ABSTRACT

ALTERATION OF SOME SEMINAL PLASMA CONSTITUENTS
DURING REPETITIVE EJACULATION
OF DAIRY BULLS

by Kenneth T. Kirton

Five Holstein bulls, ranging from 2 to 5 yr of age, were ejaculated four consecutive times at weekly intervals for a period of 4 wk. Ten to fifteen min of intensive sexual preparation was imposed prior to each ejaculation. Seminal volume, sperm concentration and pH of the fresh semen was determined for each ejaculate. The concentrations of fructose (Roe), citric acid (Saffran and Densted), protein (biuret), and free amino nitrogen (ninhydrin) were determined for each ejaculate. Zinc content of first and fourth ejaculate seminal plasma, and of blood serum for each bull was determined by the dithizone method.

Proteins of first and fourth ejaculate seminal plasma of the first and fourth week for each bull were further studied by Tiselius moving boundry electrophoresis and by agar gel double diffusion immunochemistry.

As anticipated seminal volume, sperm concentration, and total sperm per ejaculate decreased from ejaculate one to

ejaculate four ($P < .01$). A marked similarity between weeks was noticed for each of the five bulls indicating that such an ejaculation procedure may be useful to estimate a bull's sperm output capacity. The average pH of seminal plasma increased uniformly from the first to the fourth ejaculates ($P < .01$). The fructose and citric acid concentration of the seminal plasma increased from the first to the fourth ejaculate ($P < .01$), but, the total amounts per ejaculate remained relatively constant ($P \cong .20$). Since the seminal vesicles are assumed to be the primary source of seminal fructose and citric acid, these data indicated that the seminal vesicles contributed a relatively constant volume to each of the four ejaculates.

The average concentration of zinc in first and fourth ejaculate seminal plasma did not differ significantly ($P > .50$). Although the average zinc concentration of seminal plasma was considerably higher than the average of blood serum, the seminal average did not approach the comparable values previously reported for some other species.

The average seminal free amino nitrogen decreased steadily with progressive ejaculation. Based upon the reported high concentration of amino acids in epididymal secretions and the decreasing sperm concentration in

succeeding ejaculates, these results indicate a decreasing epididymal contribution from the first to the fourth ejaculates. The average concentration of protein in seminal plasma did not significantly change from first to fourth ejaculate ($P \cong .48$).

Electrophoretic analysis of seminal plasma revealed a maximum of eight protein components, and six or seven of these were found in each ejaculate. No appreciable difference was found between the number of components in first and fourth ejaculates. Electrophoretic mobilities of the major protein components were not altered by repetitive ejaculation. Based upon the electrophoretic data, the percentage composition of two of the four major protein components was altered by repetitive ejaculation ($P \cong .03$). One of the four major seminal plasma protein components had no electrophoretic counterpart in blood serum.

Immunochemical studies revealed a minimum of eight precipitating antigens in first ejaculate seminal plasma and a minimum of six in fourth ejaculate seminal plasma. However, the evidence indicated that the concentration of some minor constituents in fourth ejaculate seminal plasma was too low to be detected by this immunochemical technique.

by Kenneth T. Kirton

Correlation of the measured components in seminal plasma revealed that the totals per ejaculate were, without exception, more highly correlated than the comparable concentration values. The magnitude and similarity of the correlations between total fructose and total citric acid ($r = 0.61$), between total fructose and total free amino nitrogen ($r = 0.68$), and between total citric acid and total free amino nitrogen ($r = 0.64$) indicated a common major origin for these seminal constituents. The correlation ($- 0.81$) between the concentration of sperm and pH of the seminal plasma indicated that seminal hydrogen ions originated primarily in the ductus deferentia and epididymides.

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BIOGRAPHICAL SKETCH

Kenneth T. Kirton was born at Kingman, Kansas on June 16, 1934. He received his elementary education at Independence School and was graduated from Iola High School in June, 1952.

In September he entered Kansas State College, and received a Bachelor of Science degree in Dairy Husbandry in June, 1957. Having obtained an R.O.T.C. commission with the United States Army, he then entered upon two years active duty with a Nike-Ajax Battery in Detroit, Michigan.

After discharge from active duty in August, 1959, he operated a dairy farm at LaHarpe, Kansas until accepting a graduate assistantship at Michigan State University in September, 1961. He received the Master of Science degree in the Department of Dairy with a major in Reproductive Physiology in June, 1963.

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INTRODUCTION

Although the importance of seminal plasma to the spermatozoa contained therein has been a topic for controversy, most of the evidence indicates seminal plasma or some constituents (s) of seminal plasma is (are) responsible for improved sperm survival. The seminal plasma unquestionably serves as a vehicle for the sperm during ejaculation. There is little doubt that efficiency of natural mating would be lower without the provision of seminal plasma to serve as an effective diluent and vehicle for the closely packed epididymal spermatozoa.

Seminal plasma exerts a definite stimulatory effect on the non-motile epididymal sperm, and provides an important source of nutrition in the form of fructose (Mann, 1946). In addition, seminal plasma is capable of exerting striking biodynamic effects upon blood pressure and smooth muscles when injected intravenously (Mann, 1949). Additionally, the plasma undoubtedly has some effect upon the functional life expectancy of the sperm even though it is progressively diluted in the luminal fluids of the female genitalia at the time of mating. For example, Weil (1962) has shown that ejaculated sperm are rapidly and relatively permanently

coated with proteins of seminal plasma origin. Mann (1949), in reviewing the available evidence, alluded to the importance of the buffering capacity of seminal plasma for the sperm.

Numerous authors have cited evidence for the metabolic utilization of nutrients from seminal plasma by sperm. Dilution of sperm with seminal plasma did not effect fructolysis rate, while dilution with an artificial isotonic solution invariably decreased the rate of fructose utilization (Mann and Mann, 1951). A further indication of the beneficial effect of seminal plasma on sperm was provided by the increased fertilizing capacity of minimal numbers of sperm in the presence of increased concentrations of seminal plasma (Chang, 1947).

Desjardins and Hafs (1962) presented evidence indicating that bull sperm survive deep freezing better when frozen with high sperm cell concentrations. Kinney and VanDemark (1954) showed that sperm from second ejaculates survive freezing better than sperm from first ejaculates. Since the sperm concentration of second ejaculates is less than that of first ejaculates, the concentration of seminal plasma during freezing would be greater for second ejaculates because bull semen is usually frozen with a constant

concentration of sperm. Improved quality of sperm from second and third ejaculates has been reported by many authors. Although this improved quality could be attributable to inherent differences between the sperm populations, Desjardins and Hafs's data seem to emphasize the role of the seminal plasma.

Alterations of sperm production by repetitive ejaculation have been investigated extensively (Hale and Almquist, 1963, VanDemark, 1956, Hafs et al., 1962). In contrast, seminal plasma constituents during repetitive ejaculation have not been extensively investigated.

The purpose of this thesis was to determine whether repetitive ejaculation affected the proportions of some seminal plasma constituents of mature dairy bulls. In view of the lack of information on seminal plasma proteins and because of their potential importance to sperm physiology, they were emphasized in this thesis.

REVIEW OF LITERATURE

Behavioral Factors Affecting Seminal Volume and Sperm Output:

While artificial insemination was still in its infancy, Lagerlöf (1934) observed that bulls frequently yield first ejaculates with few or no sperm. Later, Hellstrom (1947) found that restraining bulls for a few minutes before ejaculation resulted in larger seminal volumes. Collins et al. (1951) were the first to publish quantitative information concerning pre-ejaculation sexual preparation of bulls. They concluded that restraining a bull in the vicinity of a mount animal and allowing one false mount prior to ejaculation increased seminal volume and numbers of motile sperm per ejaculate significantly. Branton et al. (1952) obtained similar increases with one false mount.

Hale and Almquist (1960) summarized previous data by stating that at collection frequencies of one or two ejaculates per week, one may reasonably anticipate that a single false mount prior to ejaculation will increase sperm output by approximately 50%. Two additional false mounts may increase output per ejaculate by another 50%, representing double the output with no preparation.

Restraining a bull for about 10 min was estimated to have essentially the same effect as several false mounts (Almquist et al., 1958, and Cromback, 1958). This work was subsequently substantiated by Hafs et al. (1962) who obtained almost 230% as many total sperm per ejaculate after 10 min of active restraint as was obtained from bulls with no sexual preparation. This increase in sperm output was accompanied by an increase of 153% in seminal volume. The same authors found that the increased sperm output obtained as a result of false mounting was primarily a factor of seminal volume, which was significantly increased by false mounting, whereas the concentration of sperm was not.

Alteration of Seminal Constituents by Repetitive Ejaculation:

Cragle et al. (1958) found a definite change in mineral content of seminal plasma of ten consecutive ejactulates taken from dairy bulls. Values for potassium and calcium decreased from 155 and 39 mg/100 ml, respectively, in the first ejaculate to 91 and 13 mg/100 ml, respectively, in the tenth ejaculate. Values for sodium and chloride increased from 289 and 154 mg/100 ml, respectively, in the first ejaculate to 325 and 411 mg/100 ml, respectively, in the tenth ejaculate. VanDemark and Boyd (1956) determined some chemical components

in twenty consecutive ejaculates. They found a progressive decrease in fructose, potassium, and calcium from the first to twentieth ejaculates.

Hopwood et al. (1961a) analyzed 16 consecutive ejaculates collected from each of three beef bulls. The semen was analyzed for Nessler nitrogen, fructose, and free amino acids. Sperm count, seminal fructose, and nitrogen steadily declined while the pH rose in successive ejaculations. Total free amino acid content progressively decreased from the first to the twentieth ejaculate. This decrease was primarily due to a lowered glutamic acid and alanine content while glycine, serine, and aspartic acid did not change appreciably.

De Groot (1961) found a decrease in seminal fructose and sperm concentration with succeeding ejaculates taken from triplet bulls subjected to an exhaustion test. The pH of the semen showed a pronounced increase as ejaculate number increased. Mean sperm motility showed little change with ejaculate number, except in the case of the first ejaculate which tended to be lower.

Successive ejaculates tend to increase in pH from an average value of 6.75 for first ejaculates (Sergin, 1936 and Anderson, 1942) to about 7.0 (Davis, 1938, Hopwood et al.,

1961a, VanDemark and Boyd, 1956, and Davis and Williams, 1939). An investigation of the buffering capacity of bull semen disclosed a well buffered system at pH 4.0 to 5.5 and 9.0 to 10.0, while the semen was poorly buffered at physiological values of 6.0 to 9.0 (Smith and Asdell, 1940). Dougherty and Ewalt (1941) found seminal pH increased for about 2 hr and then decreased steadily when fresh semen was incubated at room temperature. Mann (1949) attributed the buffering capacity of seminal plasma to the inorganic phosphate, bicarbonate, citrate, and protein components of the seminal plasma. In view of the sensitivity of sperm to an acid medium, such as occurs due to the accumulation of lactic acid during metabolism, the buffering effect of seminal plasma is of particular value.

Characterization of Seminal Constituents:

Seminal plasma is a mixture of secretions originating in the testis, epididymis, deferent duct, seminal vesicle, prostate, bulbo-urethral gland, and urethra. The seminal vesicle is the major source of seminal fluids, but the relative contributions of the other sources is not well known.

Seminal plasma differs remarkably from other body fluids in several respects. The seminal concentrations of citric

acid (Schersten, 1929), fructose (Mann, 1946), and phosphorylcholine (Lundquist, 1946) are much higher than the amounts found in other body fluids. These three substances appear to originate primarily in the seminal vesicle of most domesticated animals.

Mann (1949) has speculated that seminal fructose and citric acid are secreted independently of each other, in the sense that the two components are produced by two distinct types of cells. In support of this contention, he showed (Mann et al., 1948) three distinct cell types in the secretory epithelium of the seminal vesicle upon histological examination. While Rothchild and Barnes (1954) found a significant ($P < .01$) positive correlation between seminal fructose and citric acid, Mann (1948) found no correlation between the seminal content of these two components. Ehlers et al. (1953) found evidence of a cyclic relationship between fructose and citric acid, in that correlations were negative in some months and significantly positive in others.

Evidence for the separate origins of sperm and seminal fructose was provided by Mann (1948) who collected eight ejaculates from a bull in a period of 63 min. As expected, sperm numbers decreased from 1,664 X 10^6/ml for the first ejaculate to 98 X 10^6/ml for the eighth ejaculate. The

seminal fructose content increased from first to sixth ejaculate and then decreased in the last two ejaculates.

The fructose and citric acid content of semen declined markedly following seminal vesiculectomy (Hess et al., 1960). The average values before seminal vesiculectomy were 255 and 245 mg/100 ml for fructose and citric acid, respectively, where-as comparable values after removal were 10.4 and 21.7 mg/100 ml. These data indicate nearly all of the fructose and citric acid found in bovine semen is produced by the seminal vesicle.

Bertand and Vladesco (1921) surmised that zinc was in some way important for reproduction. This assumption was based upon the high zinc content found in semen of the stallion and human. Mawson and Fischer (1953) obtained similar values for human semen, and an exceptionally high zinc content in the dorsolateral prostate of the rat. Dyrendahl (1954) found 2.84 mg/100 ml of zinc in fresh bovine semen. Cragle et al. (1958) reported finding only 0.2 mg/100 ml and suggested the zinc concentration of bull semen fluctuates widely. However, it is not clear if this value is for whole semen or seminal plasma. Mann (1945) reported a comparable value of 0.28 mg/100 ml for ram seminal plasma.

Keilin and Mann (1940) have shown that zinc is the prosthetic metal of the enzyme carbonic anhydrase, which catalyzes the reaction;

$$H_2CO_3 \longrightarrow H_2O + CO_2.$$

Much of the zinc content found in tissue may be explained in this manner. However, only small amounts of the enzyme were found in sperm and seminal plasma (Mawson and Fischer, 1953).

Hopwood and Gassner (1962) found glutamic acid, alanine, glycine, serine, and aspartic acid in decreasing order of concentration in bovine seminal plasma. Evidence that the free amino acids were related to testicular and epididymal function was obtained by causing a drastic alteration in amino acid level by (1) removal of the testes and epididymides, (2) blockage of their products by vasectomy, or (3) interference with their function by hyperthermy (Hopwood et al., 1961b). The most pronounced change was reduction of glutamic acid to a very low level, indicating its origin to be the testis or epididymis. Analysis of the free amino acid content of ampullar and seminal vesicular fluid revealed alanine to be present in the largest quantaties. Administration of testosterone propionate in the castrated animals restored the level of alanine to near normal, but the levels of the other amino acids did not increase similarly.

These findings are not in complete agreement with those of Hess et al. (1960) who found glycine to be more concentrated than alanine in seminal plasma of normal dairy bulls. Mean concentrations of five individual seminal amino acids were not altered significantly by the removal of the seminal vesicles. Hess stated these results were similar to the findings of Brown (1952).

Huggins et al. (1942) found only a small per cent of the proteins of human seminal plasma to be heat-coagulable, and approximately 60% of the proteins were of a proteose nature and dialyzable. Electrophoretic mobilities of the undialyzable proteins corresponded to the α_1, α_2, and α_3 globulins, and serum albumin of blood. As ejaculation frequency increased, a definite decrease in seminal plasma protein content was found. Ross et al. (1942), and Ross (1946) found proteoses, blood globulins, and glycoproteins present in quantity, but little serum albumin in human seminal plasma.

Although the proteins of human seminal plasma have been extensively investigated, few studies have been published on the proteins of bovine seminal plasma. Larson and Salisbury (1954) found the nitrogen content of the total seminal plasma proteins to be 14.4%. About 90% of the total nitrogen

present in seminal plasma was found to be protein in nature and non-dialyzable. Seventy six per cent of these proteins were heat-coagulable. A weak Molish test of exhaustively dialyzed seminal plasma indicated that non-dialyzable carbohydrate constituents are present in low concentration. These results are not in complete agreement with those of Sarkar et al. (1947) who found only 12.1% nitrogen in dried bovine seminal plasma protein.

Electrophoretic examination of bovine seminal plasma protein revealed eleven components, three of which accounted for the majority of the total protein found (Larson and Salisbury, 1954). The three major components possessed mobilities similar to those of blood α globulins. Immune-globulins were found in higher concentration in the seminal plasma of older bulls. The relative proportions of the proteins found in seminal plasma did not resemble the proportions existent in blood serum. According to the authors, the three components found in largest quantities in seminal plasma had electrophoretic mobilities similar to the α_1, α_2, and α_3 globulins of blood. Proteins with mobilities of blood serum albumin were either absent from the seminal plasma or present at a very low concentration in the from of one minor component. The electrophoretic patterns of the seminal plasma

of all bulls studied were remarkably similar. Although the major components possess electrophoretic mobilities similar to blood globulins, they were found to differ by possessing a greater stability to physical treatment as well as lower carbohydrate and lipid contents.

Ultracentrifugal analysis of bovine seminal plasma proteins by Larson et al. (1954) revealed five components. The three present in largest quantities accounted for 95% of the total protein. The relative proportion of the proteins found in seminal plasma did not resemble the proportions existent in blood serum. Two components found in seminal plasma possessed sedimentation velocities similar to components found in blood serum. The authors stated that 30 to 40% of the bovine seminal plasma proteins are unlike any found in blood. Ultracentrifugal analysis of milk serum proteins revealed they resembled those of seminal plasma more closely than did proteins found in blood serum.

Immunological studies conducted by Larson et al. (1954) indicated that proteins found in seminal plasma were much more antigenic than those from milk serum or blood serum. The primary causative factors were found to be heat labile and non-dialyzable. A weak cross reaction between seminal plasma protein and milk serum protein, which was known to be

high in immune-globulin, indicated a low content of these proteins in seminal plasma. Substantiating evidence was obtained by a weak reaction between antisera to a purified preparation of immune-globulins and seminal plasma. A cross reaction obtained between blood serum and seminal plasma and their respective antisera indicated the systems have some antigens in common.

The authors suggested that the cross reactions of seminal plasma and blood serum proteins were due to components which were either antigenically weak, or present in low concentration. They further stated that with the exception of one component which appeared to be similar to lactalbumin of milk serum, "the major protein components of bovine seminal plasma are definite chemical entities which are not similar to any of the major protein components of blood or milk serum."

EXPERIMENTAL PROCEDURES

Semen Collection and Handling:

The semen used in these studies was collected from five Holstein bulls ranging from 2 to 5 yr of age. These bulls had been in routine use at Michigan Artificial Breeders Co-operative. Based upon 60- to 90-day non-return percentages of routine artificial inseminations, they had normal fertility. Four consecutive ejaculates were collected from each bull at weekly intervals for a period of 4 wk. Ten to fifteen min of intensive sexual preparation including two or three false mounts were imposed previous to each ejaculation.

Immediately after a semen sample was collected, the volume of semen was recorded and a small sample was removed to determine sperm concentration with a photelometer, and to determine pH of the fresh semen with a Beckman Zero-matic pH meter equipped with a one-drop electrode. Within 20 min, the remaining portion of the semen sample was centrifuged at 5^{o} C with a centrifugal force of 26,000 x g for 20 min. Following centrifugation, the seminal plasma was decanted from the sperm cells and placed in plastic vials. A sample was removed at this time to determine biuret protein and ninhydrin-nitrogen. The rest of the seminal plasma was frozen and

stored at -20° C for subsequent determination of citric acid, fructose, and zinc and for electrophoretic and immuno-chemical analysis of the proteins.

Free Amino Nitrogen:

The free amino nitrogen content of seminal plasma was determined by the ninhydrin reaction (Harding and Mac Lean, 1916). This test gives positive results with proteins, peptides, amino acids, and other primary amines, including ammonia. One tenth ml of seminal plasma was diluted with 0.9 ml of distilled water, 1.0 ml of 10% aqueous pyridine, and 1.0 ml of freshly prepared 2.0% solution of aqueous ninhydrin. The samples were then heated at 100° C for 20 min, cooled to room temperature, and diluted to 200 ml with distilled water. The optical density was determined at 565 mμ in a Spectronic 20 spectrophotometer and compared to a standard curve which had been previously constructed using the amino acid alanine.

Citric Acid:

Citric acid was determined by the procedure of Saffran and Denstedt (1948). Although this method is neither as accurate nor as specific as some other assays, it's relative simplicity motivated its use here. A sample of frozen

seminal plasma was thawed and 0.2 ml of the sample was deproteinized by adding 4.8 ml of 5% trichloroacetic acid at 5° C. The resultant precipitate was removed by centrifugation. Duplicate 1-ml samples of the supernatant were each mixed with 8 ml of acetic anhydride and incubated for 10 min at 60° C. One ml of pyridine was then added and the mixture was incubated at 60° C for an additional 40 min. The samples were then cooled to room temperature and the optical density was determined at 400 mμ in a Spectronic 20 spectrophotometer and compared to a previously prepared standard curve.

Fructose:

Fructose was determined by the method of Roe et al. (1934). This reaction is quite specific, the main disadvantage being that the phosphorylated esters of fructose are less reactive than the sugar itself. A sample of frozen seminal plasma was thawed and 0.2 ml of the sample was deproteinized by adding 4.8 ml of 5% trichloroacetic acid at 5° C. The resultant precipitate was removed by centrifugation. Duplicate 0.5 ml samples of the supernatant fluid were diluted with 1.5 ml of 5% trichlaroacetic acid and mixed with 2.0 ml of 30% resorcinol and 6.0 ml of 30% HCL. These samples were incubated at 80° C for 8 min, quickly cooled to room

temperature, and the optical density was determined at 490 mμ in a Spectronic 20 Spectrophotometer and compared with a previously prepared standard curve. The fructose to be used to establish the standard curve was dissolved in a saturated aqueous solution of benzoic acid to prevent deterioration with time.

Zinc:

Zinc was determined by the dithizone reaction as outlined by Johnson et al. (1959). This method is extremely sensitive, detecting as little as 0.1 μ g/ml. Because of this sensitivity, avoidance of contamination from glassware or reagents is of utmost importance. The samples of frozen seminal plasma were thawed and 0.5 ml taken from each of the four first ejaculates from each bull to obtain a 2.0 ml pooled first ejaculate for each bull. A similar procedure was followed for fourth ejaculates. Each 2.0 ml sample was wet ashed and zinc determinations were performed according to the procedure described by Johnson et al. (1959) (Appendix, Procedure A).

Total Protein:

Total protein content of the seminal plasma was determined by the biuret method of Kingsley (1939) as modified by Gornall et al. (1949). There are practically no substances other than protein in biological fluids which give the biuret reaction, the only exception being bile pigments which are not present in sufficient quantity to cause an appreciable error. One-tenth ml of seminal plasma was diluted with 1.9 ml of distilled water and 4.0 ml of biuret reagent (Gornall et al., 1949) (Appendix, Procedure B) was added. The mixture was incubated for 30 min at 38° C and the optical density was determined at 540 mμ in a Spectronic 20 Spectrophotometer and compared to a standard curve which had been previously constructed using bovine serum albumin.

Electrophoretic Analysis of Proteins:

Electrophoretic analyses were per formed at 1° to 2° C in a Tiselius electrophoresis apparatus (American Instrument Company) equipped with a cylindrical lens and a rotating slit. Seminal plasma from the first and from the fourth ejaculates of the first and fourth weeks for each bull were thawed, adjusted to a biuret protein content of 1.75 to 2.00% and a total volume of 7.5 ml by addition of sodium veronal

buffer (Appendix, Procedure C) at pH 8.6 and ionic strength 0.1. Each sample was then dialyzed in Visking cellulose casing with constant agitation for 12 hr at 5° C against each of two changes of buffer, each about 100 times the volume of the sample. The electrophoretic mobilities were calculated according to the procedure of Alberty (1948) which is outlined in the Appendix, Procedure D. The relative concentration of each of the major protein constituents in each sample was estimated by drawing the electrophoretic pattern on graph paper, cutting out the curves, and weighing the resultant paper (Tiselius, 1939).

Immuno-chemical Analysis of Proteins:

Immuno-chemical analyses were performed by injecting samples of seminal plasma and adjuvant into rabbits and analyzing the resultant antisera by the agar del double diffusion method of Ouchterlony (1958). The samples of frozen seminal plasma were thawed and 0.25 ml taken from each of the four first ejaculates from each bull to obtain a 1.0 ml pooled first ejaculate for each bull. A similar procedure was followed for fourth ejaculates.

Each sample was adjusted to a biuret protein content of 2.0% with 0.85% saline and then emulsified with an equal

quantity of Freund's complete adjuvant (Appendix, Procedure E). The mode of action of adjuvants was discussed by Freund (1947, 1951). One ml of each sample was subcutaneously injected into five intrascapular sites in each of four Dutch Belted rabbits. One week later, each rabbit was similarly injected with the same sample homogenized with Freund's incomplete adjuvant (Appendix, Procedure E).

The rabbits were bled by cardiac puncture 2 wk after the second injection and the blood was allowed to clot. The serum was decanted and then centrifuged to remove any remaining cells. Merthiolate was added to each serum sample to a concentration of 0.001% and the samples were stored at -20^{o} C. Subsequently, the antisera were thawed and the numbers of precipitating antibodies were determined by diffusing the antisera against seminal plasma samples in Ouchterlony plates (Appendix, Procedure F). The progress of precipitin line formation was followed and recorded by photographing the plate against a black background with angularly transmitted light.

Initially, one titer plate was prepared for the antisera of each immunized rabbit to determine the optimal antigen concentration for subsequent diffusions. The center well was filled with antisera, and the six peripheral wells

with serial dilutions of antigen. The presence of antibodies and the relative titer were determined in this manner. Subsequently, plates containing four wells in the H-pattern (Fox, 1959) were prepared with pooled antisera to ejaculate one in one well, pooled antisera to ejaculate four in another well, seminal plasma (antigen) from ejaculate one in a third well, and seminal plasma from ejaculate four in the fourth well. Duplicate plates of this variety were prepared for each bull.

The Bjorklund (1952) modification of Ouchterlony's double diffusion method was utilized to determine any precipitating antigens peculiar to either the first or to the fourth ejaculates. Antiserum to ejaculate one was absorbed with an equal volume of ejaculate four seminal plasma at 38^{o} C for 0.5 hr. The absorbed antisera were then allowed to diffuse against seminal plasma from ejaculate one and ejaculate four.

Cross reactions between the antisera to seminal plasma proteins and bovine blood serum were also determined by the double diffusion in gel method.

RESULTS AND DISCUSSION

Ejaculate Volume and Sperm Numbers:

The average volume of semen for each of the four consecutive ejaculates for each bull is listed in Table I, where it is apparent that the volume of semen declined progressively from the first to the fourth ejaculate ($P < .01$).

Table 1. Milliliters of semen per ejaculate.

Bull	Ejaculate			
	1	2	3	4
Remark	8.8	8.5	7.9	7.3
Climax	9.4	8.9	7.1	5.9
Imperial	8.5	6.8	5.2	5.5
Explorer	10.4	7.4	6.4	5.3
Pure Gold	5.4	4.5	4.3	4.4
Avg	8.5	7.2	6.2	5.7

The average number of sperm per milliliter of semen for each of the four consecutive ejaculates for each bull is listed in Table 2. Although there is considerable variation among the bulls, the average concentration of sperm progressively declined from the first to the fourth ejaculate for

each bull ($P < .01$). This fact indicates that the epididymal contribution decreases with repetitive ejaculation.

Table 2. Billions of sperm per ml of semen.

Bull	Ejaculate			
	1	2	3	4
Remark	1.390	1.137	0.686	0.571
Climax	1.527	0.945	0.688	0.471
Imperial	2.130	1.469	0.742	0.650
Explorer	2.031	1.050	0.561	0.419
Pure Gold	1.911	1.563	0.789	0.698
Avg	1.798	1.233	0.693	0.562

The total sperm in each ejaculate for each bull is presented in Table 3. As was the case for volume of semen and for concentration of sperm, the average number of total sperm per ejaculate declined progressively from the first to the fourth ejaculates for each bull ($P < .01$). However, the decline from first to fourth ejaculate was considerably greater for some bulls than for others ($P < .01$). Total sperm production for each bull was remarkably constant among weeks for each of the four ejaculates and for the total sperm in the four ejaculates. These data agree with previous reports providing evidence that the "exhaustion trial" is an

Table 3. Billions of sperm per ejaculate for each ejaculate.

Bull	Week	Ejaculate				
		1	2	3	4	Sum
Remark	1	10.5	9.4	6.8	3.9	30.6
	2	14.8	9.3	4.8	3.8	32.8
	3	10.6	9.5	5.4	3.7	29.1
	4	13.0	9.6	4.6	4.9	32.1
	Avg	12.2	9.5	5.4	4.1	31.2
Climax	1	14.0	8.1	5.8	2.8	30.8
	2	12.2	7.5	6.5	2.8	28.8
	3	12.5	9.0	3.8	3.7	29.1
	4	19.3	8.2	3.6	1.1	32.2
	Avg	14.5	8.2	4.9	2.6	30.2
Imperial	1	17.3	13.9	5.8	4.7	41.6
	2	17.3	7.0	3.7	3.2	31.2
	3	18.9	9.9	1.4	4.3	34.5
	4	16.6	8.8	5.0	1.3	31.8
	Avg	17.5	9.9	4.0	3.4	34.8
Explorer	1	17.0	12.1	4.9	2.0	35.9
	2	26.0	5.2	4.3	1.2	36.6
	3	18.8	7.8	2.9	3.0	32.6
	4	22.6	6.2	2.3	2.6	33.7
	Avg	21.1	7.8	3.6	2.2	34.7
Pure Gold	1	12.3	8.9	2.8	1.8	25.7
	2	8.5	5.9	2.8	2.5	19.7
	3	7.5	7.4	4.5	4.2	23.5
	4	13.1	6.4	3.0	3.7	26.2
	Avg	10.3	7.1	3.3	3.0	23.8
Avg		15.1	8.5	4.2	3.1	30.9

excellent means of estimating the sperm output capacity of bulls (Walton and Edwards, 1938, and Hale and Almquist, 1960).

Hydrogen Ion Concentration:

The average pH of the fresh semen for each ejaculate for each bull is listed in Table 4. These data revealed a uniform increase in the pH from the first to the fourth ejaculate for each bull ($P < .01$). The bulls were remarkably similar in this regard ($P > .75$). This progressive increase in pH has been reported by other workers (Rimoldi and Brigatti, 1945, and Davis and Williams, 1939). The average pH of first ejaculates was 6.26, somewhat lower than some reports, but well within the range reported for normal bovine semen (Anderson, 1942). Values obtained for each bull seemed characteristic for what particular animal, with little fluctuation between weeks. Several randomly chosen pH determinations following freezing and thawing of the seminal plasma showed no appreciable change from the fresh semen values previously obtained for the same semen sample. This indicated that bicarbonate probably was not a major factor in the buffering system of seminal plasma.

Table 4. Averages of pH values for the fresh semen.

Bull	Ejaculate			
	1	2	3	4
Remark	6.39	6.52	6.66	6.77
Climax	6.36	6.63	6.82	7.09
Imperial	6.19	6.41	6.68	6.96
Explorer	6.15	6.35	6.74	6.91
Pure Gold	6.21	6.41	6.75	6.85
Avg	6.26	6.46	6.73	6.92

Free Amino (Ninhydrin) Nitrogen:

The concentration of free amino nitrogen in the seminal plasma, and the total free amino nitrogen per ejaculate are listed in Table 5. The average concentration of ninhydrin nitrogen declined progressively from the first to the fourth ejaculate ($P < .01$) a trend which was magnified when presented in terms of total free amino nitrogen per ejaculate ($P < .01$). If we assume that the epididymides and ductus deferentia are the primary sources of free amino nitrogen (Hess et al., 1960, and Hopwood et al., 1961b), the data in Table 5 indicate that the epididymides and ductus deferentia contribute declining proportions to the seminal plasma of successive ejaculates.

Table 5. Average seminal plasma free amino nitrogen values.

Units of measurement	Bull	Ejaculate 1	2	3	4
µg/ml	Remark	1,023	945	1,038	813
	Climax	976	928	856	834
	Imperial	574	563	470	375
	Explorer	930	850	787	708
	Pure Gold	695	697	649	625
	Avg	840	797	760	671
mg/ejac	Remark	9.02	8.05	8.45	6.30
	Climax	9.20	8.13	6.05	4.80
	Imperial	4.77	3.73	2.45	1.83
	Explorer	9.67	6.35	5.03	3.70
	Pure Gold	3.63	3.15	2.77	2.80
	Avg	7.26	5.88	4.95	3.88

Fructose and Citric Acid:

The concentrations of fructose and citric acid in the seminal plasma and the total amounts per ejaculate are presented in Table 6. The concentration of both of these constituents progressively increased with successive ejaculation ($P < .01$). The similarity of the values for concentration of fructose in third and fourth ejaculates indicates that further ejaculations would not have increased fructose concentration appreciably. However, the total amounts of fructose and citric acid per ejaculate were quite constant ($P \cong .20$) relative to the comparable values for free amino nitrogen

Table 6. Average seminal plasma fructose and citric acid values.

Constituent	Bull	Mg/100 ml				Mg/ejac			
		Ejaculate				Ejaculate			
		1	2	3	4	1	2	3	4
Fructose	Remark	703	774	892	887	62.0	65.0	68.5	67.2
	Climax	745	924	976	977	79.2	85.2	69.8	49.5
	Imperial	445	655	699	479	37.0	44.2	36.5	24.5
	Explorer	1,000	1,267	1,410	1,353	104.2	92.8	89.8	70.8
	Pure Gold	513	775	887	890	26.2	35.5	38.5	39.2
	Avg	681	879	973	917	61.8	64.6	60.7	50.2
Citric Acid	Remark	553	547	627	760	48.5	46.2	49.0	56.8
	Climax	417	403	423	431	39.2	40.2	28.0	28.0
	Imperial	465	505	541	526	41.5	33.8	28.2	27.0
	Explorer	726	663	724	803	76.0	48.8	46.5	41.8
	Pure Gold	437	470	505	575	23.8	21.2	21.8	26.2
	Avg	520	518	564	619	45.8	38.0	34.7	36.0

presented above. From the standpoint of the welfare of the sperm, the concentration values are probably more important.

We may assume that the seminal vesicles are the primary source of fructose and citric acid and that the concentration of these two constituents in the seminal vesicular contribution to the first through the fourth ejaculates was constant (Mann, 1949). Then the data in Table 6 indicate that the total seminal vesicular contribution was quite constant from the first to the fourth ejaculates. However, the relative contribution of the seminal vesicles increased from the first to the fourth ejaculate, presumably because of declining contributions from the other accessory sex organs. This contention is supported by the free amino nitrogen values presented above and by the zinc values presented below.

Zinc:

The average concentrations of zinc in seminal plasma from first and fourth ejaculates and in blood serum from each of the five bulls are listed in Table 7. The average concentration of zinc in blood serum was considerably less than that in seminal plasma ($P < .05$). The average concentrations in ejaculates one and four did not differ greatly ($P > .50$).

The tissues from one bull (Remark) were available for analysis at the end of the experiment. The prostate tissue had an average zinc concentration of 29 μg/gm of fresh tissue a value only about 10 to 30% of those found in other species. The seminal vesicular secretions had an average zinc concentration of 6.5 μg/ml. These facts in addition to the relatively small size of the bovine prostate indicate that the seminal vesicles were the primary source of seminal plasma zinc, at least in this bull.

Table 7. Micrograms of zinc per milliliter of seminal plasma and blood serum.

Bull	Ejaculate		Blood serum
	1	4	
Remark	6.0	9.0	4.0
Climax	11.3	10.2	5.5
Imperial	8.3	6.6	7.3
Explorer	8.4	7.7	4.5
Pure Gold	13.1	10.5	8.3
Avg	9.4	8.8	5.9

Biuret Protein:

The average concentrations of protein in seminal plasma are listed in Table 8. Repetitive ejaculation failed to

influence the concentration of protein ($P \cong .48$). This relative uniformity in protein concentration seems indicative of a homeostasis that may be important to sperm survival. The total amount of protein per ejaculate declined with ejaculate number ($P < .02$) - a result which reflects declining seminal volume.

Table 8. Average seminal plasma biuret protein values.

Unit of measurement	Bull	Ejaculate			
		1	2	3	4
mg/100 ml	Remark	8.29	8.85	8.44	7.97
	Climax	8.55	9.16	9.63	8.71
	Imperial	8.14	7.89	7.45	7.15
	Explorer	5.31	5.74	5.36	4.62
	Pure Gold	5.42	5.68	6.47	6.85
	Avg	7.14	7.47	7.47	7.06
mg/ejac	Remark	0.730	0.747	0.663	0.590
	Climax	0.805	0.810	0.687	0.507
	Imperial	0.700	0.545	0.373	0.417
	Explorer	0.547	0.425	0.345	0.235
	Pure Gold	0.283	0.260	0.280	0.303
	Avg	0.613	0.557	0.469	0.410

Electrophoretic Analysis of Proteins.

Typical Tiseulius electrophoretic patterns for the first and fourth ejaculates from each bull are presented in figure

1. Enlargement of these patterns revealed six or seven components in each pattern. The components were classified by electrophoretic mobility and are listed in Table 9. Repetitive ejaculation failed to alter the mobilities for components 2, 4, 5, 6, or 7 ($P > .15$). However, the average mobility of 6.70 for component eight in fourth ejaculates was significantly less than the corresponding average of 6.88 in first ejaculates ($P < .01$). The full meaning of this result is not clear. No statistical procedure was applied to components 1 or 3, because they were found only occasionally.

Electrophoretic components 4, 5, 6, and 7 were present in sufficient amounts to estimate the relative proportion of these components from the patterns. These proportions are listed in Table 10. Repetitive ejaculation did not affect the proportion of components 5 or 7 ($P > .17$). However, fourth ejaculates contained an average of 28.8% of component 4, significantly more than the average of 22.0% for first ejaculates ($P \cong .03$) and fourth ejaculates contained an average of 30.4% of component 6, significantly less than the 35.3% for first ejaculates ($P \cong .02$). Thus, although the total protein content of seminal plasma was not measurably altered by repetitive ejaculation, the proportion

of the protein constituents was significantly altered.

Electrophoretic analysis of blood serum proteins from the Remark bull revealed eight components (figure 2)- in excellent agreement with comparable results by Larson and Salisbury (1954). The number of electrophoretic components for seminal plasma listed by those authors ranged from six to ten, somewhat higher than the numbers obtained in the present experiment (Table 9). The present author is of the opinion that the patterns presented by Larson and Salisbury do not provide adequate evidence for as many as ten electrophoretic components in seminal plasma. Consequently, regarding the number of components, their results were very similar to those presented here.

In agreement with the data presented by Larson and Salisbury (1954), the present data indicate that three components comprise the great majority (95 to 97%) of the protein in seminal plasma. Larson and Salisbury claimed that "the major components of the seminal plasma exhibit mobilities similar to those of the α_1-, α_2-, and α_3- globulins of blood." The data in the present experiment indicate that two of the major components (components 4 and 6) of seminal plasma exhibited mobilities similar to those of the α_2- and α_3- globulins of blood. The third major

component (component 5) appeared to have no electrophoretic counterpart in blood serum.

Figure 3 pictures an electrophoretic pattern of the protein components in seminal vesicular fluid from Remark. Seminal vesicular fluid contained all of the components for seminal plasma displayed in figure 1 and listed in Table 9. The relative proportions of the components in seminal vesicular fluid were very similar to those for ejaculated seminal plasma.

Immuno-chemical Analysis of Proteins:

Photographs of Ouchterlony titer plates representing the first and the fourth ejaculates from each of the five bulls are displayed in figure 4. The number of visible precipitin lines obtained when antisera from each rabbit was reacted with its antigen in Ouchterlony titer plate is presented in Table 11. There was considerable variation in the measurable antibody response from rabbit to rabbit within a particular seminal plasma sample, probably due to a lower sensitivity of the reticulo-endothelial systems of certain rabbits. The rabbit yielding the maximum number of precipitin lines for a particular seminal plasma sample demonstrated the minimum number of antigens in that sample. Additional antigens may not have been detected due to the

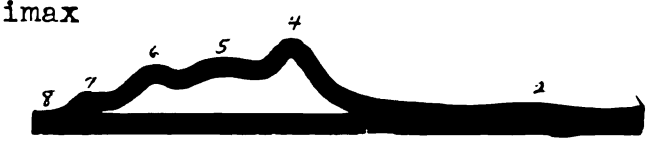
Figure 1. Electrophoretic Patterns of Seminal Plasma.

Remark

Ejaculate 1

Ejaculate 4

Climax



Ejaculate 1

Ejaculate 4

Imperial

Ejaculate 1

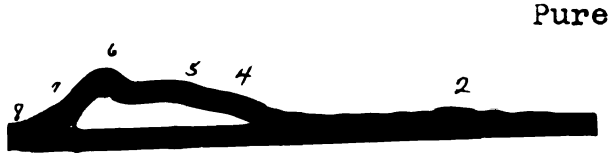
Ejaculate 4

Explorer

Ejaculate 1

Ejaculate 4

Pure Gold



Ejaculate 1

Ejaculate 4

Numbered components correspond to electrophoretic components listed in Table 9 and 10.

Table 9. Electrophoretic mobilities* of measurable seminal plasma proteins.

Bull	Week	Ejac	Electrophoretic component							
			1	2	3	4	5	6	7	8
Remark	1	1	___	1.8	___	4.1	5.0	5.7	6.2	7.1
		4	___	1.8	___	4.4	5.0	5.7	6.2	7.0
	4	1	___	1.9	___	4.1	4.8	5.6	6.2	7.1
		4	___	1.9	___	4.3	5.0	5.7	6.3	6.8
Climax	1	1	___	2.0	___	4.2	5.0	5.8	6.3	7.1
		4	1.5	2.6	___	4.0	4.6	5.3	6.0	6.5
	4	1	___	1.5	3.2	3.8	4.5	5.0	5.5	6.4
		4	___	1.7	3.0	3.8	4.5	5.0	5.7	6.4
Imperial	1	1	___	2.3	___	4.0	4.9	5.5	6.0	6.8
		4	___	1.9	___	4.0	4.9	5.6	6.2	6.8
	4	1	___	2.3	___	3.9	4.4	5.0	5.5	6.4
		4	1.0	2.3	___	3.9	4.2	5.0	5.3	6.3
Explorer	1	1	1.8	2.9	___	4.2	5.0	5.6	6.2	7.2
		4	___	2.1	___	4.1	4.9	5.6	6.1	7.0
	4	1	___	1.9	___	4.0	4.7	5.5	5.9	6.7
		4	1.6	2.7	___	3.8	4.7	5.4	5.9	6.5
Pure Gold	1	1	___	1.8	___	4.3	4.9	5.5	6.0	6.8
		4	___	2.0	3.5	4.1	4.8	5.3	5.8	6.7
	4	1	0.4	2.4	___	4.2	4.8	5.4	6.1	7.3
		4	1.3	2.9	___	4.2	4.7	5.2	5.7	7.0
Avg		1	1.1	2.1	3.2	4.1	4.8	5.5	6.0	6.9
		4	1.7	2.2	3.2	4.1	4.7	5.4	5.9	6.7

* Mobilities in (cm^2/volt sec) (x 10^{-5}).

Table 10. Average* percentage composition of major protein constituents of seminal plasma.

Bull	Ejac	Electrophoretic component			
		4	5	6	7
Remark	1	24.7	38.1	35.1	2.5
	4	31.7	35.1	30.1	3.2
Climax	1	31.9	40.7	24.9	2.4
	4	42.6	34.5	21.3	1.7
Imperial	1	16.3	40.7	40.2	2.9
	4	12.9	44.9	40.3	2.1
Explorer	1	24.8	43.1	28.7	3.5
	4	28.3	42.9	25.7	3.2
Pure Gold	1	11.9	37.7	47.5	2.9
	4	28.3	34.4	34.4	2.8
Avg	1	22.0	40.1	35.3	2.8
	4	28.8	38.3	30.4	2.4

* Entries are averages of first and fourth week samples

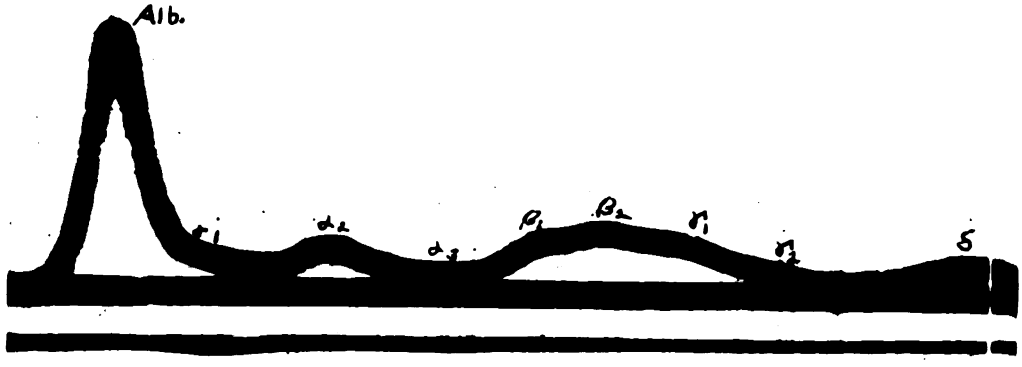


Figure 2. Electrophoretic pattern of bovine blood serum.

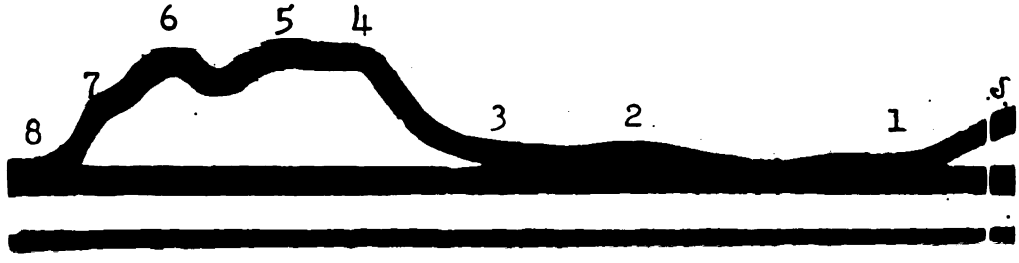


Figure 3. Electrophoretic pattern of seminal vesicular fluid.

Numbered components correspond to similarly numbered components in seminal plasma.

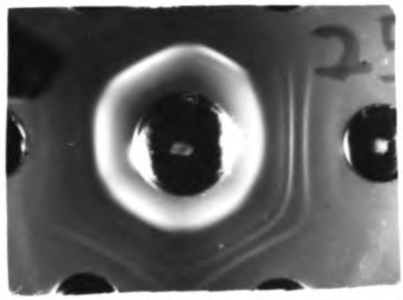
Figure 4. Ouchterlony titer plates.

Each plate was prepared in the following manner:

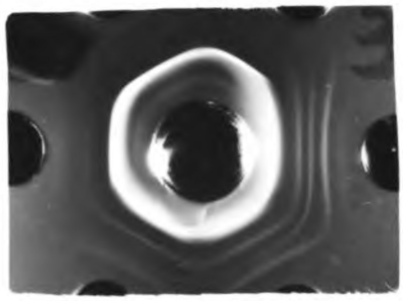
Center well: rabbit anti-bovine seminal plasma immune-serum

Outer wells: serial dilutions of bovine seminal plasma, well 1 at three o'clock 1:1, to well 6 at one o'clock 1:63

A. Remark, ejaculate 1
B. Remark, ejaculate 4
C. Climax, ejaculate 1
D. Climax, ejaculate 4
E. Imperial, ejaculate 1
F. Imperial, ejaculate 4
G. Explorer, ejaculate 1
H. Explorer, ejaculate 4
I. Pure Gold, ejaculate 1
J. Pure Gold, ejaculate 4



A



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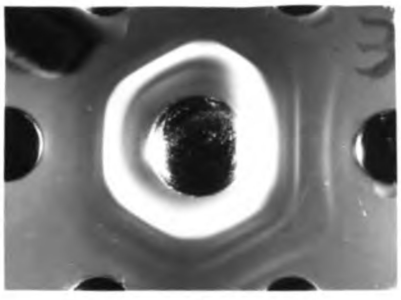

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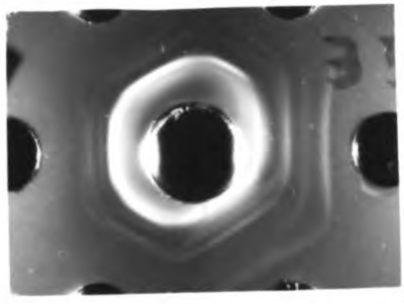

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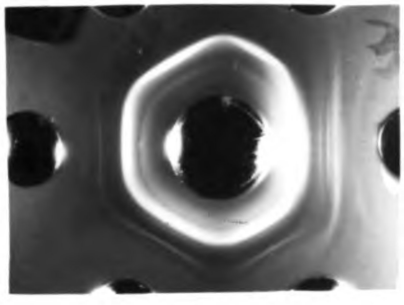

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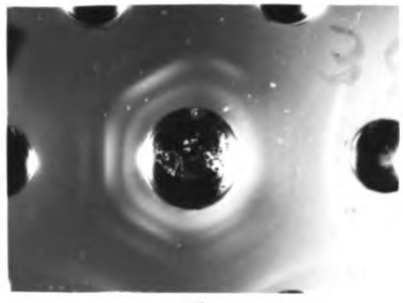

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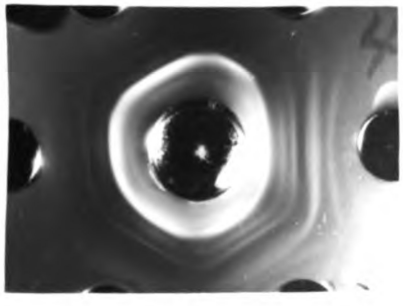

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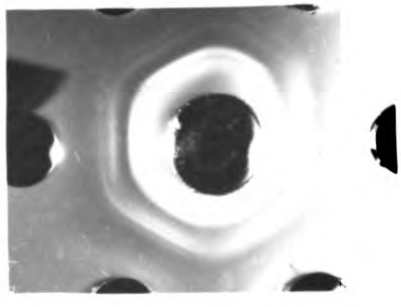

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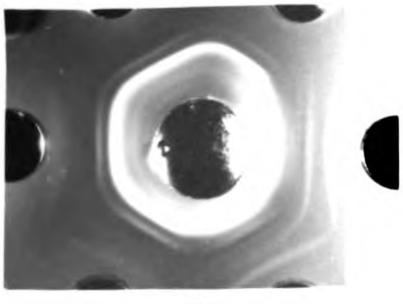

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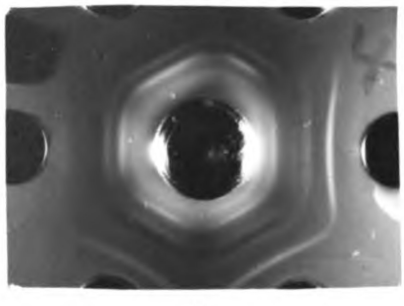


J

critical nature of the concentrations of the reactants necessary to obtain a precipitin line. It is also possible that some antigen-antibody complexes were soluble and consequently undetected.

Table 11. Number of precipitin lines visible in titer plates

Bull	Ejac	Rabbit				Maximum no. of lines
		1	2	3	4	
Remark	1	5	8	3	4	8
	4	5	4	5	7	7
Climax	1	8	7	7	7	8
	4	6	5	7	7	7
Imperial	1	6	8	7	-	8
	4	5	3	5	5	5
Explorer	1	4	7	6	8	8
	4	6	5	6	-	6
Pure Gold	1	4	8	5	7	8
	4	4	5	5	-	5

The average maximum of eight precipitin lines for seminal plasma from first ejaculates was significantly greater than the comparable average of six for fourth ejaculates

($P < .01$). The number of protein components demonstrated in the seminal plasma from first ejaculates by this immunochemical technique is in good agreement with the number of components demonstrated electrophoretically. However, the concentrations of some of the proteins from fourth ejaculates apparently were too low to be detected by the immunochemical technique, since seven electrophoretic components were found in seminal plasma from fourth ejaculates.

A photograph of a typical Ouchterlony "H-pattern" plate is displayed in figure 5. A continuous precipitin line such as that marked in figure 5 demonstrated a common protein antigen in the seminal plasma from first and fourth ejaculates. All precipitin lines appeared to be continuous when examined under magnification. These results indicated that there were no antigens in first ejaculate seminal plasma that were not also present in fourth ejaculate seminal plasma. However, as concluded above, the concentration of some of the proteins from fourth ejaculates were apparently too low to be detected by this technique.

The Bjorklund inhibition studies substantiated these results.

Figure 5. Typical "H" pattern Ouchterlony plate.

Well 1 (upper) Rabbit anti-sera to ejaculate one seminal plasma.

Well 2 (right) Ejaculate one seminal plasma.

Well 3 (lower) Rabbit anti-sera to ejaculate four seminal plasma.

Well 4 (left) Ejaculate four seminal plasma.

An antibody formed as the result of an antigen common to first and fourth ejaculate seminal plasma resulted in a continuous precipitin line (e.g., 1).

Figure 6. Cross reaction of bovine seminal plasma, blood serum, and their respective anti-sera.

Well 1 (upper) Rabbit anti-seminal plasma sera.

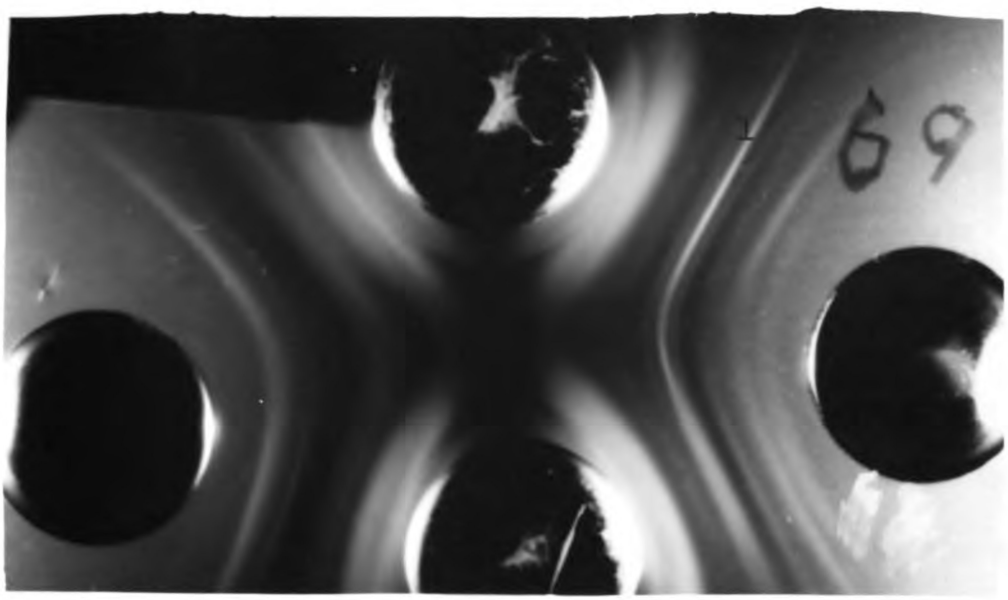
Well 2 (right) Pooled ejaculate one and ejaculate four seminal plasma.

Well 3 (lower) Rabbit anti-blood sera.

Well 4 (left) Bovine blood serum.

1. Precipitin line indicating an antigen common to seminal plasma, and blood serum.

2. Precipitin line indicating an antigen present in seminal plasma, but not in blood serum.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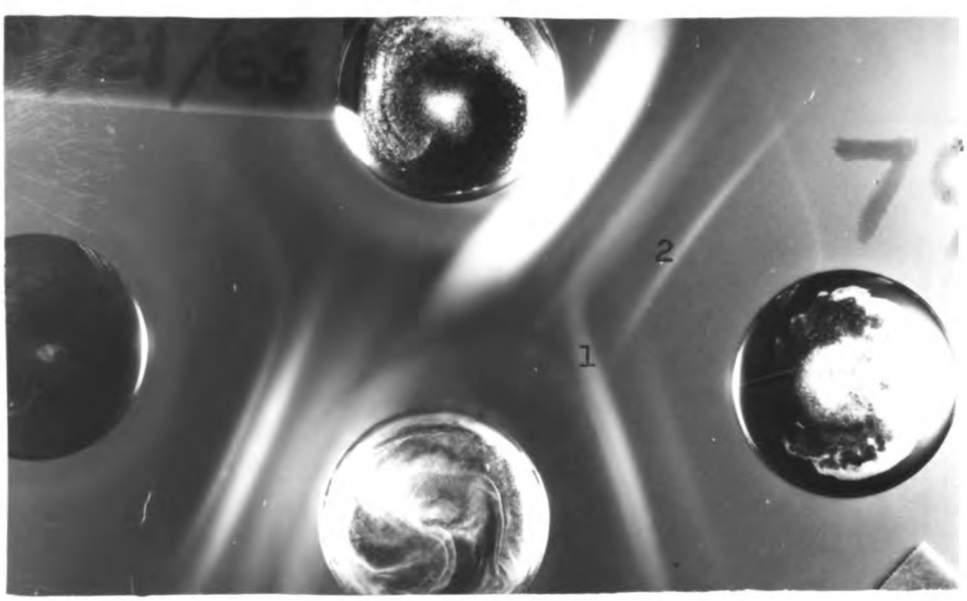
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Relationship Between Some Seminal Constituents:

When the average values for each ejaculate number were summarized, as in Table 12, there appeared to be a relationship between certain components. For example, the concentrations of fructose and citric acid appeared to be quite parallel, where-as the concentrations of ninhydrin-nitrogen and fructose appeared to have a negative relationship. Since such relationships could have value in determining the origin of certain constituents, correlation coefficients were determined by pairing values obtained for each ejaculate from each bull in each week, resulting in 80 paired values from which to estimate each correlation coefficient.

Table 12. Summary of averages of each seminal component measured in each ejaculate number.

Seminal Component	Ejaculate			
	1	2	3	4
Ml of semen/ejac	8.51	7.22	6.15	5.68
Sperm/ml of semen ($x10^9$)	1.80	1.23	0.69	0.56
Sperm/ejac ($x10^9$)	15.14	8.51	4.24	3.06
pH	6.26	6.47	6.75	6.92
Ninhydrin-N (µg/ml)	840	797	760	671
Ninhydrin-N (mg/ejac)	7.26	5.88	4.78	3.78
Fructose (mg/100 ml)	681	879	973	917
Fructose (mg/ejac)	61.8	64.6	60.8	50.2
Citric acid (mg/100 ml)	520	518	564	619
Citric Acid (mg/ejac)	45.8	38.0	34.7	36.0
Protein (gm/100 ml)	7.1	7.5	7.5	7.1
Protein (mg/ejac)	61.3	55.8	47.0	41.0

The correlation coefficients between the concentration of seminal constituents and volume of semen are listed in Table 13 with their significance levels. These values, while demonstrating some significant relationships, generally are too low for predictive purposes, with the single possible exception of the correlation of -0.81 between concentration of sperm and pH.

Although the correlations for the concentration values listed in Table 13 have more importance in terms of the welfare of ejaculated sperm, the comparable correlations for the total amounts of each seminal constituent per ejaculate (Table 14) have more significance in terms of the origin of the constituents and in terms of the relative contribution of the adnexal sex glands. The correlations shown in Table 14 are all considerably larger than their counterparts in Table 13.

The correlation of 0.94 between total protein and total ninhydrin-nitrogen indicated that the majority of each of these components has a common origin. On the other hand, since the ninhydrin test measured all free amino nitrogen, including that exposed on proteins, the correlation of 0.94 is higher than would be expected if the protein had been eliminated before the ninhydrin test was performed.

Table 13. Summary of correlation coefficients for the concentration of some seminal constituents and volume of semen.

	Sperm conc.	Citric acid	Fructose	Protein	NH_2-N	pH
Seminal vol.	0.29[a]	0.07	0.02	0.32[a]	0.41[a]	0.32[a]
Sperm conc.		-0.22[b]	-0.40[a]	-0.14	0.07	-0.81[a]
Citric acid			0.36[a]	-0.33[a]	0.13	0.02
Fructose				-0.27[b]	0.26[b]	0.19
Protein					0.16	0.14
NH_2-N						-0.27[b]

[a] $P_I < .01$

[b] $P_I < .05$

Table 14. Summary of correlation coefficients for the amount of some seminal constituents per ejaculate.

	Total sperm	Total citric acid	Total fructose	Total protein	Total NH_2-N
Seminal volume	0.69[a]	0.68[a]	0.64[a]	0.84[a]	0.82[a]
Total sperm		0.39[a]	0.26[b]	0.44[a]	0.53[a]
Total citric acid			0.61[a]	0.41[a]	0.64[a]
Total fructose				0.41[a]	0.68[a]
Total protein					0.94[a]

[a] $P_I < .01$

[b] $P_I < .05$

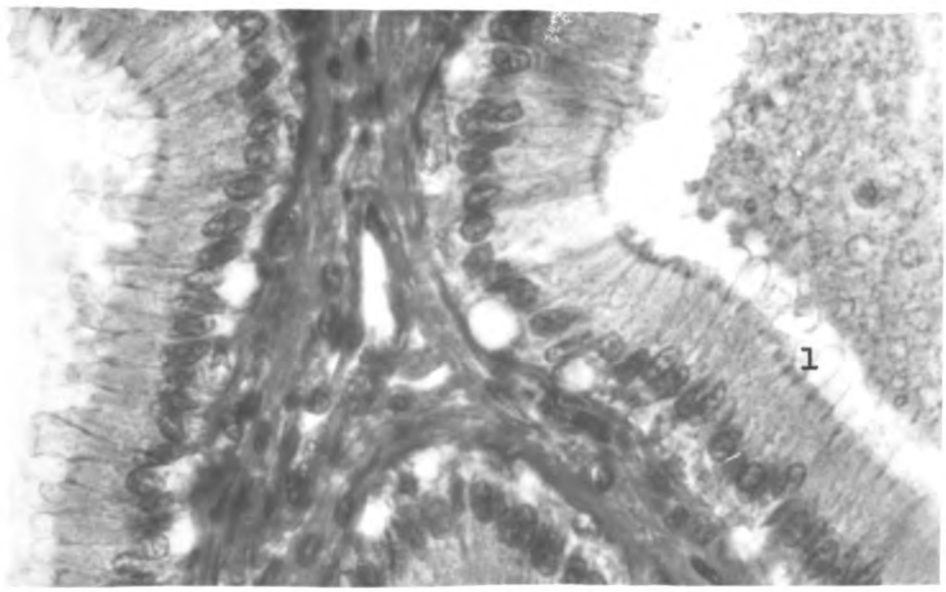
The magnitude and similarity of the correlation coefficients between total fructose and total citric acid, between total fructose and total ninhydrin-nitrogen, and between total ninhydrin-nitrogen and total citric seem indicative of a common origin for these three seminal constituents. However, each of these correlation coefficients account for less than half of the total variance associated with them and this fact strongly implicates multiple origins for at least two of the three constituents.

Some Histologic Observations on Remark:

Tissues from the various parts of the reproductive tract of the Remark bull were obtained at the time of slaughter and fixed in Bouin's fixative preparatory to histologic examination. Based upon these examinations, the testes, epididymides, ductus deferentia, prostate, and bulbo-urethral glands all closely resembled those repeatedly described in the literature (Bailey, 1958).

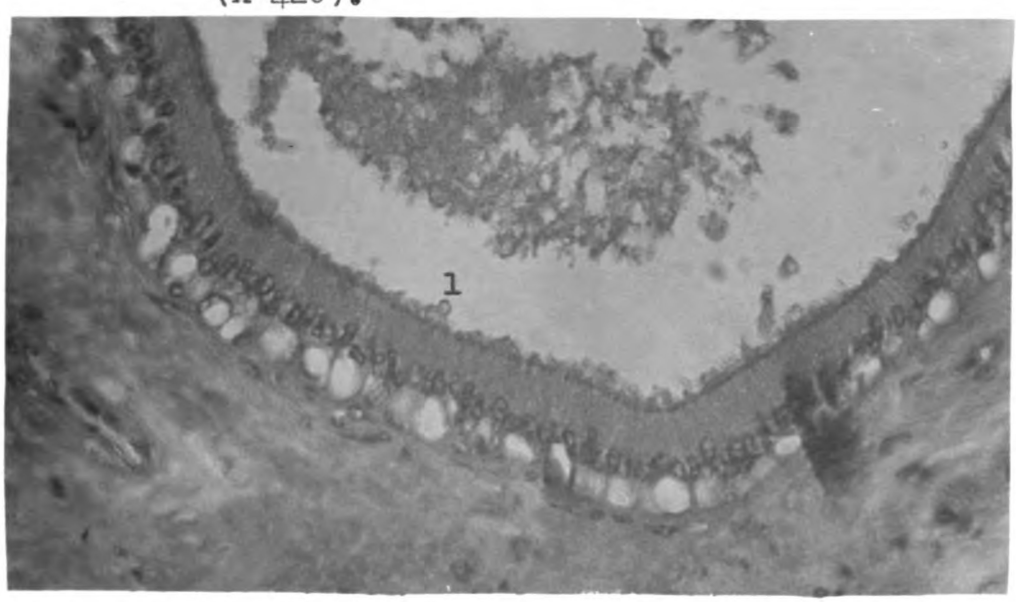
Micromorphologic examination of the seminal vesicles revealed three cell types corresponding to those described by Mann et al. (1948). The three cell types are identified in the photomicrograph of a seminal vesicle displayed in figure 7. Secretion blebs comparable to those mentioned by

Figure 7. Photomicrograph of bovine seminal vesicle (X 736).



1. Secretion bleb.

Figure 8. Photomicrograph of ampulla of ductus deferens (X 420).



1. Secretion bleb.

Mann et al. (1948) were present at the luminal extremity of many of the cells in the seminal vesicle. These blebs are pictured in figure 7 and appeared to represent an apocrine type of secretion.

Micromorphologic examination of the ampullae of the ductus deferentia revealed a striking similarity between the epithelium of this organ with that of the seminal vesicles. The photomicropgraph of an ampulla displayed in figure 8 identifies the same three cell types described for the seminal vesicles. Although this anatomical similarity has not been previously reported, Mann et al. (1948) reported that ampullar secretions were quite similar to seminal vesicular secretions.

SUMMARY

Five Holstein bulls, ranging from 2 to 5 yr of age, were ejaculated four consecutive times at weekly intervals for a period of 4 wk. Seminal volume, pH, and sperm concentration were determined and the sperm were removed by centrifugation. The concentrations of fructose, citric acid, protein, free amino nitrogen, and zinc in the seminal plasma were determined. The proteins were also studied electrophoretically and immunochemically.

As anticipated, seminal volume, sperm concentration, and total sperm per ejaculate decreased from ejaculate one to ejaculate four. There was a marked similarity in sperm output from week to week for each of the five bulls, indicating that such an ejaculation procedure could be used to predict a bull's sperm producing capacity.

The average pH of seminal plasma from first, second, third, and fourth ejaculates was 6.26, 6.46, 6.73, and 6.92, respectively ($P < .01$). Comparable averages for fructose in seminal plasma were 681, 879, 973, and 917 mg/100 ml, respectively ($P < .01$), and for citric acid were 520, 518, 564, and 619 mg/100 ml, respectively ($P < .01$). Neither the total fructose nor the total citric acid in the first, second, third, and fourth ejaculates differed significantly

($P > .20$). These data indicate that the seminal vesicle contributed a relative constant volume to each ejaculate. However, the relative contribution of the seminal vesicle increased from the first to the fourth ejaculates, presumably because of declining contributions from the other accessory sex organs.

The average concentration of zinc in first and fourth ejaculate seminal plasma did not differ significantly ($P > .50$). Although the average zinc concentration of 9.1 μ g/ml of seminal plasma was considerably larger than the average of 5.9 μ g/ml of blood serum, the seminal average did not approach the comparable values previously reported for some other species.

Average seminal free amino nitrogen, in first, second, third, and fourth ejaculates were 840, 797, 760, and 671 μ g/ml, respectively ($P < .01$). In view of the reportedly high concentration of amino acids in epididymal secretions, these results indicate that the epididymis contributes progressively lower proportions to the seminal plasma of successive ejaculates. The average concentration of protein in seminal plasma did not significantly change from the first to the fourth ejaculates.

Electrophoretic analysis of seminal plasma revealed eight protein components and six or seven of these were found in each ejaculate. No appreciable difference was found between the number of components in first and fourth ejaculates. Electrophoretic mobilities of the major protein components were not altered by repetitive ejaculation. Based upon the electrophoretic data, the percentage composition of two of four major protein components was altered by repetitive ejaculation ($P \cong .03$). One of the four major seminal plasma protein components had no electrophoretic counterpart in blood serum.

Immunochemical studies revealed a minimum of eight precipitating antigens in first ejaculate seminal plasma and a minimum of six in fourth ejaculate seminal plasma. However, the evidence indicated that the concentration of some minor constituents in fourth ejaculate seminal plasma was too low to be detected by this immunochemical technique.

Correlation of the measured components in seminal plasma revealed that the totals per ejaculate were, without exception, more highly correlated than the comparable concentration values. The magnitude and similarity of the correlations between total fructose and total citric acid ($r = 0.61$), between total fructose and total free amino nitrogen

(r = 0.68), and between total citric acid and total free amino nitrogen (r = 0.64) indicated a common major origin for these seminal constituents. The correlation between the concentration of sperm and pH was -0.81, suggesting that seminal hydrogen ions and sperm have similar origins.

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APPENDIX

Procedure A. Dithizone Zinc Determination (Johnson et al., 1959):

Preparation of reagents:

A. Ammonium hydroxide solution:

(1) 1N: Prepare a solution slightly stronger than required, then standardize against standard acid and dilute as required.

(2) 0.01 N: Dilute 10 ml solution (1) to one liter.

B. Ammonium citrate solution:

(1) Solution I (0.05 M): Dissolve 226 g ammonium citrate in 2,000 ml distilled water. Add concentrated ammonium hydroxide until the pH of the solution is 8.5-8.7 (80 to 85 ml).

(2) Solution II: Add 70 ml of I N ammonium hydroxide to 500 ml of Solution I (0.05 M) and dilute to 2,000 ml.

C. Carbamate solution:

Dissolve 1.25 g sodium diethyldithiocarbamate in distilled water and dilute to 1,000 ml. Store at 5^{o} C.

D. Diphenylthiocarbazone (dithizone):

(A) Concentrated solution: Dissolve 0.0375 g dithizone in 500 ml CCl_4. (Prepare fresh daily.)

(B) Dilute solution: Add one volume of the concentrated solution to four volumes of carbon tetrachloride.

Zinc determinations:

Twenty-five ml of concentrated HNO_3 was added to 2 ml of seminal plasma in a 150 ml Pyrex glass beaker. Each beaker was covered with a watch glass and allowed to react at room temperature for 15 min. The beakers were then transferred to a hot plate and heated to approximately 100° C with caution to prevent excessive frothing. Digestion was continued for about 2 hr until the color was light yellow.

Beakers were removed from the heat and cooled to room temperature, before addition of 6 ml of 70-72% perchloric acid. The samples were then refluxed vigorously until a colorless or pale yellow solution remained. If necessary, additional perchloric acid was added to prevent the samples from going to dryness. Upon completion of digestion, the samples were evaporated to dryness by placing the cover glass slightly to one side. The beakers were then removed

from the source of heat, and the cover glass and sides of the beaker washed down with distilled water.

The solution was made just basic to litmus with concentrated ammonium hydroxide, then just acid with concentrated hydrochloric acid, and two drops of acid were added in excess. The solution was transferred to a 100 ml volumetric flask and the beaker was washed several times with distilled water. One ml of 1.0 N HCl was added and the flask was filled to volume with distilled water. The pH was 1.8 to 2.2. Thirty to thirty-five ml of the solution from the volumetric flask was measured into a 1 X 8 in glass-stoppered Pyrex test tube. Ten ml of concentrated dithizone was added and the tube stoppered and shaken 1 min. The layers were allowed to separate. If the carbon tetrachloride layer was red or purple it was removed with a pipette and the dithizone extraction was repeated.

The following reagents were added to a 125 ml red, glass stoppered, Erlenmeyer flask in the order listed:

(1) 50 ml ammonium citrate solution II

(2) 5 ml carbamate reagent

(3) 15 ml of aqueous sample solution from the test tube

(4) 25 ml dithizone solution B.

The flask was stoppered, shaken vigorously for 1 min, the

layers were allowed to separate, and then the aqueous phase was decanted and discarded. Fifty ml of 0.01 N ammonium hydroxide was added to the flask and the flask was stoppered, shaken 30 sec, and the layers were allowed to separate. The aqueous phase was decanted and discarded. The ammonium hydroxide extraction was repeated and the aqueous phase was decanted just prior to determining the absorption of the organic phase.

A cuvette was filled with the organic phase and the absorption was read at 535 mμ. Two blanks were processed with the samples and all readings corrected for the average values obtained for the blanks. The corrected values were then compared to a previously constructed standard curve.

Procedure B. Preparation of Biuret Reagent (Gornall et al., 1949):

Place 1.5 g copper sulfate and 6.0 g sodium potassium tartrate in a 1,000 ml flask and add sufficient water to dissolve. Slowly add 300 ml 2.5 N sodium hydroxide. Add 1.0 g potassium iodide and dilute to 1,000 ml. Store anaerobically at 5^{o} C and discard when a dark precipitate forms.

Procedure C. Preparation of Sodium Veronal Buffer:

Add 5.594 g Barbitol and 41.2 g sodium Barbitol to 800 ml boiling water in a 2,000 ml vol flask and add glass distilled water to volume.

Procedure D. Computation of Electrophoretic Mobilities (μ):

Mobilities of the seminal plasma proteins were calculated by the formula:

$$\mu = \frac{v\ q\ c}{I\ \ R},$$

Where: μ mobility in (cm^2 /volt sec) ($\times 10^{-5}$)

v = velocity in cm/sec

q = cross section of cell (cm^2)

c = cell constant

I = amperes of current applied

R = resistance of protein solution (ohms)

Photographs used to compute mobilities were taken with a Polaroid camera. The starting boundry was recorded after compensation but before power was applied to the cell. Patterns were photographed twice during, and upon completion of each run. The negatives were projected with an enlarger, the patterns were traced on paper, and the displacement of each component from the reference boundry was measured. Migration of a particular component was determined

by the distance from the initial boundry to a point which bisected the curve representing the component. Velocity (v) was calculated by determining the migration (cm) per unit of time (sec).

Procedure E. Composition and Use of Adjuvant:

Immunization was accomplished using Freund's complete adjuvant and incomplete adjuvant (Bacto-Adjuvant, Difco Laboratories). The complete adjuvant contained 1.5 ml mannide monooleate, 8.5 ml paraffin oil, and 5 mg killed and dried Mycobacterium butyricum. The incomplete adjuvant contains the same quantities of mannide monooleate and paraffin oil, but no bacteria.

An oil in water emulsion of the antigen and adjuvant was prepared by emulsification in a Serval Omnimixer.

Appendix F. Preparation of Ouchterlony Plates:

Plates to be used for the double diffusion technique of Ouchterlony (1958) were prepared as follows:

1. Eight strips of filter paper (Whatman No. 1), measuring 1.0 by 0.5 in., were placed evenly around the rim of a 90 mm petri dish. A 24 in. 26 ga stainless steel wire was coiled so as to hold the

paper strips around the periphery of the petri dish.

2. Thirty ml of melted 0.85% agar[a], dissolved in phosphate-buffered saline, was poured into each plate. The phosphate-buffered saline was prepared by dissolving 1.4198 g of Na_2HPO_4 and 0.3233 g of KH_2PO_4 in 2,475 ml of 0.85% sodium chloride.
3. "H-pattern" wells were cut with a template and tube cutter (figure 9). Seven-well patterns were cut with a Feinberg No. 1801 agar cutter (figure 9). The bottoms of the wells were sealed with a few drops of molten agar.
4. Antigen and antibody solutions were added to the wells and the plates held at 5° C while the reactants diffused.

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[a] Agar Agar, No. 3 (Consolidate Labs.)

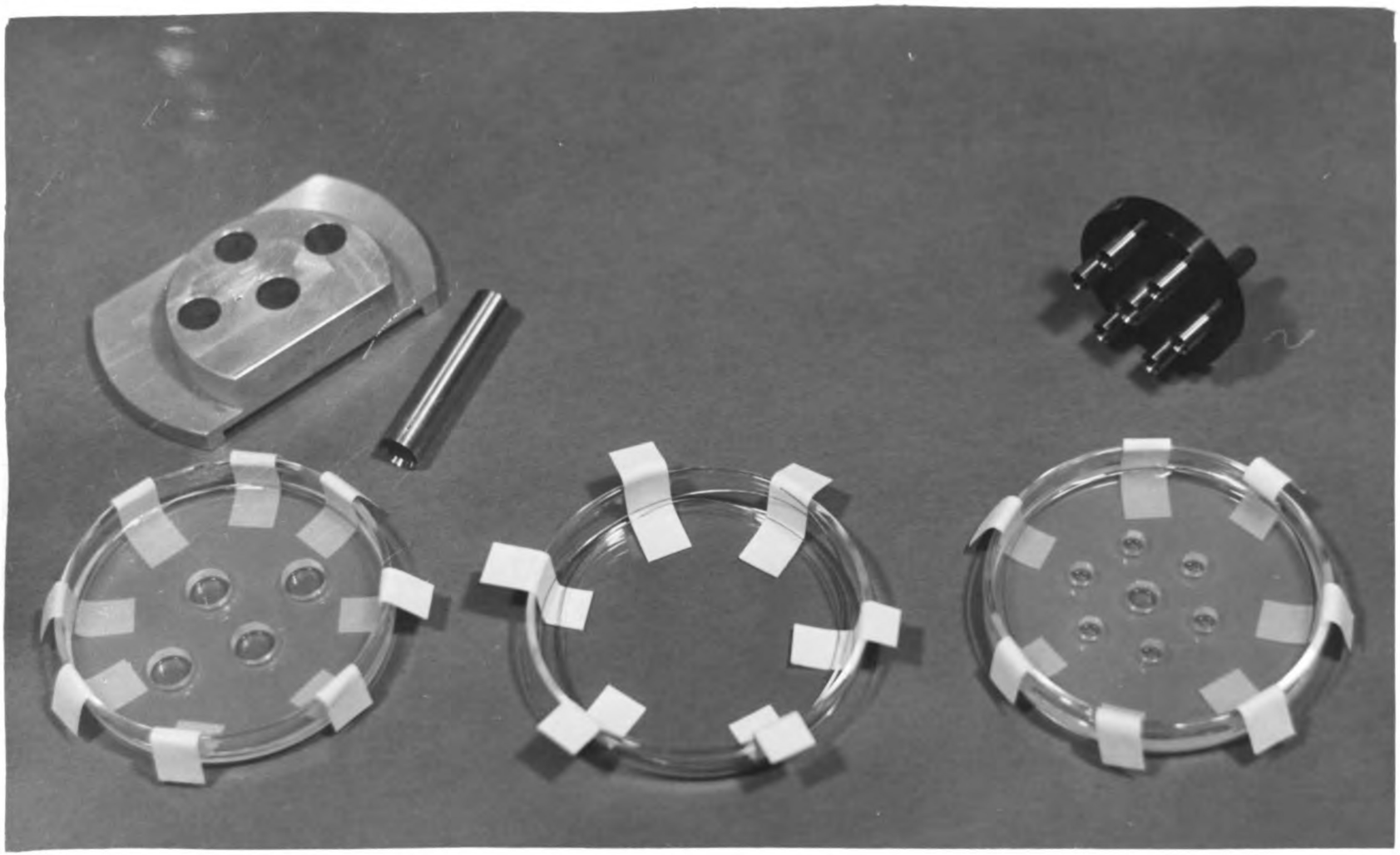

Figure 9. Templates and cutter used to prepare Ouchterlony plates.