent. The results of both the aerobic and anaerobic trials were similar with regard to increased metabolic activity associated with increasing concentrations of AMP.

### Effect of ATP on Respiration and Anaerobic Glycolysis of Bovine Spermatozoa.

As mentioned in the introduction, a number of investigators studied the relationship between ATP and sperm metabolism but they did not report quantitative measurements of the effects of ATP on the metabolic rate of spermatozoa. Several preliminary experiments were conducted to study whether ATP added to whole semen would eventually affect motility. In these preliminary experiments, E.Y.C. diluted bovine semen samples stored for several days at 4° C were subjected to various concentrations of ATP, ranging from 0.006 M to 0.026 M. The results showed a rather rapid response in increased motility upon the addition of ATP. In addition to this observation, it was found while comparing motility in samples to which ATP had been added and in their controls, that usually the controls showed a much better motility after prolonged storage. This would suggest an exhaustion of ATP due to the temporary increase in metabolic activity, leaving the spermatozoa improverished in ATP stores for subsequent glycolysis.

As shown in Fig. 5, the increased anaerobic glycolytic activity following the addition of ATP is of relatively short duration with the metabolic rates returning to approximately that of the controls. These results are in agreement with the observations indicating a reduction in semen storage time as a result of added ATP and thus it appears most likely that the addition of a substance which markedly stimulates the metabolic rate of spermatozoa, at the same time causes a more rapid exhaustion of sperm activity. The length of time after addition of ATP required for the metabolic rates to return to

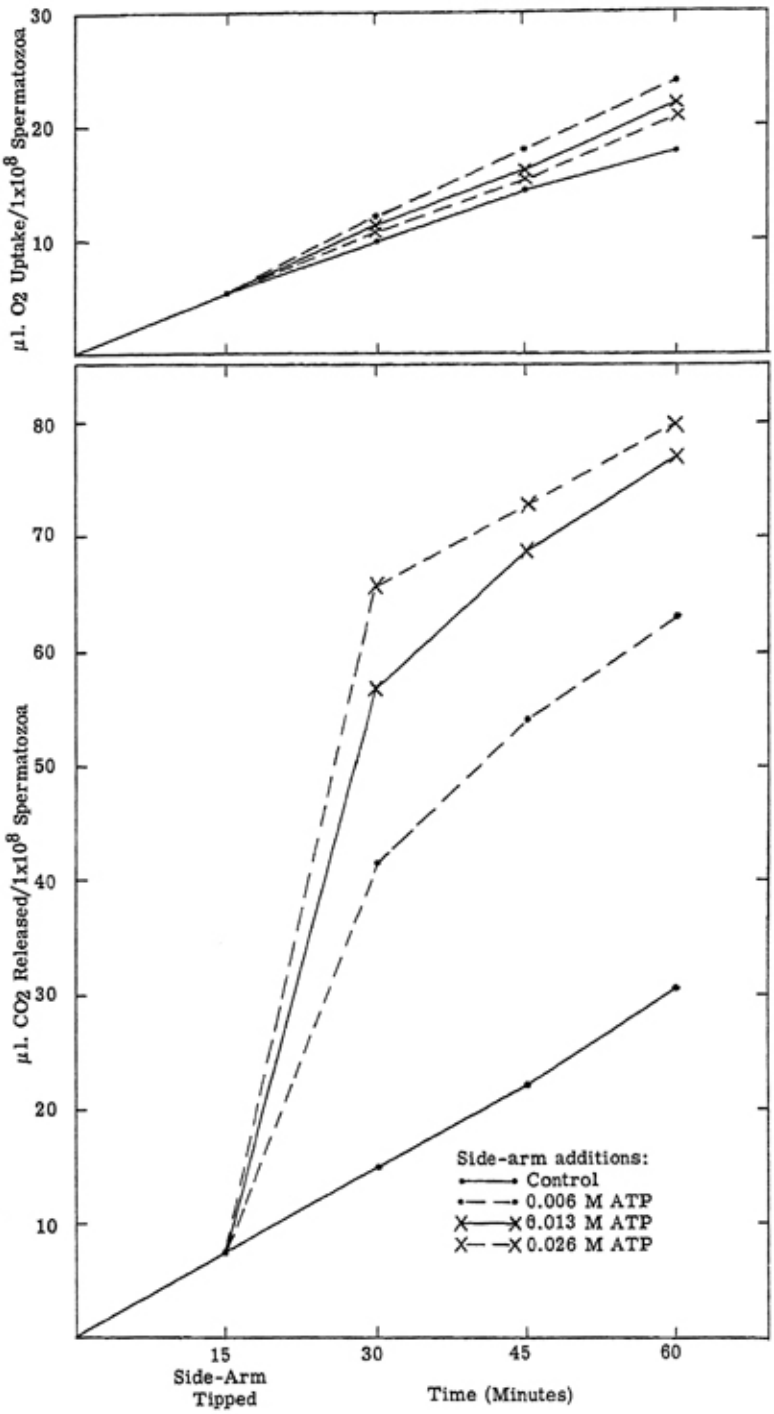


Fig. 5—Effect of supplementary ATP on the metabolic activity of washed spermatozoa.

that of the controls varied with different samples and it is believed that the varying responses of individual samples to ATP addition is most probably associated with the enzyme ATP-ase, which in turn would affect the energy mechanism involved in sperm motility. In all trials, motility was checked

immediately upon the termination of manometric measurement but no differences in motility could be observed when the spermatozoa in the control flasks were compared with those to which ATP was added. The explanation for this lies in the fact that motility ratings were made only at the end of the experiment when the metabolic rate was more or less equal in all flasks. Only twice were motility ratings made when the experiment was stopped at a point at which increased metabolic activity due to ATP addition appeared to be still effective. When this procedure was followed, the spermatozoa to which ATP had been added were found to exhibit a higher rate of motility.

Experiments on the respiratory rate of washed spermatozoa did not indicate significant changes upon addition of ATP. However, all of the three different concentrations increased the rate of respiration slightly above that of the controls. It was interesting to note that the lowest concentration of 0.006 M ATP resulted in a greater response than the higher concentrations, whereas the highest concentration gave the smallest response. In subsequent experiments, concentrations lower than 0.006 M ATP were used but none of these were as effective as the 0.006 M level in increasing the respiratory rate of the spermatozoa.

### Effect of EDTA on the Metabolic Activity of Spermatozoa.

The apparent close relationship between spermatozoan motility and enzymatic hydrolysis of ATP emphasized the need for more information on the relationship of the enzyme to metabolic activity and motility of bovine spermatozoa. Previous trials in which anaerobic glycolysis was rather significantly increased by the addition of ATP, gave rise to the question whether a compound inhibiting ATP-ase could be utilized in showing that the added ATP is directly associated with the observed increased glycolysis. As the enzymic function of the ATP-ase is known to be greatly dependent on the presence of calcium ions,[4, 6] it was thought that the addition of a calcium chelating compound such as ethylenediamine tetra-acetate would be a good indication of this effect.

Fig. 6 illustrates the inhibiting effect exerted by the addition of the calcium chelating EDTA, on the anaerobic glycolysis of spermatozoa to which ATP had been added. However, EDTA did not affect the glycolysis of the sperm without added ATP.

This latter observation is difficult to explain, except that it would indicate that the added ATP is utilized by a metabolic path or mechanism different from that of the "in-vivo" or intracellular ATP.

### ATP-ase Activity in Bovine Semen at Different Levels of Sperm Motility.

The presence of ATP splitting enzymes in seminal fluid has been demonstrated repeatedly by several workers.[6, 18, 25] In spermatozoa, even though

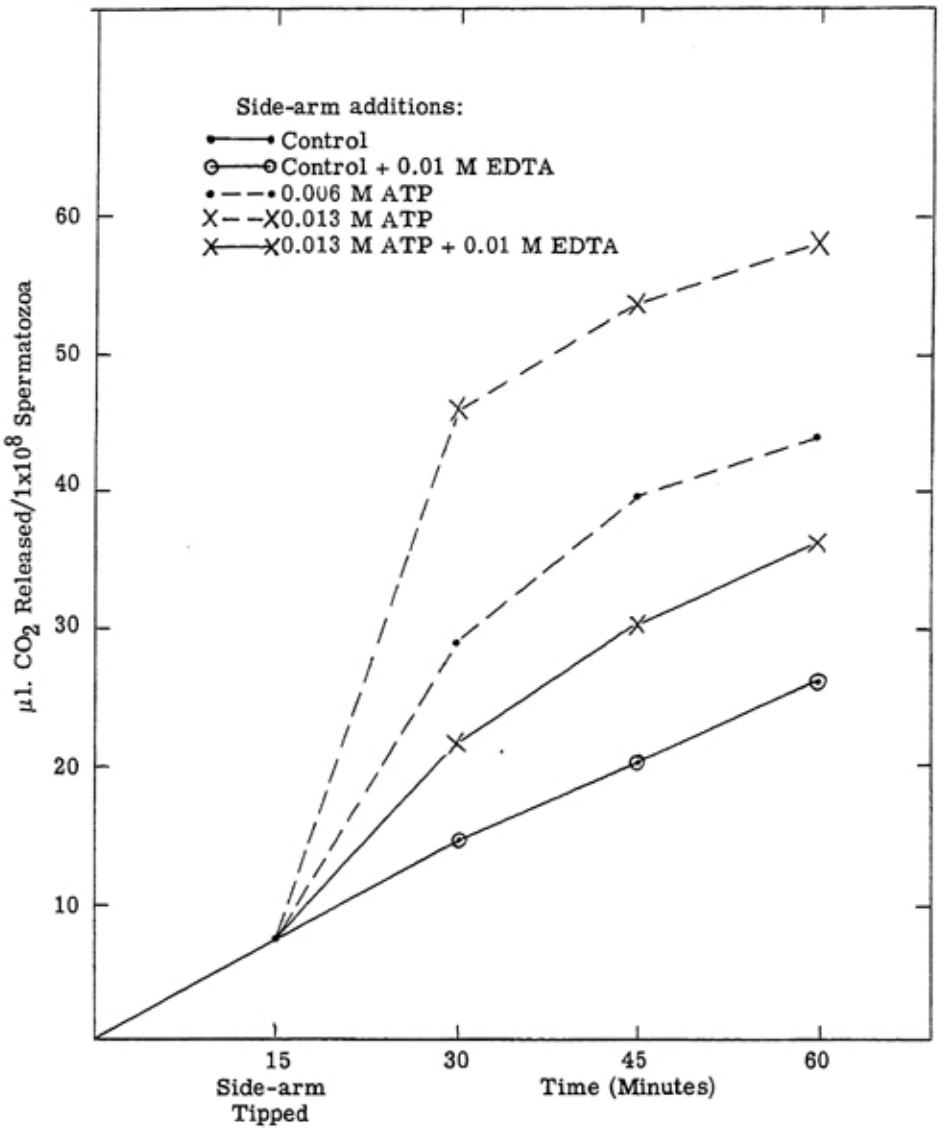


Fig. 6—Effect of ethylenediamine tetra-acetate on anaerobic glycolysis of washed spermatozoa.

washed several times to remove the phosphatases present in the seminal fluid, a high phosphatase activity against ATP is exhibited which, together with all other evidence available, points to the close relationship between sperm ATP-ase, motility, and survival of the sperm cells. With respect to ATP, it has been shown by Mann[22] that this compound is continuously utilized and resynthesized until some interference with intermediary enzymic reactions occurs which prevents the breaking down and building up of ATP, resulting in a decrease of both metabolism and motility. In conjunction with the gradual loss of ATP it became of interest to investigate whether a corresponding decrease in the ATP splitting enzyme could be demonstrated in spermatozoa kept under storage (4° C) and which could be related to a decrease in motility.

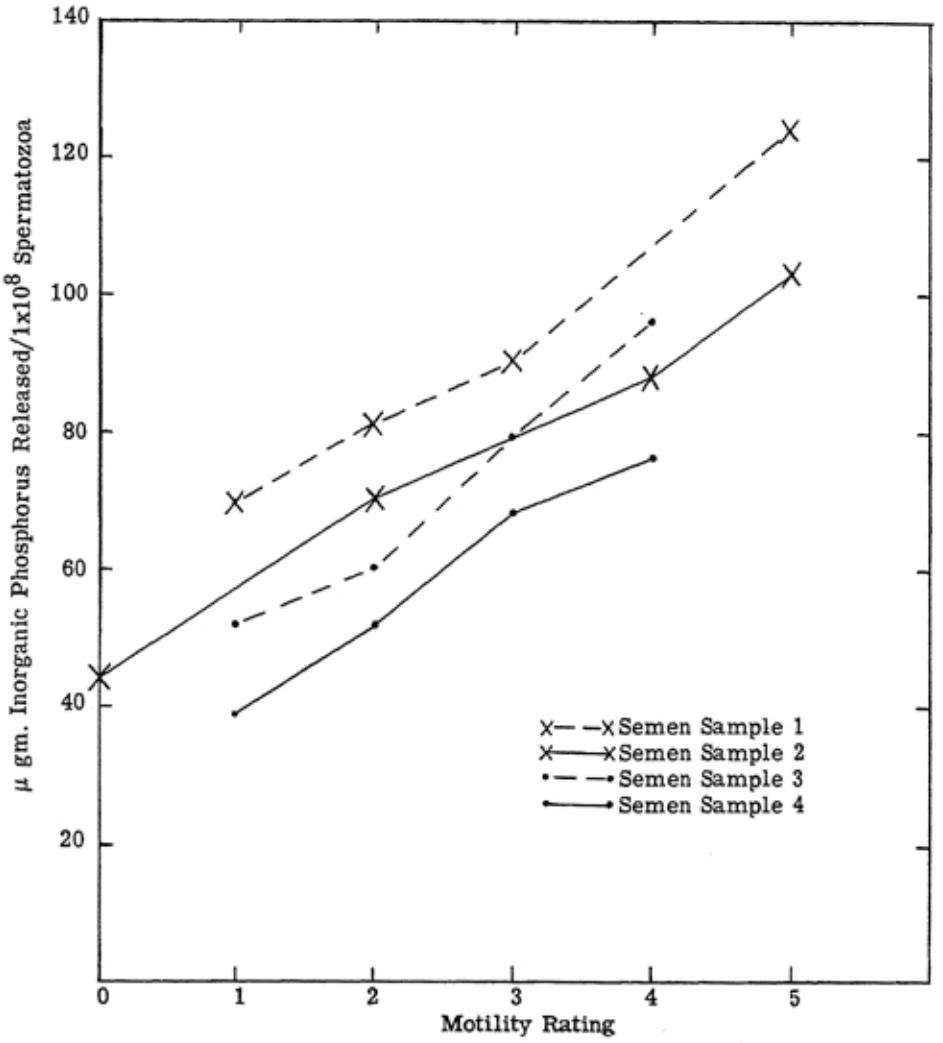


Fig. 7—The relationship between spermatozoan motility and ATP-ase activity.

Fig. 7 shows the results of ATP-ase assays for four different semen samples. Aliquots of each one of the four samples were assayed at different stages of storage, to observe any change in ATP-ase activity with decreasing motility.

The results seem to indicate a close relationship between the decreased motility of stored semen and ATP-ase activity, as measured by μgm. of inorganic P liberated from ATP by $1x10^8$ sperm cells incubated for 15 minutes at 37° C. The values obtained were more linear with motility measurements than with time of storage, as the decrease in sperm motility with storage time varies from sample to sample. Only one of the samples in this experiment was assayed when the observed motility was 0, as it appeared from preliminary experiments that the enzymic activity drops rapidly and disproportionally once sperm motility has ceased completely and unless there is continuous observation it is impossible to establish the point at which cessation of motility occurs.

# DISCUSSION

The first series of experiments was concerned with measuring the effects of supplementary amounts of various compounds, constituents of phosphocreatine biosynthesis, on bovine spermatozoan longevity. Results indicated that both arginine and glycine favored the maintenance of a higher level of sperm motility and thus were in general agreement with those of Knoop and Krauss,[10] Marcus and Bishop,[24] and Tyler and Tanabe,[29] all of whom reported favorable effects for glycine.

In a recent study, White[32] could not detect improved motility by adding glycine or arginine to diluted bull semen. He remarks, however, that the use of glass-distilled water which he employed in his experiments may account for his inability to reproduce the results of other workers. On the other hand, his method of evaluating the beneficial effects of glycine or arginine differed from that of the other investigators, in that he measured motility at hourly intervals over a four-hour period whereas the marked effects of these added amino-acids would be evident only after a prolonged storage time and in turn express themselves primarily in terms of extended longevity rather than as increased motility.

The theory advanced by Tyler and Tanabe[29] to explain the beneficial effects of glycine or arginine addition on bull semen is similar to that brought foward by Tyler and Rothschild,[28] who, from their studies of metabolism in sea-urchin spermatozoa, suggested that one way in which these amino-acids might act in promoting the life span of spermatozoa could be by binding certain highly toxic heavy metals ordinarily present in trace amounts in the diluents. The latter workers also based their hypothesis on the fact that while using 1-$C^{14}$-glycine, they could not find evidence that the sperm cells metabolically utilized the amino acids to any great extent. Although experimental evidence offers considerable support for this theory, there exists another distinct possible mode of action for arginine and glycine, that of direct contribution in the synthesis of phosphocreatine.

Varying responses among the different individual ejaculates, as well as the effect of added creatine, would indicate the possibility that the effectiveness of adding methionine or arginine and glycine to semen is governed to a considerable extent by the available reserves of these compounds and/or the conversion of creatine to creatinine.

The manometric studies, in which the effect of added amino acids on the rate of respiration and anaerobic glycolysis were measured, supported the results of the motility studies.

The mechanism by which AMP added to semen may promote metabolic activity can best be described on the basis of Mann's[19] investigations which showed that energy-rich ATP losing its terminal labile phosphate group in the initial phase of glycolysis, is later reconstituted by way of AMP receiving

phosphate groups from phosphoglyceric acid. In the trials where AMP was added to washed spermatozoa, the increase in metabolic activity was related to the increased concentration of AMP. The observed differences in metabolic response to AMP between whole semen and washed spermatozoa (Figs. 3 and 4) apparently are related to the presence or absence of seminal fluid components.

Results obtained with supplementary ATP, in both the motility and manometric experiments, indicated a rather immediate utilization of this compound by washed spermatozoa. This stimulation leads to apparent "spermatozoan fatigue," as indicated by the reduced longevity of ATP supplemented semen samples and the faster return to control level of anaerobic glycolysis. The fact that anaerobic utilization of ATP added to spermatozoa appears to be completed with a relatively short period of time and is altered by EDTA chelation, suggests that the utilization of "in-vitro" or added ATP occurs in a manner different to and/or lacks the controls imposed on "in-vivo" or intracellular ATP.

Assays for ATP-ase activity in semen samples with varying motility ratings indicate that at least one of the primary reasons for decline in spermatozoan motility is associated with inactivation of the ATP-ase system.

## SUMMARY

Experiments were conducted to study the effects of various phosphocreatine constituents, AMP, and ATP on the motility and metabolic activity of bovine spermatozoa.

1. Of the phosphocreatine constituents (glycine, arginine, methionine and creatine) added to whole semen, the combination of arginine and glycine favored the maintenance of the highest level of spermatozoan motility. Arginine and methionine were the most effective in stimulating respiration and anaerobic glycolysis.

2. Additions of AMP and ATP to spermatozoa washed free of seminal fluid resulted in definite increases in both respiration and anaerobic glycolysis. Addition of AMP to whole semen had no apparent effect on the rate of respiration and depressed anaerobic glycolysis.

3. Decreasing spermatozoan motility was found to be proportional to decreases in ATP-ase activity .

## BIBLIOGRAPHY

1. Anderson, J. *The Semen of Animals and its Use for Artificial Insemination.* Imperial Bureau of Animal Breeding and Genetics. Edinburgh. 1945.

2. Baldwin, E. *Dynamic Aspects of Biochemistry.* Cambridge Univ. Press 2nd ed. p. 405. 1952.
3. Bloch, K. and R. Schoenheimer. "The Biological Precursors of Creatine." *J. Biol. Chem.* 138:167. 1941.
4. DuBois, K. P. and U. R. Potter. "The Assay of Tissues for Respiratory Enzymes. III. Adenosine Triphosphatase." *J. Biol. Chem.* 150:185. 1943.
5. Engelhardt, W. A. "Phosphoric Acid and Cell Functions." *Akad. Nauk. Bull. Classe de Sci. U.S.S.R.* No. 2:195. 1945.
6. Heppel, L. A. and R. J. Hillmoe. "Mechanism of Enzymatic Hydrolysis of Adenosine Triphosphate." *J. Biol. Chem.* 202:217. 1953.
7. Herman, H. A. and F. W. Madden. *The Artificial Insemination of Dairy Cattle.* Lucas Bros. Columbia, Mo. Rev. ed. p. 20. 1953.
8. Ivanov, I. I., Kassavina, B. S. and L. D. Fomenko. "Adenosine Triphosphate in Mammalian Spermatozoa." *Nature* 158:624. 1946.
9. Kaplan, N. O. "Thermodynamics of the Phosphate Bond." *The Enzymes.* Vol. II. J. B. Sumner and K. Myrback ed. Academic Press, Inc. New York. 1951.
10. Knoop, C. E. and W. E. Krauss. "Storage of Bovine Spermatozoa in Diluents Containing Certain Amino-Acids." *J. Dairy Sci.* 27:657. 1944.
11. Lardy, H. A. and P. H. Phillips. "Inhibition of Sperm Glycolysis." *J. Biol. Chem.* 148:343. 1943.
12. Lardy, H. A. and P. H. Phillips. "Effect of pH and Certain Electrolytes on the Metabolism of Ejaculated Spermatozoa." *Am. J. Physiol.* 138:714. 1943.
13. Lardy, H. A. and P. H. Phillips. "Recent Observations on the Preservation of Bull Spermatozoa." *J. Dairy Sci.* 27:658. 1944.
14. Lardy, H. A., R. G. Hansen and P. H. Phillips. "The Metabolism of Bovine Epididymal Spermatozoa." *Arch. Biochem.* 6:41. 1945.
15. Lardy, H. A., D. Ghosh and G. W. E. Plaut. "A Metabolic Regulator in Mammalian Spermatozoa." *Science.* 109:365. 1949.
16. Lohmann, K. "Uber die Enzymatische Aufspaltung der Kreatinphosphorsaure." *Biochem. Z.* 271:264. 1934.
17. Lowry, O. H. and J. Lopez. "The Determination of Inorganic Phosphate in the Presence of Labile Phosphate Esters." *J. Biol. Chem.* 162:421. 1946.
18. Mann, T. "Studies on the Metabolism of Semen. I. General Aspects, Occurrence and Distribution of Cytochrome, Certain Enzymes and Coenzymes." *Biochem. J.* 39:451. 1945.
19. Mann, T. "Studies on the Metabolism of Semen II. Glycolysis in Spermatozoa." *Biochem. J.* 39:458. 1945.
20. Mann, T. "Anaerobic Metabolism of Spermatozoa." *Nature* 156:80. 1945.
21. Mann, T. "Metabolism of Semen." *Adv. Enzyme.* 9:329. 1949.

22. Mann, T. *The Biochemistry of Semen.* John Wiley & Sons, Inc. New York. p. 155. 1954.
23. Mann, T. and C. Lutwak-Mann. "Studies on the Metabolism of Semen. IV. Aerobic and Anaerobic Utilization of Fructose by Spermatozoa and Seminal Vesicles." *Biochem. J.* 43:266. 1948.
24. Marcus, A. R. and W. H. Bishop. "Effect of Glycine on the Survival of Bull Spermatozoa." *Nature* 174:746. 1954.
25. MacLeod, J. and W. H. Summerson. "The Phosphatase Activity of Human Spermatozoa." *J. Biol. Chem.* 165:533. 1946.
26. Melrose, D. R. and C. Terner. "The Metabolism of Pyruvate in Bull Spermatozoa." *Biochem. J.* 53:296. 1953.
27. Ryan, W. L. *A Metabolic Study of Cold Shock in Mammalian Spermatozoa.* Thesis. Univ. of Missouri. 1953.
28. Tyler, A. and Lord Rothschild. "Metabolism of Sea Urchin Spermatozoa and Induced Anaerobic Motility in Solutions of Amino-Acids." *Proc. Soc. Exper. Biol. and Med.* 76:52. 1951.
29. Tyler, A. and T. Y. Tanabe. "Motile Life of Spermatozoa in Glycine and Yolk-Citrate Diluents at High and Low Temperatures." *Proc. Soc. Exper. Biol. and Med.* 81:367. 1952.
30. Umbreit, W. W., Burris, R. H. and J. F. Stauffer. *Manometric Techniques and Tissue Metabolism.* Burgess Publishing Co. 1949.
31. White, I. G. "The Effect of Washing on the Motility and Metabolism of Ram, Bull and Rabbit Spermatozoa." *J. Exper. Biol.* 30:200. 1953.
32. White, I. G. "The Effect of Some Seminal Constituents and Related Substances on Diluted Mammalian Spermatozoa." *Austral. J. of Biol. Sci.* 7:379. 1954.