

The Effect
of *Escherichia coli*
on the Semen of Bulls,
Rams and Rabbits

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ACKNOWLEDGMENTS: The authors thank Professor Walter B. Bollen of the Bacteriology Department, and Professor Ralph Bogart and particularly Professor Hugo Krueger, of the Beef Improvement Project, Animal Husbandry Department, who read the manuscript patiently and made invaluable suggestions. We are grateful to Mr. Ira F. Roberts, Chief Technician of the Oregon Dairy Breeders' Association, for providing dairy bull semen samples.

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Conclusions

In vitro studies revealed that *Escherichia coli* (isolated from dairy bull semen or obtained from the American Type Culture Collections) had a depressing effect on spermatozoan motility in semen of dairy bulls, beef bulls, rams, and rabbits. This depressing effect was noticeable usually about four hours after the semen, diluted in egg-yolk sodium citrate medium, was incubated with the *E. coli* at 38° C., or about 96 hours at 5° C.

It has been found that the oxygen uptake in spermatozoa-*E. coli* or semen-*E. coli* suspensions was increased above the sum of the oxygen uptake of spermatozoa alone plus the oxygen uptake of *E. coli* alone. This increase has been found consistently in semen from dairy bulls, beef bulls, rams, and rabbits. Washing of spermatozoa or of *E. coli* cells did not eliminate the increase in oxygen consumption; heat treatment of *E. coli* (65° C. for 30 minutes), however, did eliminate the increase.

The pH in semen-*E. coli* suspensions was consistently higher than the pH of semen in egg-yolk sodium citrate medium without the addition of *E. coli*. The pH changes brought about by *E. coli* did not appear to be a contributing factor in depressing spermatozoan motility. Likewise, carbon dioxide did not seem to be a prime factor.

The depressing effect of *E. coli* on spermatozoan motility was reversed by streptomycin, glucose and catalase. This suggested that the main factor involved in the depressing effect of *E. coli* on spermatozoan motility was an accumulation of hydrogen peroxide in the semen-*E. coli* suspensions. The reversal by streptomycin of the effect of *E. coli* on spermatozoan motility was tentatively explained as an interference with the terminal respiration process of the *E. coli* cells by streptomycin and thus the provision of more pyruvate for the sperm cells to react with hydrogen peroxide. The reversal of glucose on the depressing effect of *E. coli* on spermatozoan motility was suggested as due to a block of amino acid oxidase formation in the *E. coli* cells and elimination of the deamination of amino acids and reduced formation of hydrogen peroxide from amino acids. Glucose may also have provided more pyruvate to the spermatozoa to react with hydrogen peroxide.

The reversal of the depressing effect of *E. coli* on spermatozoan motility by catalase confirmed the suggestion that the motility depressing effect of *E. coli* on spermatozoa was chiefly due to an accumulation of hydrogen peroxide in semen-*E. coli* suspensions in an amount sufficiently high to interfere with glycolysis and to inhibit the motility of spermatozoa.

The Effect of *Escherichia coli* on the Semen of Bulls, Rams and Rabbits

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The detrimental effect of microorganisms on semen or spermatozoa may be one of the important factors contributing to reduced fertility. As *Escherichia coli* was one of the organisms found in the majority of semen ejaculates of bulls giving comparatively greater difference between the 50-80 days and 8-month nonreturn of estrus rates, it was suspected of being a factor in prenatal death (Wu, Elliker, and McKenzie, 1952). Another possible effect of *E. coli* might be due to its reducing the viability of spermatozoa in such a way as to reduce fertility.

There are many chances for fecal contamination in the female tract with this organism. The opportunity for such infection is enhanced by natural mating or artificial insemination. An investigation was therefore undertaken in an effort to obtain information concerning the specific effect of this organism on spermatozoa. *E. coli* was found to inhibit sperm motility and further investigations were made to elucidate the mechanism involved in this motility depressing effect of *E. coli* on spermatozoa.

Review of Literature

The literature to be reviewed has been classified under five subheadings: Effects of microorganisms on sperm motility, physiological aspects of mammalian spermatozoa, physiological aspects of *E. coli*, the toxic effects of hydrogen peroxide on spermatozoa, and the mode of action of streptomycin on *E. coli*.

Effect of microorganisms on spermatozoan motility

In a study of bacteria in bovine semen, Edmondson, Tallman and Herman (1949) found that the addition of micrococci, non-hemolytic streptococci or yeasts to fresh diluted semen caused a substantial increase in the duration of sperm motility, while other bacteria (including *Bacillus*, coliform bacteria, hemolytic streptococci, *Pseudomonas*, *Actinomyces* and *Proteus*) brought about some decrease in the livability of the spermatozoa. This was especially true of the *Actinomyces* and hemolytic *Bacillus*.

Mathews and Buxton (1951) reported that, of the bacteria isolated from endocervical cultures of 100 women attending the

sterility clinic (Sloane Hospital for Women, New York), *E. coli*, *Streptococcus viridans* and hemolytic streptococci were highly spermicidal, whereas diphtheroid bacteria, bacillary streptococci, hemolytic and nonhemolytic streptococci, *Bacillus subtilis* and *Pseudomonas aeruginosa* were entirely innocuous to spermatozoa. Mathews and Buxton believe that the pH variation which occurred in their experiments did not affect the longevity of the sperm. They found that streptomycin, acting on sperm-depressing *E. coli*, resulted in a definite prolongation of viability of the spermatozoa.

Physiological aspects of mammalian spermatozoa

MacLeod (1939, 1941a, 1941b) believed that metabolism of human spermatozoa was almost exclusively glycolytic. Aerobic lactic acid production was 80 per cent of the anaerobic, and aerobic lactic acid production fell off with time. Anaerobic glycolysis on the other hand was constant over a period of seven hours. Maximum motility was maintained for many hours under anaerobic conditions but showed a marked tendency to decrease when the spermatozoa were exposed to pure oxygen. Respiratory inhibitors, such as cyanide, azide and carbon monoxide, did not depress motility, whereas monoiodoacetate and fluorides inhibited glycolysis and destroyed motility. MacLeod (1943) demonstrated the presence of a complete cytochrome system and succinic dehydrogenase in human spermatozoa.

Ivanov (1931, 1935) believed that sperm derived energy from some sources other than oxidation and that motility did not depend on glycolysis. He found that sperm remain motile in a medium containing sufficient cyanide to inhibit respiration almost completely, and motility was retained in Ringer's solution containing sufficient monohaloacetic acid to inhibit lactic acid formation from glycolysis.

Lardy and Phillips (1941a, b, c) found that sperm separated from semen maintained motility in Ringer-phosphate solution only in the presence of oxygen. There was practically no motility in a nitrogen atmosphere, but with addition of glucose (or maltose, fructose, or mannose) motility was maintained as long as three hours in both air and nitrogen atmospheres. During storage of semen, inorganic phosphate remained constant, acid soluble phosphate and ester phosphate increased, lipid phosphate decreased. The decrease in lipid phosphate paralleled the decrease in motility. Lardy and Phillips concluded that the energy for sperm motility was obtained from the oxidation of intra-cellular phospholipids or from glycolysis. The anaerobic mechanism is sufficient when sugar is available and lessens the demand on the oxidative mechanism.

Physiological aspects of *E. coli*.

E. coli is widely distributed in nature and is a normal inhabitant of the intestinal tract of almost all vertebrates. Sometimes it is associated with infections of the genito-urinary tract. It is facultatively anaerobic with an optimum growth temperature of 30°-37° C. Growth takes place at temperatures as low as 10° C. and as high as 45° C. Usually, it is destroyed in 30 minutes at 60° C. (Breed, Murray and Hitchens, 1948). Heat resistant strains, however, have been reported (Ayers and Johnson, 1914; Stark and Patterson, 1936).

Nutritionally, *E. coli* is heterotrophic. It can thrive on very simple media containing carbon and nitrogen sources together with the appropriate salts (Anderson, 1946). As is true of a number of heterotrophes, traces of carbon dioxide are prerequisites for *E. coli* growth, and it has been reported that the formation of succinic acid was higher when fermentation was carried out in the presence of carbon dioxide. *E. coli* is known to possess a number of dehydrogenases. It has cytochromes a_1 , a_2 and b_1 (Keilin and Harkley, 1941) and the coenzyme systems identical with those in animal and yeast cells. It also has the same series of enzymes as those involved in the production of lactic acid by muscle. Washed *E. coli* suspensions, which are deprived of cozymase, no longer produce lactic acid from glucose (Gale, 1948).

McLeod and Gordon (1922) failed to detect the formation of hydrogen peroxide in *E. coli* from the indirect evidence of not forming a green zone in "chocolate" agar. They found that *E. coli* is a strong producer of catalase (McLeod and Gordon, 1923). Stephenson (1949) stated that the media containing cyanide but no methylene blue permit "anoxytrophic" growth of *E. coli* in the presence of oxygen. But *E. coli* failed to grow anoxytrophically in the presence of oxygen when methylene blue was added to the cyanide-containing media, because methylene blue (reduced to the leuco base through hydrogen acceptance) was subsequently oxidized by oxygen back to methylene blue with the formation of hydrogen peroxide. Catalase, normally present in *E. coli*, being inactivated by cyanide, was not available to decompose the peroxide and the growth was prevented.

Toxic effect of hydrogen peroxide on spermatozoa

The biological formation of hydrogen peroxide has often been postulated, particularly in connection with the presumed function of catalase and peroxidase. Its actual detection in cultures of bacteria has been successful (McLeod and Gordon, 1922, 1923a, 1923b, 1925; Avery and Neil, 1924; Sevag and Maiweg, 1934). It has been found

in molds (Pearce, 1940; Coulthard, et al. 1944) and can be produced by certain enzymic oxidations *in vitro*. The formation of hydrogen peroxide requires molecular oxygen as hydrogen acceptor. By using egg-yolk medium Tomic and Walton (1946) have demonstrated chemically the formation and elimination of hydrogen peroxide by spermatozoa and the toxic effect of hydrogen peroxide on sperm cells.

MacLeod (1946) reported that the motility of human spermatozoa failed in 95 per cent oxygen and that even in extremely low concentrations, oxygen has these toxic effects. He believed that the oxygen effect was due to the formation of hydrogen peroxide by the sperm cells. He found that the addition of sodium succinate to a suspension of human spermatozoa producing lactic acid from glucose in the presence of oxygen resulted in a considerable reduction (40 per cent) in lactic acid production and concomitant with the reduction in glycolysis there was a failure of sperm motility. The addition of very dilute hemoglobin, an analogue of catalase, to the sperm suspension in the presence of succinate prevents the loss of motility.

Mode of action of streptomycin on *E. coli*

Streptomycin is primarily a biochemical antagonist intervening in one or more metabolic reactions of the cell by disturbing the normal chain of events through which carbon, hydrogen, oxygen, nitrogen, phosphorus or sulfur containing residues are absorbed from the environment and incorporated into energy rich linkages from which the cell ultimately derives the energy necessary to sustain its life (Dufrenoy, 1949; Benedict and Langlykke, 1947). Tests with phenolphthaleine phosphate showed that alkaline phosphatase activity is blocked in cells exposed to streptomycin (Henry, et al. 1948). Some evidence also points to the conclusion that streptomycin intervenes in ribonucleic acid metabolism by blocking a reaction preceding the formation of mononucleotides. This interference permits the redox potential to rise to a level that is incompatible with proper functioning of dehydrogenase and other enzyme systems involving the transfer of hydrogen (Cohen, 1947; Berkman et al., 1948).

Umbreit (1947) has postulated that in *E. coli* the mode of action of streptomycin was to inhibit the terminal respiration process involving a pyruvate-oxalacetate condensation.

Material and Methods

Reagents

All reagents used were chemically pure. Whenever advisable, the solutions were sterilized by autoclaving at 121° C. under 15

pounds pressure for 20 minutes. The solutions were kept in screw-capped bottles in the refrigerator.

- ▶ Saline: 0.85 per cent sodium chloride.
- ▶ Sodium citrate: 4.76 per cent (by dissolving 4.76 grams of $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 5\frac{1}{2}\text{H}_2\text{O}$ and making up to 100 ml. with distilled water).
- ▶ Streptomycin: Dihydrostreptomycin sulfate (Merck) was dissolved in sodium citrate solution (to provide a concentration of 2 mg. per ml.) before mixing with egg-yolk.
- ▶ Catalase: Catalase (Armour) was suspended in sodium citrate solution to provide a concentration of 100 micrograms per ml. before mixing with egg-yolk.

Egg-yolk sodium citrate medium

This was prepared by mixing 1 part of sodium citrate solution with one part of egg-yolk from freshly laid hen's eggs.

Semen

The semen samples were collected from bulls, rams, and rabbits by means of an artificial vagina essentially according to the methods described by Lambert and McKenzie (1940). Sperm suspensions were prepared by washing the semen twice with saline through centrifugation and resuspension in the desired media.

Sperm count was made by the haemocytometer; and whenever desirable the concentration of the sperm suspensions were standardized to contain 1,000,000 spermatozoa per cmm of the suspension.

***E. coli* cultures**

Two cultures of *E. coli* were used for this study: one was isolated from dairy bull semen (DE1); the other was obtained from the American Type Culture Collection (sucrose positive, Levine). Nutrient broth cultures were grown at 37° C. for 24 hours. After centrifuging, the cells were washed twice with sterile saline before resuspending in the desired medium. The number of cells was estimated by photometric determination of optical density and checked by plate count.

Motility of the spermatozoa

The motility of the spermatozoa was estimated microscopically under high power of magnification (430 X). A drop of semen mixture was placed on a clean slide, a cover glass placed over it and the preparation held at 37.8° C. on a thermostat. Motility of the spermatozoa was graded from 10 to zero, where 10 denotes the best motility. The grades of motility were essentially as shown in Table 1.

The pH

The pH of the semen-*E. coli* suspensions was measured by means of pH meter (Beckman Model H) after 10 hours of incubation. The semen control or the semen-*E. coli* mixtures were suspended in egg-yolk sodium citrate medium; 3.5 ml. of the suspension were placed in screw-capped culture tubes (capacity 30 ml.); the air space above the suspension was therefore 26.5 ml. in each tube. In some experiments a filter paper, moistened with NaOH, was placed in a center tube to absorb carbon dioxide.

Oxygen uptake

The oxygen uptake was measured manometrically, using the Aminco-Lardy Rotatory Type manometrical apparatus according to the methods described by Umbreit et al. (1949). Usually 2 ml. of egg-yolk sodium citrate medium were placed in each flask together with 0.5 ml. of semen (or spermatozoa suspension) or 0.5 ml. of *E. coli* suspension or both. The semen or spermatozoa suspensions were usually standardized to contain 1,000,000 spermatozoa per cmm or a total of 500,000,000 spermatozoa per flask. In the flask which contained only spermatozoa or only *E. coli* suspensions, 0.5 ml. of distilled water was placed in the side arm of the flask in order to have the same V_r in the flasks. Air was the gas phase. The water bath was held at 34° C. The carbon dioxide produced was absorbed by a roll of filter paper (Whitman No. 40) placed in the center cup which contained 0.2 ml. of a 40 per cent KOH solution. The manometers were shaken by oscillation at 100 excursions per minute. After 15 minutes equilibration the zero point was adjusted and the system closed by turning the taps. Experiments were run in duplicate and readings were taken at 10-minute intervals for a period of 2½ hours.

Experimental Findings

Both *E. coli* cultures used in these studies showed consistent effects in reducing sperm motility when the organisms were incubated with sperm at 38° C. This inhibitory effect can be observed in most cases after incubation for 4 hours. The decrease in sperm motility caused by *E. coli* has been observed with ram semen (Figures 1 and 8), dairy bull semen (Figures 2, 4, 5, 6, and 7) and rabbit semen (Figure 9). The motility depressing effect of *E. coli* on spermatozoa was influenced to a great extent by the temperature of incubation. As shown in Figure 1, at 5° C. the motility of ram spermatozoa did not show any appreciable depression over the control until after 48 hours of incubation with the microorganism. The depres-

sion in motility was slight and not until 96 hours did the depression in sperm motility occur in all samples incubated with *E. coli* at 5° C. At 38° C., however, the motility of the spermatozoa incubated with *E. coli* rapidly decreased and no motile sperm could be observed at the end of 6 hours.

The pH of semen-*E. coli* suspension

In considering the mechanism involved in the motility depression of spermatozoa by *E. coli*, the question arises whether there was a shift toward a pH unfavorable to spermatozoan motility in the spermatozoa-*E. coli* suspensions. As shown in Figure 16, with one exception, all samples of semen from dairy bulls, rams, and rabbits had pH values for the semen in egg-yolk sodium citrate medium 0.2 to 0.3 unit higher when incubated with *E. coli* than in the semen mixture without the addition of this microorganism.

Carbon dioxide

Since a depressing effect of carbon dioxide on spermatozoan motility has been discussed by some investigators (Roemmele, 1927; Shettles, 1939), an increase in carbon dioxide tension in semen incubated with *E. coli* conceivably could have been one of the factors contributing to the motility-depressing effect. The data obtained in this study indicated, however, that the decrease in motility was the same in semen samples incubated with *E. coli* regardless of whether or not carbon dioxide had been absorbed from the air space above the spermatozoa-*E. coli* mixture (Figures 7, 8, and 9).

Oxygen uptake

For the first hour, the oxygen consumption of the final suspensions of ram semen in egg-yolk sodium citrate medium (500,000,000 spermatozoa) ranged from 30 to 68 microliters per hour. The oxygen consumption of *E. coli* suspensions ranged from 170 to 225 microliters per hour per 500,000,000 cells. The initial number of *E. coli* in the egg-yolk sodium citrate medium ranged from 500,000,000 to 700,000,000.

The oxygen consumed by spermatozoa and *E. coli* was increased when they were placed together, consumption by the mixture being greater than the sum of the oxygen consumption by the spermatozoa and the *E. coli* alone. This increase in oxygen consumption in the semen-*E. coli* suspension (or sperm-*E. coli* suspension) was very consistent for semen from dairy bulls (Figure 10) and for semen or spermatozoa from rams (Figures 11, 12, 13, 14, and 15). Beef bull and rabbit spermatozoa were not studied. All oxygen uptakes were measured in duplicate at 34° C.

The augmentation of oxygen consumption when ram spermatozoa and *E. coli* were placed together occurred even if streptomycin (1 mg. per 3 ml. of the suspension) was added to the spermatozoa-*E. coli* suspension (Figure 15).

Washing of spermatozoa or of *E. coli* cells twice with physiological saline did not prevent the increase in oxygen consumption of the combination (Figures 11 and 12) regardless of whether the suspension medium was sodium citrate or egg-yolk sodium citrate. The increased oxygen uptake developed in fresh semen and spermatozoa or stored (5° C.) semen and spermatozoa. Heat treatment of *E. coli* (by holding the bacteria suspension at 65° C. in a water bath for 30 minutes), however, abolished the increase in oxygen uptake. This indicated that heat-killed *E. coli* had no effect on the oxygen consumption of spermatozoa but the heat-killed sperm still had a stimulating effect on the oxygen consumption of *E. coli* (Figure 13). It was also found that neither the heat-killed spermatozoa nor the heat-killed *E. coli* had a measurable oxygen consumption.

Reversal by streptomycin, penicillin, glucose, and catalase of the motility depressing effect of *E. coli* on spermatozoa

The motility of spermatozoa was found to be depressed by *E. coli*. However, in the presence of streptomycin, or streptomycin together with penicillin, the addition of *E. coli* to the semen led to an increase in sperm motility. As shown in Figure 4, in dairy bull semen, the sperm motility was much better in semen where streptomycin, penicillin and *E. coli* were added simultaneously than in the control semen or in semen with the addition of the antibiotics alone. In the absence of the antibiotics the addition of *E. coli* to semen resulted in complete immobilization of almost all the sperm cells at the end of six hours incubation at 38° C., while in the presence of the antibiotics the sperm motility in semen where *E. coli* had been added was even better than in the control semen without the addition of this microorganism. As shown in Figure 4, when dairy bull semen was incubated with antibiotics at 38° C., those with *E. coli* added had an average motility rating of 7.4 after 10 hours of incubation while at the same time the average motility rating of the control semen, without antibiotics and without *E. coli*, dropped to 2.9.

Glucose seemed to have an effect similar to streptomycin in reversing the motility-depressing effect of *E. coli* on spermatozoa. As shown in Figure 5, semen with the addition of both *E. coli* and glucose at the level of 1 mg. of glucose per 3 ml. of diluted bull semen, maintained a better sperm motility for a longer period of time

than did the control semen or semen with glucose or with *E. coli* added separately.

Catalase added to semen at the level of 100 micrograms per 3 ml. of diluted bull semen (1 part of semen in 100 parts of egg-yolk sodium citrate medium) also altered the effect of *E. coli* on sperm motility. A very high motility was shown by spermatozoa when catalase was added. Subsequent addition of *E. coli* to the combination of catalase plus sperm slightly lowered the motility, but the motility was still much higher than that of the untreated sperm cells. As shown in Figure 6, the motility of spermatozoa was highest in semen with catalase added, although the motility in semen with addition of *E. coli* in the presence of catalase was better than that of control semen or in semen where only *E. coli* had been added. Thus, catalase at the level used merely reduced the inhibitory effect of *E. coli* on sperm motility but did not completely abolish the detrimental effect of this microorganism. The sequences of combinations (Table 2) yielding decreasing motilities indicate such explanation.

Discussion

Many factors may be involved in the depression of spermatozoan motility by *E. coli*. Thus, the nutritive substances available to the spermatozoa may be exhausted more rapidly in the presence of *E. coli*. The metabolites of this microorganism may be harmful to spermatozoa. The physical or chemical characteristics of the medium may be altered by *E. coli* and become unfavorable to the survival of the spermatozoa. Data of this study indicated, however, that an accumulation of hydrogen peroxide in semen-*E. coli* suspension was the main factor involved.

The pH change in the semen-*E. coli* suspension

Moore, Mayer and McKenzie (1940) reported the importance of pH on motility and metabolism of ram semen. Nordby and Bollen (1930) found that a difference of pH in the order of 0.3 unit in direct semen samples was associated with infertility in a boar. Moore and Mayer (1941) concluded that the factor which influenced the motility and glycolysis in ram semen was the degree of acidity of the medium surrounding the sperm cells. They found, however, that no detectable changes occur in motility until the pH reaches a value of 6 or below and the motility ceases at pH values of 5.5 to 5.3.

From the results of this study, it has been found that when semen was incubated with *E. coli* for 24 hours at 5° C. and then 10 hours at 38° C., at the end of this incubation the pH values ranged

from 6.7-6.8 in bull semen, 6.6-6.91 in ram semen, and 6.8 in rabbit semen. The pH of semen in egg-yolk sodium citrate medium without *E. coli* ranged from 6.45 to 6.5 in bull semen, 6.55 to 6.85 in ram semen, and 6.7 in rabbit semen. According to the data of Moore and Mayer (1941), the motility of spermatozoa in the present study should not have been affected by these relatively small differences in acidity of the surrounding medium having pH values within the ranges observed. The motility-depressing effect of *E. coli* on spermatozoa is therefore apparently not due to the pH change of the medium brought about by the presence of these bacteria. Yet, the observation of Nordby and Bollen (1930) indicates that further studies are necessary to adequately interpret the pH data and that there may be species differences in the sensitivity to pH.

Carbon dioxide tension in semen-*E. coli* suspensions

Roemmele (1927) observed that spermatozoan motility was lower in media covered up than when exposed to air, and believed that this was due to carbon dioxide retention rather than lack of oxygen. Shettles (1939), in studying the metabolism of human spermatozoa, found that carbon dioxide produced complete immobilization of human spermatozoa within a few minutes. He suggested that the toxic effect of carbon dioxide for human spermatozoa was dependent neither upon its acidic character nor upon anoxia, but upon other properties as yet unknown. Becker (1936) believed that the toxic effect of carbon dioxide on protoplasm represented a specific lethal action coupled with an ability to penetrate cell boundaries with extreme rapidity.

The data of this study show that carbon dioxide was probably not the factor contributing to the inhibitory effect of *E. coli* on sperm motility. As shown in Figures 7, 8, and 9, the spermatozoan motility was invariably inhibited when semen was incubated with *E. coli* at 38° C., regardless of whether or not alkali has been used to absorb the carbon dioxide in the air space of the tubes. It is possible that in this study the carbon dioxide tension created by the *E. coli* and the spermatozoa was not sufficiently high to affect the motility. It has been pointed out by Anderson (1944) that carbon dioxide concentration of approximately 20 per cent in an atmosphere of oxygen was required to immobilize sperm in a hanging drop. It would be desirable to amplify the data by direct gas analysis of the *E. coli*-spermatozoa mixture and of the air space above the mixture to establish the role or lack of role of carbon dioxide in depressing spermatozoan motility.

Oxygen uptake

The oxygen consumed by spermatozoa and *E. coli* was increased when they were placed together. This increase in oxygen consumption may have caused a more rapid exhaustion of the cellular reserves of the spermatozoa and consequently a more rapid drop in motility of spermatozoa in semen-*E. coli* suspensions than of spermatozoa alone. The increase in oxygen uptake may involve both the spermatozoa and the *E. coli* cells. One of the factors contributing to the increase may be the provision of certain enzymes by the spermatozoa for the *E. coli* cells or vice versa, so that the respiratory enzyme systems become reciprocally supplemental when the spermatozoa and *E. coli* are incubated in the same medium. It is known that *E. coli* has cytochromes a_1 , a_2 and b_1 (Keilin and Harkley, 1941), while the sperm cells have a complete cytochrome system (MacLeod, 1943; Mann, 1945).

Toxic effect of hydrogen peroxide on spermatozoan motility

The similarity in action of streptomycin, glucose, and catalase in reversing the depressing effect of *E. coli* on sperm motility suggested that the mechanism was largely an accumulation of hydrogen peroxide in the semen-*E. coli* suspensions. The accumulated hydrogen peroxide would interfere with the glycolytic activity of the sperm cells and thus depress the motility.

Hydrogen peroxide production has been demonstrated in bovine spermatozoa. It has been suggested that the reaction is an enzymic dehydrogenation and deamination of certain amino acids in the presence of molecular oxygen as hydrogen acceptor (Tosic and Walton, 1946; Tosic, 1947). MacLeod (1946) believed that hydrogen peroxide also was formed in human spermatozoa as an intermediate product from the oxidation of succinic acid to fumaric acid and water by succinic dehydrogenase. As *E. coli* is known to break down glycine, 1-alanine, and 1-glutamic acid by oxidative deamination, it was likely that hydrogen peroxide may have been produced in a larger amount with semen-*E. coli* suspended in egg-yolk medium than with semen in egg-yolk medium without the addition of *E. coli*. Further, as *E. coli* possesses the enzyme succinic dehydrogenase, hydrogen peroxide also could be produced through the oxidation of succinic acid to fumaric acid in the manner postulated by MacLeod (1946) in human spermatozoa.

Based on the assumption that hydrogen peroxide is the main factor responsible for the depression of spermatozoan motility by *E. coli*, the reversal by streptomycin could be explained as a removal

of hydrogen peroxide due to the high concentration of pyruvate developing when streptomycin interferes with conversion of pyruvate to succinate. As it has been pointed out (Umbreit, 1947), the mode of action of streptomycin with *E. coli* was to inhibit the terminal respiration process involving a pyruvate oxalacetate condensation. Thus, in the presence of streptomycin, a larger amount of pyruvate could accumulate. Pyruvate and hydrogen peroxide react together chemically in such a way as to destroy hydrogen peroxide. It has been suggested (Gale, 1940) that pneumococci can be protected in the presence of air by pyruvate. On the other hand, in the absence of streptomycin, pyruvate will be converted by *E. coli* to succinate with the subsequent formation of hydrogen peroxide as an intermediate product proceeding from the formation of fumarate. In the presence of streptomycin, more pyruvate would be available to the spermatozoa for destroying the hydrogen peroxide produced by semen-*E. coli* combinations. Consequently, the sperm motility would be still better with semen-*E. coli*-streptomycin than in semen suspension alone. In the absence of streptomycin, the hydrogen peroxide would accumulate in a larger amount in semen-*E. coli* suspensions than in the suspension containing sperm alone. The smaller amount of hydrogen peroxide formed in the untreated semen suspension may be more easily decomposed by the sperm catalase and consequently the sperm motility would be much better than that when *E. coli* has been added.

In considering the reversal of the sperm motility depression by *E. coli* with glucose, it is suggested that glucose interfered with the deamination process of amino acids by *E. coli* and thus led to a decrease in hydrogen peroxide formation. Further, the pyruvate accumulated during the breakdown of glucose may also have served in eliminating the inhibitory effect of *E. coli* on sperm motility. It has been pointed out (Stephenson and Gale, 1937) that the principal effect of glucose on the oxidative deamination of glycine, d-1-alanine and l-glutamic acid by *E. coli* was to inhibit the formation of the enzymes during growth. Glucose in a concentration of 2 per cent eliminated the formation of 95 per cent of the three amino acid oxidases in *E. coli* strain 1.

There are a few objections to the explanation just given. For instance, McLeod and Gordon (1922) failed to detect the formation of hydrogen peroxide by *E. coli* growth on "chocolate" agar. They also found that *E. coli* is a strong catalase producer (McLeod and Gordon, 1923). Pearce (1940) found that hydrogen peroxide is formed in *Aspergillus niger* as a result of a primary oxidation reaction of glucose catalyzed by glucose oxidase.

However, these observations are not necessarily contradictory. First of all, the failure to demonstrate the formation of hydrogen peroxide in one medium will not exclude the possibilities that the hydrogen peroxide has been formed in that medium. As mentioned by Oppenheimer and Stern (1939), in the case of cells containing catalase but possessing a cyanide resistant respiration system, e.g., *Chlorella*, the attempt to detect hydrogen peroxide (even after the inhibition of catalase by cyanide) has failed to produce positive results. Secondly, even though *E. coli* is a strong producer of catalase, it does not necessarily mean that the catalase produced by *E. coli* will be available and sufficient to reduce the hydrogen peroxide accumulated in semen-*E. coli* suspension enough to eliminate the inhibitory effect of hydrogen peroxide on sperm motility. Further, the fact that hydrogen peroxide may be formed as a result of a primary oxidation of glucose catalyzed by glucose oxidase does not necessarily mean that the addition of glucose in semen-*E. coli* suspension will result in an increase in hydrogen peroxide formation and cause a depression of sperm motility. On the contrary, Salisbury (1946) has shown that in bovine semen there was a beneficial effect on sperm motility with each addition of glucose at levels of 0, 116, 232, and 464 mg. per 100 ml. of diluted semen. This indicated that hydrogen peroxide was not necessarily produced in glucose utilization.

Hydrogen peroxide accumulation as the cause of the motility-depressing effect of *E. coli* on spermatozoa was strongly supported by the fact that catalase reversed the *E. coli* effect on sperm motility. While other interpretations may eventually be developed, all of the evidence points to hydrogen peroxide as a key substance in the system. It suggests that the depressing effect of *E. coli* on sperm motility was chiefly due to hydrogen peroxide accumulation in semen-*E. coli* suspension in an amount sufficiently high to interfere with glycolysis and to inhibit the motility of spermatozoa.

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Tables and Charts

Table 1. MOTILITY RATINGS OF SPERMATOZOA

Motility rating	Motility observed under high magnification (430 X)
10	Most spermatozoa are very active, motile and progressive
9	Most spermatozoa are active, motile and progressive
8	Many spermatozoa are active, motile and progressive
7	Many spermatozoa are motile and slowly progressive
6	Some spermatozoa are motile but only slowly progressive
5	Some spermatozoa are motile but only weakly progressive
4	Many spermatozoa are motile but have only oscillatory motion
3	Some spermatozoa with oscillatory motion
2	Only a few spermatozoa with very weak oscillatory motion
1	Only 1 or 2 spermatozoa with very weak oscillatory motion
0	No motile spermatozoa can be found

Table 2. REVERSAL OF THE MOTILITY DEPRESSING EFFECT OF *Escherichia coli* ON SPERMATOZOA BY STREPTOMYCIN, GLUCOSE, OR CATALASE

Reagents	Suspensions	Motility
STREPTOMYCIN	a. Semen- <i>E. coli</i> -Streptomycin	Most active
	b. Semen	↓
	c. Semen-Streptomycin	Least active
	d. Semen- <i>E. coli</i>	
GLUCOSE	a. Semen-Glucose- <i>E. coli</i>	Most active
	b. Semen	↓
	c. Semen-Glucose	Least active
	d. Semen- <i>E. coli</i>	
CATALASE	a. Semen-Catalase	Most active
	b. Semen- <i>E. coli</i> -Catalase	↓
	c. Semen	Least active
	d. Semen- <i>E. coli</i>	

EFFECT of *E. coli* ON RAM SPERM MOTILITY

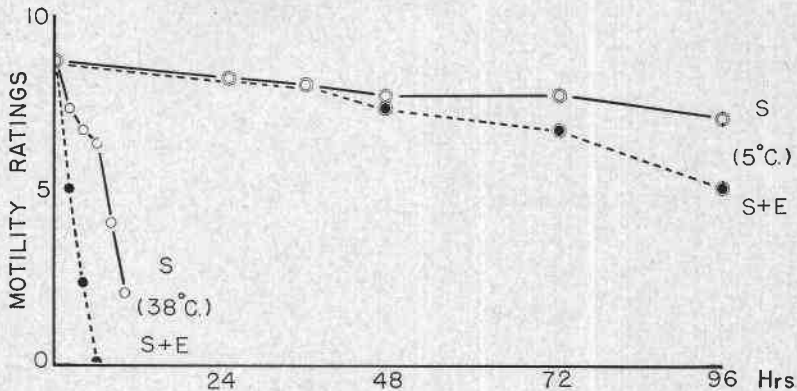


Figure 1. The solid lines show the changes in spermatozoan motility in semen alone (S); the dotted lines show the motility changes in semen-*E. coli* mixtures (S + E). At 5° C. (refrigerator temperature) the depressing effect of *E. coli* on spermatozoan motility was not noticeable until after 48 hours of incubation, whereas at 38° C. (approximately vaginal temperature) the spermatozoan motility dropped much more rapidly in the semen-*E. coli* mixture than in the semen alone.

EFFECT OF *E. coli* ON DAIRY BULL SPERM MOTILITY

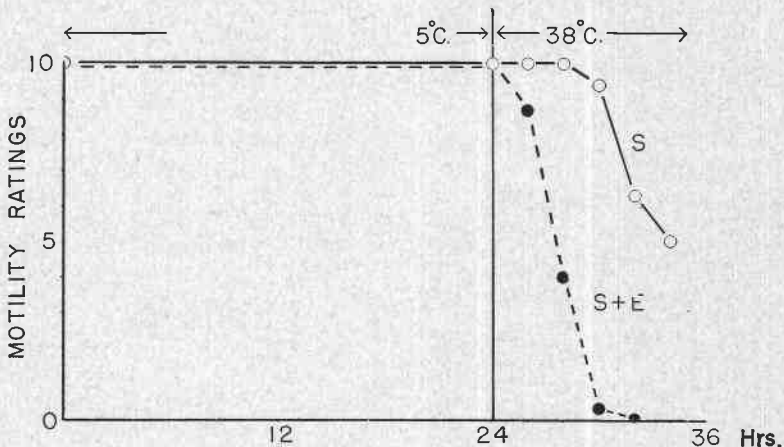


Figure 2. The solid line shows the changes in spermatozoan motility in semen alone (S); the dotted line shows the motility changes in semen-*E. coli* mixture (S + E). No noticeable decrease in spermatozoan motility was observed when semen or semen-*E. coli* mixtures were kept at 5° C. for 24 hours, but when changed to 38° C., the spermatozoan motility decreased, and the decrease was much more rapid in the semen-*E. coli* mixture.

EFFECT OF *E. coli* ON BEEF BULL SPERM MOTILITY
(Streptomycin Reversal)

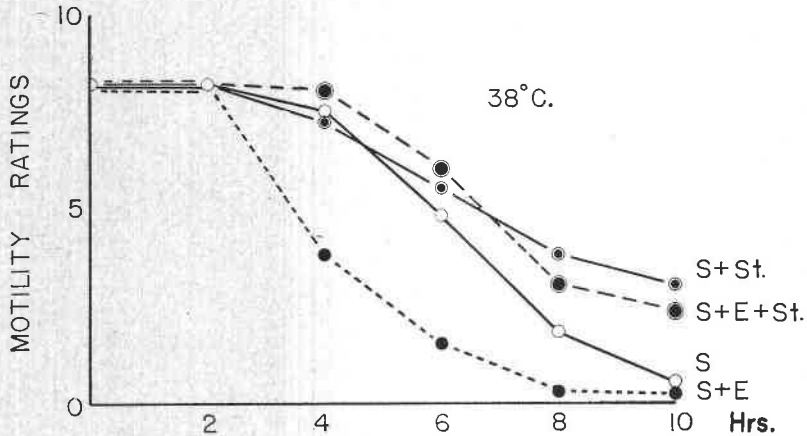


Figure 3. The solid lines show the changes in spermatozoan motility in beef bull semen plus streptomycin (S + St.) or the semen alone (S); the dotted lines indicate the motility changes in semen-*E. coli* mixtures plus streptomycin (S + E + St.) or semen-*E. coli* mixture alone (S + E). *E. coli* depressed the spermatozoan motility whereas streptomycin reversed the motility depressing effect of *E. coli*.

EFFECT OF *E. coli* ON DAIRY BULL SPERM MOTILITY
(Streptomycin and Penicillin Reversal)

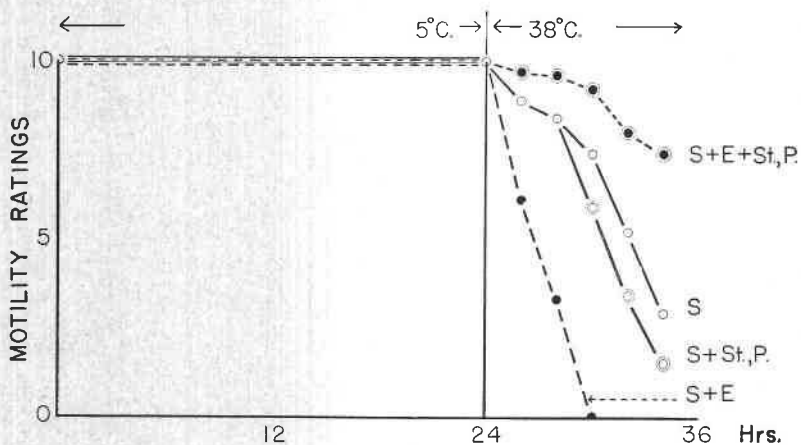


Figure 4. The solid lines show changes in spermatozoan motility in semen plus streptomycin and penicillin or semen alone; dotted lines indicate changes in semen-*E. coli* mixtures plus streptomycin and penicillin or in semen-*E. coli* mixture alone. Sperm motility was not altered in the mixtures at 5° C. At 38° C. sperm motility decreased and the decrease was more rapid in semen-*E. coli* mixture than in semen alone. Simultaneous addition of streptomycin and penicillin reversed the motility depressing effect of *E. coli*.

EFFECT OF *E. coli* ON DAIRY BULL SPERM MOTILITY
(Glucose Reversal)

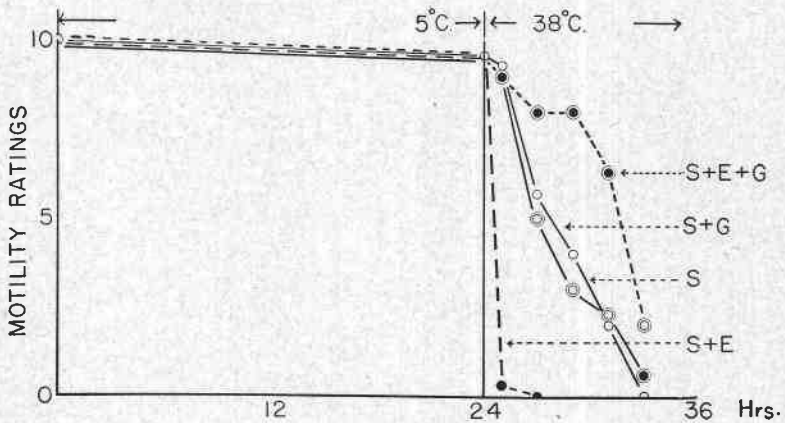


Figure 5. The solid lines show the changes in spermatozoan motility in semen plus glucose (S + G) or in semen alone (S); the dotted lines indicate the motility changes in semen-*E. coli* mixtures plus glucose (S + E + G) or in semen-*E. coli* mixture alone (S + E). At 5° C. for 24 hours, only a very slight decrease in spermatozoan motility was observed in all four different treatments, but when the temperature was increased to 38° C., the motility of spermatozoa decreased and the decrease was much more rapid in the semen-*E. coli* mixture than in any of the other three treatments. The depressing effect of *E. coli* on spermatozoan motility was reversed by glucose.

EFFECT OF *E. coli* ON DAIRY BULL SPERM MOTILITY
(Catalase Reversal)

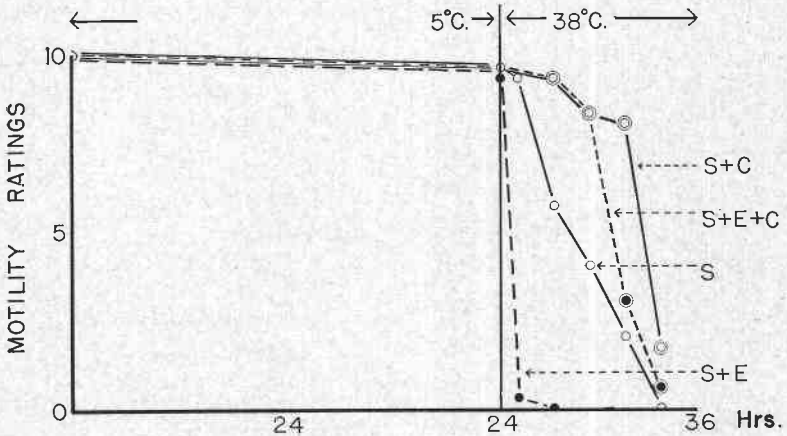


Figure 6. The solid lines show the changes in spermatozoan motility in semen plus catalase (S + C) or in semen alone (S); the dotted lines indicate the motility changes in semen-*E. coli* mixtures plus catalase (S + E + C) or in semen-*E. coli* mixtures alone (S + E). The motility depressing effect of *E. coli* was reversed by catalase.

EFFECT OF *E. coli* ON DAIRY BULL SPERM MOTILITY

(Role of Carbon Dioxide)

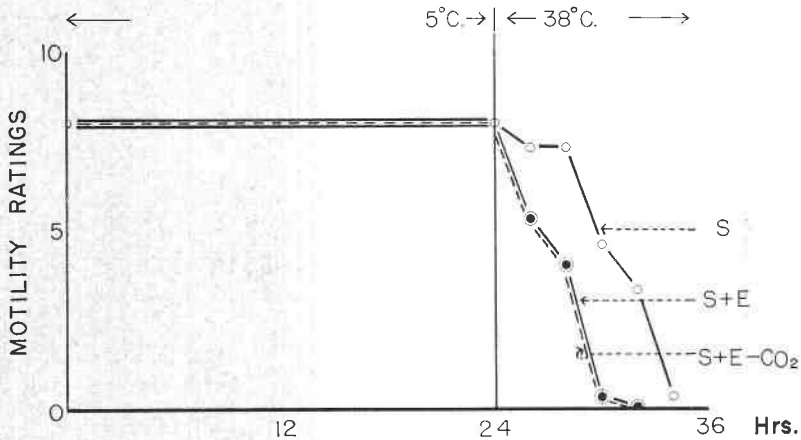


Figure 7. The solid lines show the changes in spermatozoan motility in dairy bull semen (S) and in semen-*E. coli* mixtures (S + E); the dotted line indicates the motility changes when carbon dioxide had been removed (S + E - CO₂) from the semen-*E. coli* mixtures. The removal of carbon dioxide did not alter the motility depressing effect of *E. coli* on dairy bull spermatozoa.

EFFECT OF *E. coli* ON RAM SPERM MOTILITY

(Role of Carbon Dioxide)

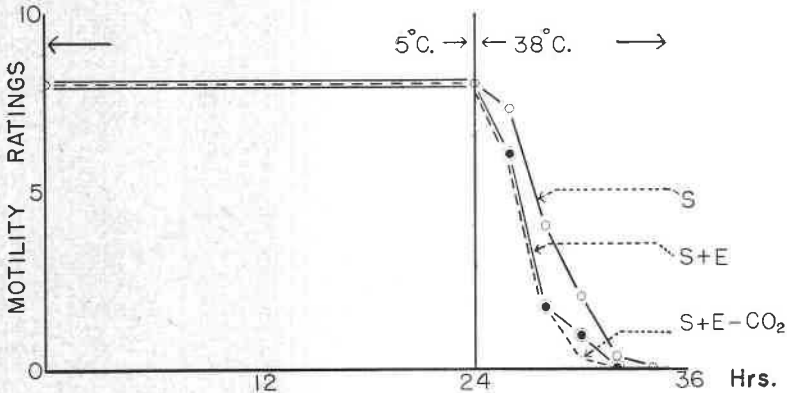


Figure 8. The solid lines show the changes in spermatozoan motility in ram semen (S) and in the semen-*E. coli* mixtures (S + E); the dotted line indicates the motility changes in the semen-*E. coli* mixtures when carbon dioxide had been removed (S + E - CO₂). The removal of carbon dioxide did not alter the motility depressing effect of *E. coli* on ram spermatozoa.

EFFECT OF *E. coli* ON RABBIT SPERM MOTILITY (Role of Carbon Dioxide)

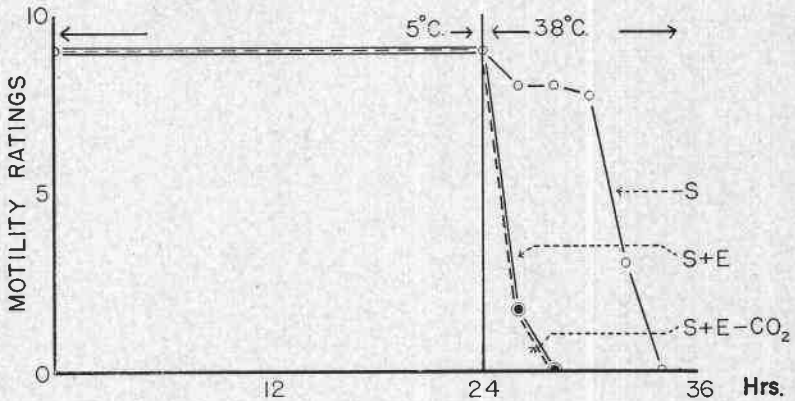


Figure 9. The solid lines show the changes in spermatozoan motility in rabbit semen (S) and in the semen-*E. coli* mixture (S + E); the dotted line indicates the motility changes in the semen-*E. coli* mixture when carbon dioxide had been removed (S + E - CO₂). The removal of carbon dioxide did not alter the motility depressing effect of *E. coli* on rabbit spermatozoa.

OXYGEN UPTAKE IN DAIRY BULL SEMEN-*E. coli* SUSPENSION

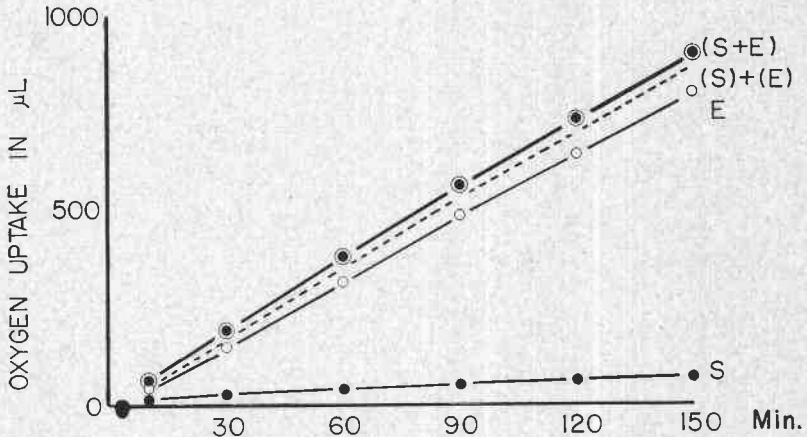


Figure 10. The oxygen uptake of dairy bull semen (S) was less than the oxygen uptake of *E. coli* (S). The oxygen consumption obtained experimentally with the semen-*E. coli* mixture (S + E) was greater than that predicted from the simple sum (S) + (E) of the oxygen uptakes obtained with *E. coli* alone and semen alone.

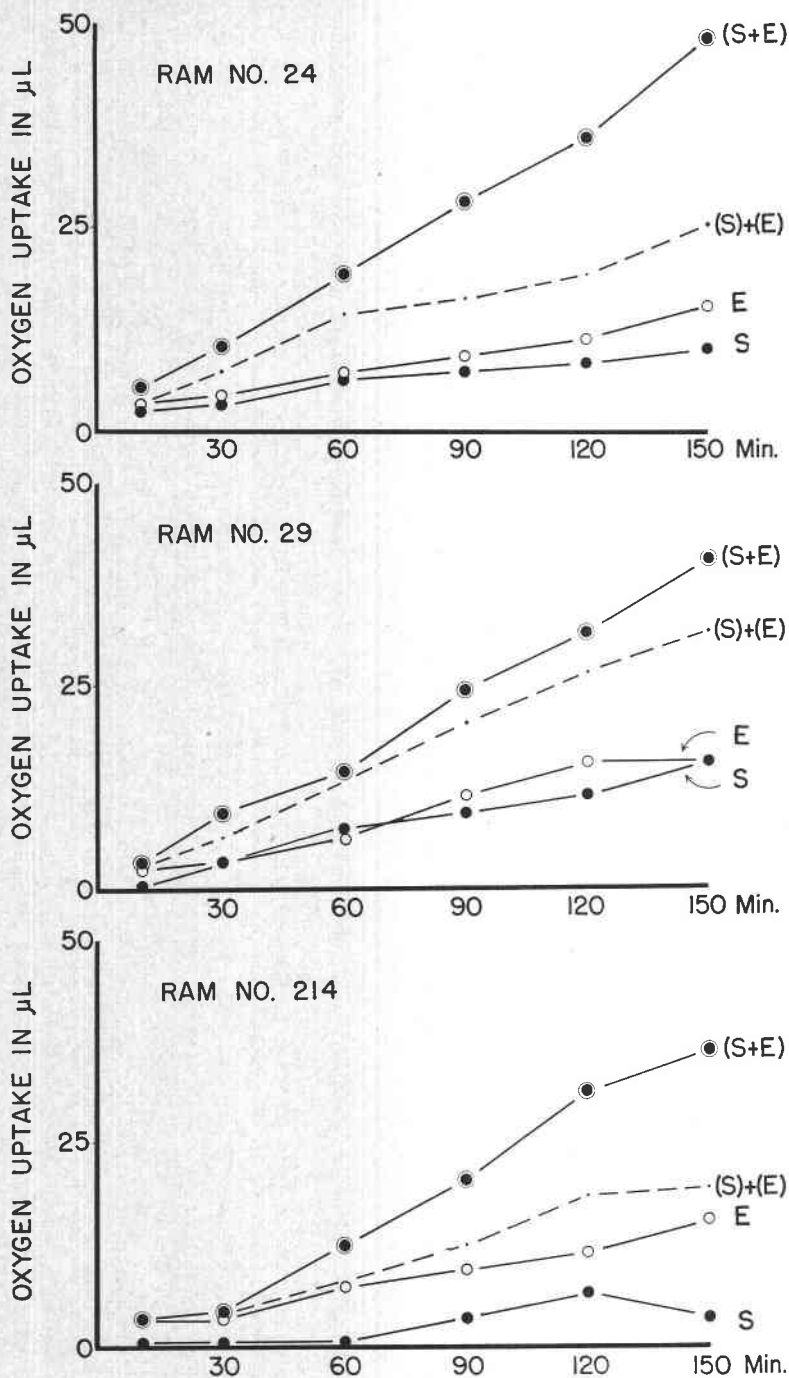


Figure 11. The oxygen uptake of spermatozoa, washed twice with physiological saline and immediately suspended in sodium citrate, varied considerably from ram to ram. The variations in oxygen uptakes of spermatozoa from different ejaculates of the same ram were not studied. The oxygen uptake in the sperm-*E. coli* mixture (S + E) was greater than the oxygen uptake predicted from the simple sum (S) + (E) of the oxygen uptakes by semen (S) and *E. coli* (E) alone. A comparison with Figure 10 indicates that the washing of spermatozoa twice with physiological saline did not eliminate the increase in oxygen uptake in (S + E) over that of (S) + (E). The oxygen uptakes of spermatozoa and *E. coli* were much lower in sodium citrate than in egg-yolk sodium citrate. Note change in ordinates between Figure 11 and Figure 12.

OXYGEN UPTAKE OF RAM SPERM-*E. coli* SUSPENSION, IN EGG-YOLK SODIUM CITRATE
(AFTER STORAGE AT 5° C. FOR 24 HOURS)

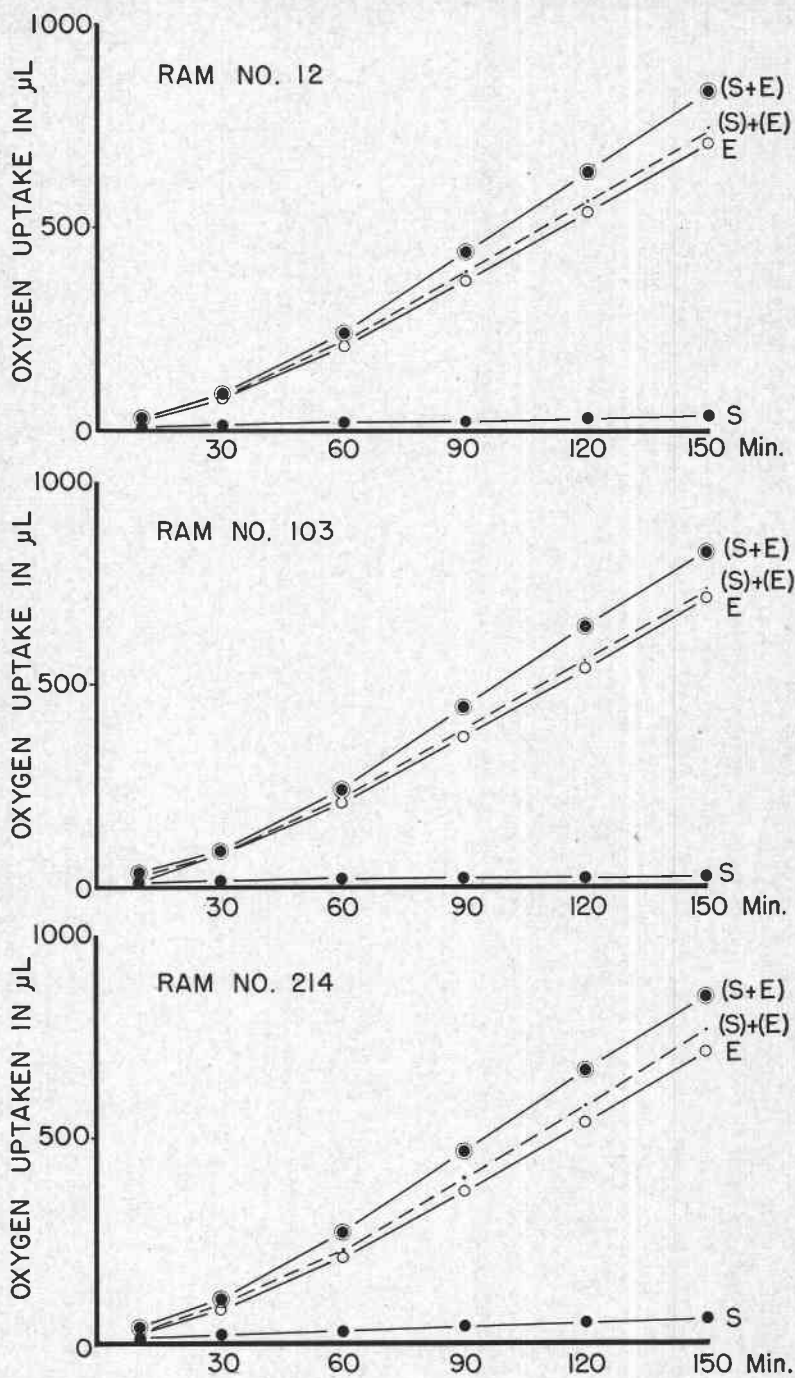


Figure 12. When the oxygen uptake of ram sperm alone (S) and the oxygen uptake of *E. coli* alone (E) were added together the sum of the two (S) + (E) was less than the oxygen uptake of the sperm-*E. coli* mixture (S + E). Figure 11 was concerned with sperm washed and immediately suspended in sodium citrate. Figure 12 differs in that the semen was stored at 5° C. for 24 hours before washing and suspending in egg-yolk sodium citrate.

OXYGEN UPTAKE ON RAM SEMEN-*E. coli* SUSPENSION, EFFECT OF HEAT TREATMENT
(65° C., 30 MINUTES)

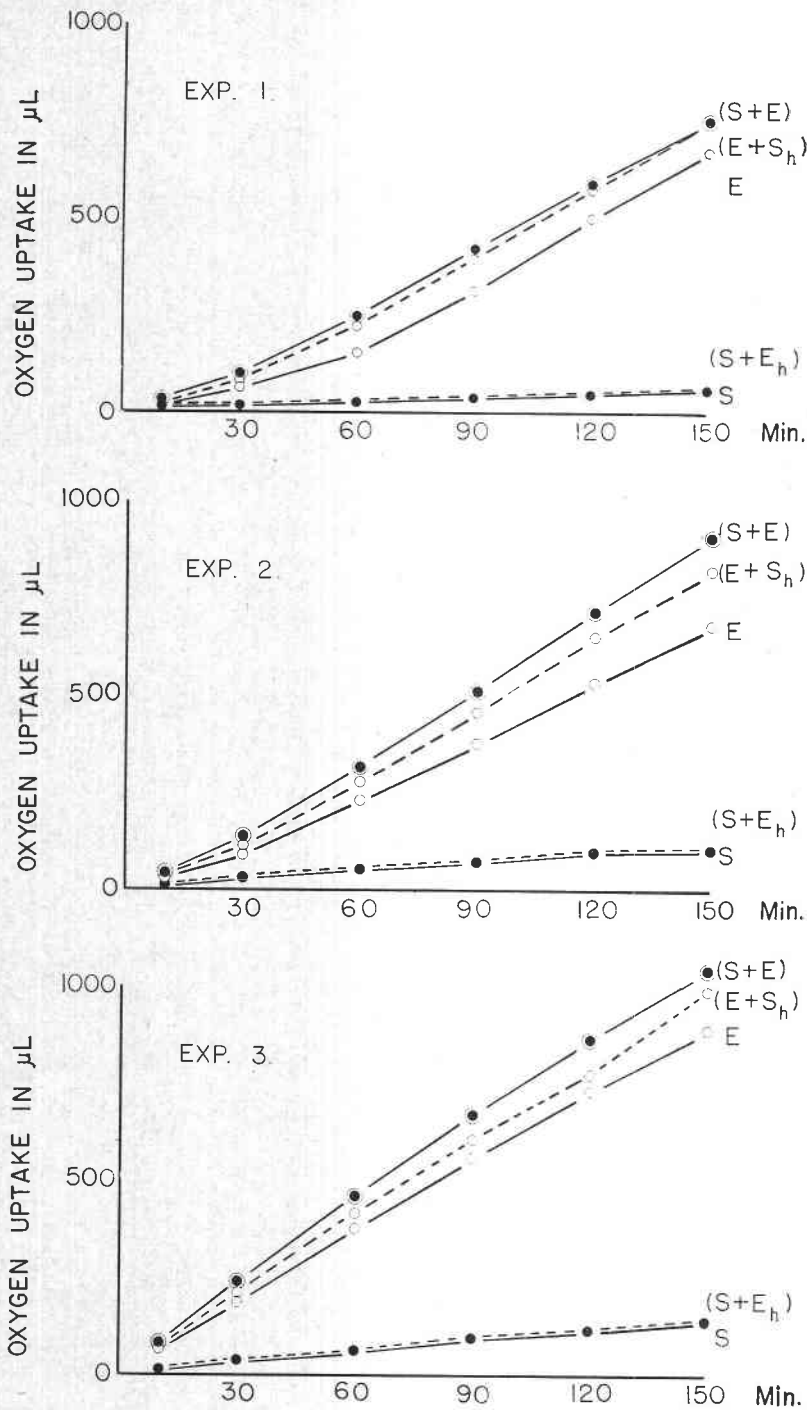


Figure 13. Heat treatment eliminated the oxygen uptake of semen or of *E. coli* suspensions. If plotted, the oxygen uptake of heat treated semen (S_h) and that of the heat treated *E. coli* (E_h) would coincide with the zero abscissa. Heat treatment of semen did not eliminate the increase in oxygen uptake in semen-*E. coli* mixtures ($E + S_h$) over the sum (E) + (S_h) of oxygen uptakes of heat treated semen and the oxygen uptake of *E. coli*. On the other hand, heat treatment of *E. coli* did eliminate this increase as indicated by the superimposition of the lines representing the oxygen uptake of semen alone (S) and that for the mixture of semen and heat treated *E. coli* ($S + E_h$).

OXYGEN UPTAKE OF RAM SEMEN-*E. coli* SUSPENSION (IN THE ABSENCE OF STREPTOMYCIN)

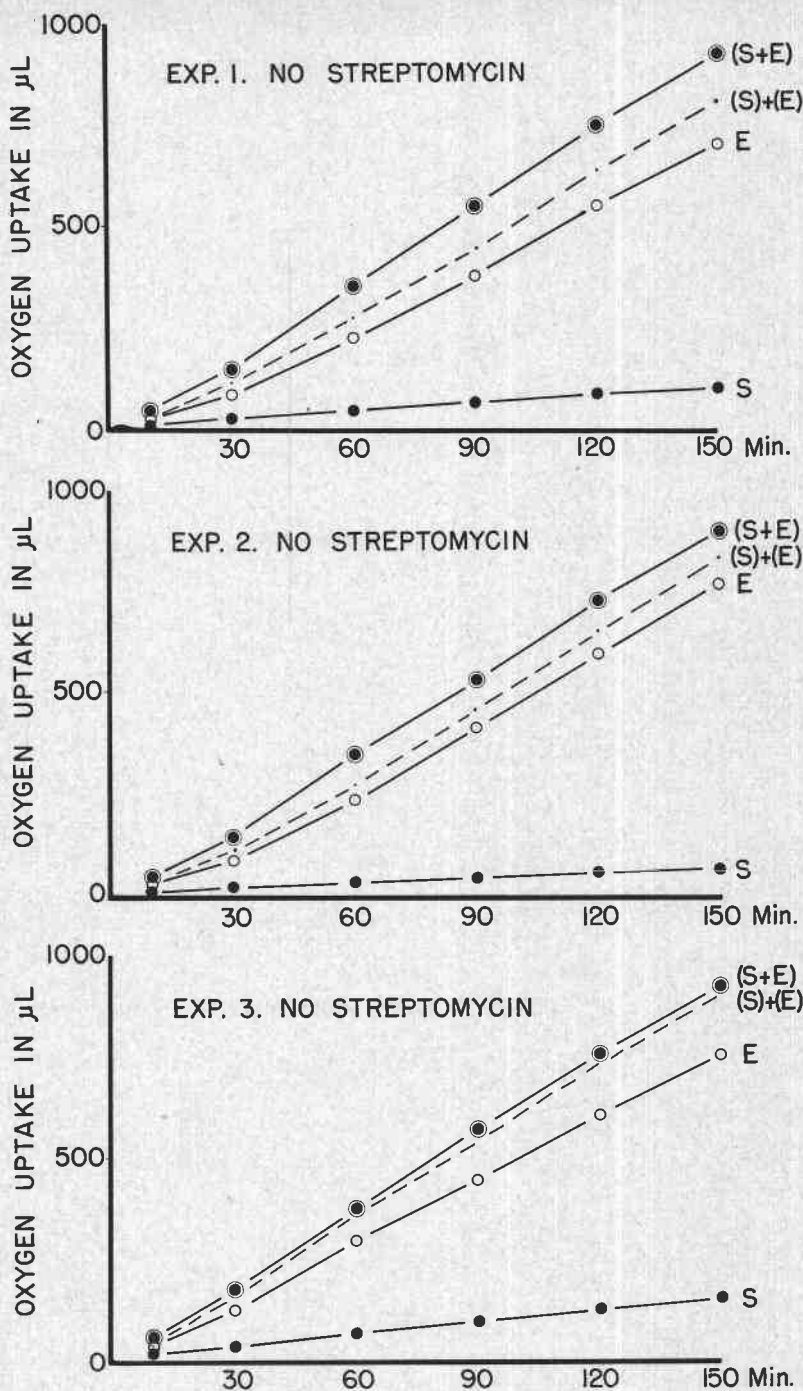


Figure 14. The symbols used are the same as in Figures 10 and 12. The charts compare the augmentation of oxygen uptake in semen-*E. coli* mixtures without the addition of streptomycin with the same preparations when streptomycin was added. (See Figure 15.)

OXYGEN UPTAKE OF RAM SEMEN-*E. coli* SUSPENSION (IN THE PRESENCE OF STREPTOMYCIN)

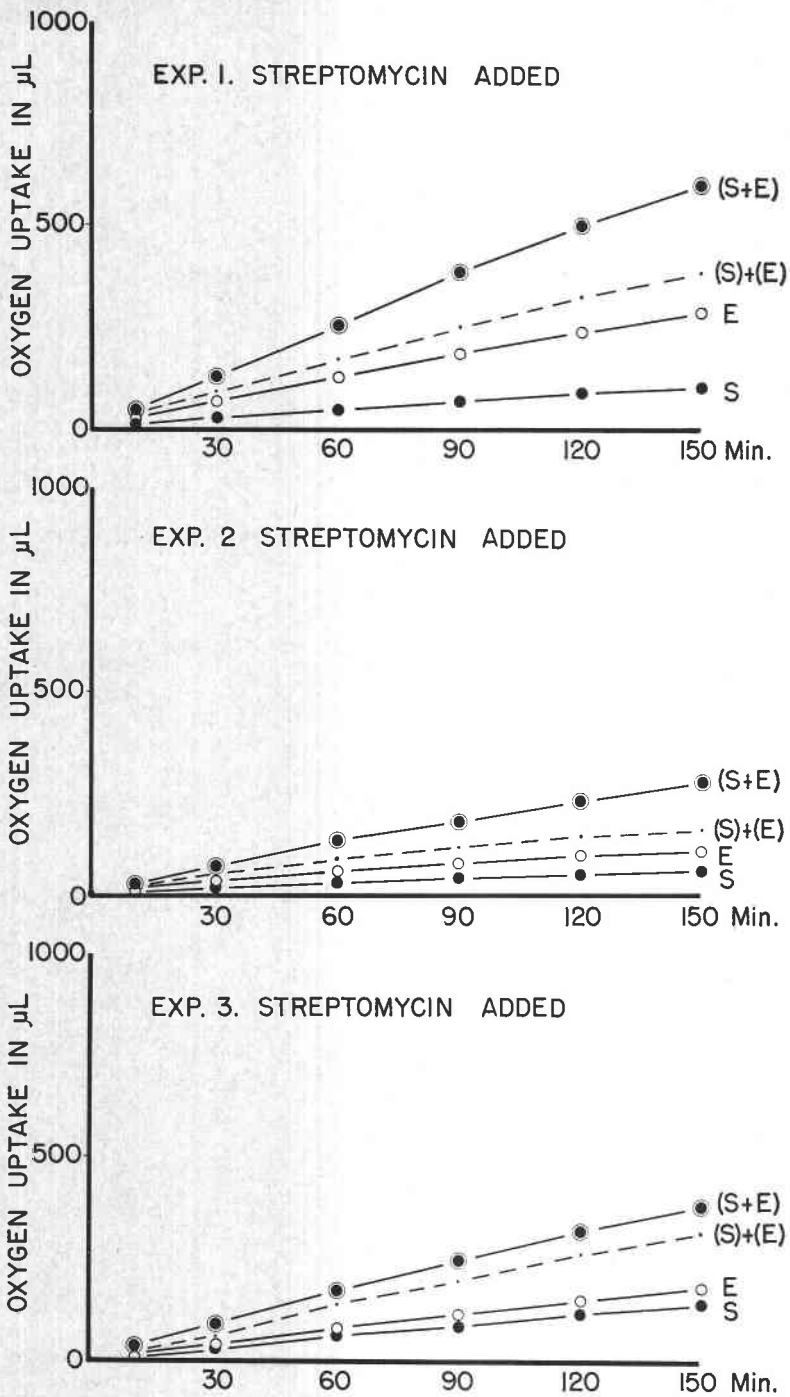


Figure 15. The symbols used are the same as in Figures 10 and 12. The augmentation of oxygen uptake in semen-*E. coli* mixtures (S + E) over the simple sum (S) + (E) of the oxygen uptake of semen alone (S) plus the oxygen uptake of *E. coli* alone (E) was consistent even in the presence of streptomycin. Streptomycin depressed the oxygen uptake of *E. coli* (E) considerably but not of the semen (S). (See Figure 14.)

EFFECT OF *E. coli* ON pH CHANGES IN SEMEN

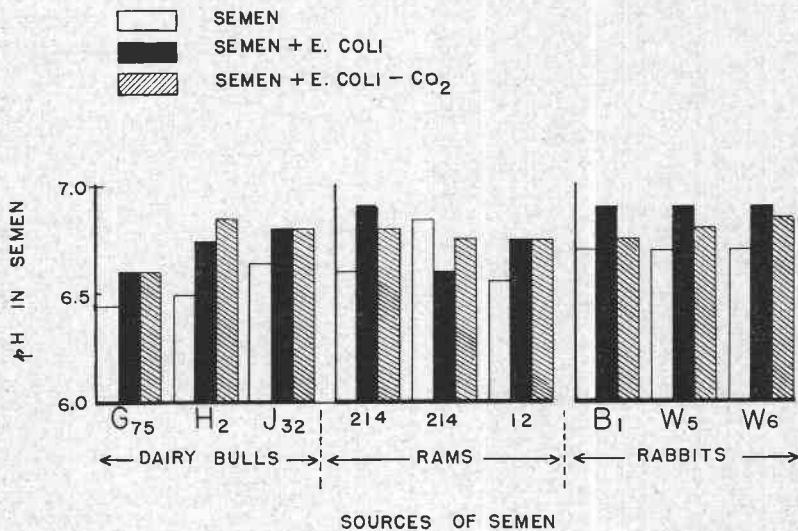


Figure 16. The figure shows the pH changes in semen and in semen-*E. coli* suspensions with and without the removal of carbon dioxide (all in egg-yolk sodium citrate medium). The pH in semen-*E. coli* mixtures was usually 0.1 to 0.3 higher than in semen alone. The removal of carbon dioxide did not alter the pH of the semen-*E. coli* mixtures more than 0.1.