EVALUATION OF THE SPERMATOZOA AND SEMINAL PLASMA OF THE INFERTILE MALE

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WITH present-day tests, the reasons for male infertility may be easily discernible, obscure, or impossible of detection. While it is true that azoospermia, oligospermia, asthenospermia, and the presence of a large number of abnormal forms are readily recognized causes of infertility, other reasons are less obvious. Biochemical or cytochemical technics have demonstrated other abnormalities of semen, the clinical implications of which are not completely understood, and corrective measures are not generally available. However, information is being accumulated, and with continuing research there is reason to hope that the problem will be solved.

The clinical study of the infertile couple as a unit was recently reviewed by Ward.[1] The present discussion is concerned solely with laboratory studies of spermatozoa and seminal plasma, the functions of the constituents of semen which are known, and how abnormalities of unit parts of whole semen may contribute to male infertility. For the sake of completeness, mention is made of the routine semen analysis; however, the primary purpose of this paper is to bring into focus other aspects of semen physiology which, when found to be abnormal, should certainly be considered as possible reasons for infertility.

Routine Semen Analysis

The semen analysis as it is customarily performed consists of the following steps: (1) measurement of volume, (2) determination of pH, (3) wet smear motility evaluation, (4) sperm count, (5) stained smear morphologic study, (6) a viability test 4 or more hours after the initial motility appraisal.[2-4]

Such an analysis will occasionally suffice to reveal the cause of infertility without question, as is the case when azoospermia is consistently found on repeated semen examination. Less confidently, one is suspicious of those specimens that repeatedly show gross reduction in sperm count and/or motility, or a large percentage of morphologically abnormal sperm. The outlook is especially bleak when all three of these defects repeatedly coexist. It is occasionally noticed that the ejaculate contains numerous spermatids, in which case suspicion is aroused that the infertility is due to faulty spermatogenesis.[5]

The contribution of all of these factors to male infertility is self-evident and does not need elaboration. Of decidedly more interest are those individuals whose

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semen examinations consistently indicate a good fertility potential but who remain infertile. If we direct our attention to cytochemical and biochemical studies of semen it becomes evident that many potential reasons for infertility exist which are rarely if ever routinely considered.

Cytochemical Demonstration of Inorganic and Organic Constituents of Spermatozoa

In 1954, Van Diujn[6] published a comprehensive study of the cytochemistry of the human spermatozoon. By special methods of microscopy, staining technics, protein digestion, and separation of components, he was able to identify and to pinpoint the location of the various organic and inorganic chemicals contained within the cell. The chemical substances and their locations within various parts of the spermatozoon are listed in *Table 1*. Although the constituents given are those he was able to identify by the methods he used, the list is undoubtedly far from complete as there is a notable lack of enzymes identified; nonetheless this considerable piece of work is a definite contribution to our knowledge of the chemical makeup of the sperm cell. It is probable that as our understanding expands, and knowledge of the enzyme systems within the cell increases, we shall discover enzyme deficiencies, such as are known for other metabolic systems, which might account for some cases of infertility.

Nuclear content of desoxyribosenucleic acid. Of singular importance to the normal physiology of any cell whether it be gametic or somatic is its nuclear content of desoxyribosenucleic acid (DNA). In accordance with its haploid state, the normal spermatozoon should have one half the amount of DNA of the normal diploid cell. Since the haploid cell should contain one half the chromosomal material of the diploid cell, deviations from this expected ratio would indicate loss of or faulty synthesis of DNA in the primordial germ cell, or unequal distribution, nondisjunction, or deletion of chromosomes during the reduction divisions. In order to determine the amount of DNA in individual spermatozoa of men of questionable fertility and men known to be fertile, Leuchtenberger, Schrader, Weir, and Gentile[7] employed quantitative cytochemical microspectrophotometric methods utilizing the Feulgen procedure for this purpose. Only cells that appeared to be cytologically normal were chosen for study. The results of this work showed that fertile men have a constant and uniform amount of DNA per sperm within and among individuals of this group. The mean amount of DNA in arbitrary units per sperm was 1.22 ± 0.005, or slightly less than one half that found in human somatic cells, which was 2.66 ± 0.05.

In contrast to this relatively constant amount of DNA in sperm of fertile men, the "infertile" group showed wide variations in amount of DNA. Since this study was based on the sterile couple and not on the male partner exclusively, it is not

Table 1.—*Location of chemical constituents of spermatozoa**

	Head						
	Nucleolus	Nucleus	Cyto-plasm	Neck	Midpiece	Axial filament	Tail†
Desoxyribonucleic acid	+	+	−	−	−	−	−
Ribonucleic acid	+	+	+	−	+	+	−
Arginine	+	+	+	−	−	−	−
Histidine	−	+	−	−	−	−	−
Histone	−	+	−	−	−	−	−
Tryptophane	−	−	+	−	−	−	−
Tyrosine	−	−	+	−	−	−	−
Sulfhydryl groups / Disulfate groups	+	−	−	+	+	−	+
Cholesterol	−	+	−	−	−	−	−
Lipoprotein	−	+	−	−	+	−	−
Lipoid	−	−	+	−	−	−	−
Lipids	−	−	−	−	−	−	+
Phospholipid	−	−	−	−	+	−	−
Mucoprotein	−	−	+	−	+	−	+
Protein	−	−	−	+	−	−	+
Fructose	−	−	+	−	−	−	−
Polysaccharose	−	−	−	+	−	−	−
Calcium	−	−	+	+	+	−	+
Copper	−	+	−	+	+	−	−
Iron	−	+	−	−	+	−	+
Magnesium	−	−	+	−	−	−	−
Phosphate	−	+	+	−	+	−	−
Potassium	+	−	+	−	+	−	+
Peroxidase	−	+	−	−	−	−	−

*+ = present; − = absent.
†The periodic structure of the tail was noted and was related to locomotor function.

surprising that a few of the individuals in the suspected group had normal DNA values; however, one half of them had sperm with impaired motility, which in itself may be a cause of sterility. The rest had abnormally low DNA values ranging from 0.76 to 1.05 arbitrary units per sperm or excessively high values in a range of 1.54 to 1.98 arbitrary units. These findings suggested a causal relationship between male infertility and abnormal DNA content of sperm cells. It should be pointed out that a number of these individuals would have been judged normal on the basis of routine semen analysis which was a concomitant part of this investigation. Going a step further and employing the same methods, Leuchtenberger, Weir, Schrader, and Leuchtenberger[8] demonstrated that the DNA content of primary spermatocytes, secondary spermatocytes, and spermatids of fertile men showed the expected values of 4 DNA, 2 DNA, and 1 DNA, respectively. In contrast, the infertile group had abnormal amounts of DNA in the primary spermatocytes; however, the secondary spermatocytes and spermatids while equally abnormal maintained their ratios of one-half and one-quarter DNA respectively of the primary spermatocytes. Thus the defect leading to infertility in these individuals did not seem to be based on faulty reduction divisions but rather on abnormal synthesis of DNA before meiosis.

Unfortunately, the determination of DNA by microspectrophotometry requires expensive equipment and highly skilled technicians; it is, therefore, not a practical test that can be done in the average laboratory. This should not prevent us from keeping in mind that there are recorded cases of infertility due to abnormal DNA synthesis.

Another cause of infertility related to DNA, and therefore to the chromosomes, appears to be due to unequal distribution of sex chromosomes of the four spermatids. Ferguson-Smith[9] recently reported on five distinct sex chromosome types leading to a clinical picture of Kleinfelter's syndrome or gonadal dysgenesis. Quite possibly a larger number of individuals than we suspect have sex chromosome anomalies contributing to or accounting for their infertility.

Seminal Plasma

The seminal plasma is a mixture of secretions from the accessory sex organs, which are under the hormonal control of the testes and hence secondarily the pituitary gland. Aside from being a vehicle in which to transport the sperm, the plasma is a complex mixture of chemical substances necessary to the health and well-being of the cells. A number of important constituents of this fluid have been identified, and in some cases their function has been elucidated. Chemical methods for analysis of some of these constituents have been outlined by Mann.[10] At least two important components of semen in addition to the germ cells come from the testes; these are the enzyme hyaluronidase and the three natural estrogenic hormones.

Hyaluronidase. Hyaluronic acid is depolymerized and hydrolyzed by hyaluroni-

dase. The follicle cells of the ovary are surrounded by and are embedded in a viscous gel believed to be composed of hyaluronic acid. This gel presumably must be removed to permit penetration of the ovum by the sperm. Hyaluronidase, prepared from several sources, when allowed to act directly on recently ovulated ova causes dispersion of the follicle cells and leaves the ovum denuded.[11]

This finding suggested that human semen deficient in hyaluronidase might be a cause of sterility. Kurzrok, Leonard, and Conrad[12] demonstrated on more than 400 semen specimens that hyaluronidase concentration increased directly with sperm count up to a cell population of 100 million sperms per milliliter. Beyond this number, hyaluronidase concentration did not increase proportionately. They also found that specimens containing less than 50 million cells per milliliter rarely contained the enzyme. Some specimens with excellent cell count had no hyaluronidase and were considered to be infertile; however, attempts to improve fertility by addition of hyaluronidase to oligospermic semen have not been successful. More recently, it has been shown in laboratory animals that the sperm penetrates the egg before there is complete dispersal of follicular cells;[13] therefore, at least in some animal species, complete denudation of the egg is not necessary for fertilization. In addition to this it has been shown that ovulation can be blocked with hyaluronidase inhibitors, and it has been suggested that the presence of these inhibitors, either in the semen or in the female genital tract, can cause sterility.[14] Yamane[15] has postulated from his studies that the intracellular action of hyaluronidase is probably of more importance than its extracellular role as a spreading factor. He has shown that hyaluronic acid exists in the egg cytoplasm. After the first maturation division the viscosity of the cytoplasm increases at the animal pole and the secondary oocyte is arrested at metaphase. The penetrating sperm carries with it hyaluronidase, which induces a local fall in viscosity of the cytoplasm at the animal pole, resulting in production of the second polar body. Thus, hyaluronidase appears to be indispensible to the maturation divisions of the germ cells; therefore, its absence in the sperm cell can prevent maturation from going to completion and can effectively inhibit the formation of the zygote.

The presence of hyaluronidase can be determined by several methods[16] with relative ease and there is no need for special equipment; however, the conditions of the assay method must be rigidly controlled and standardized in each laboratory to assure reproducible results.

Estrogens. The question of the nature of the second testicular hormone, that is, a hormone that would inhibit pituitary follicle-stimulating hormone, has been the subject of considerable speculation. Extracts of animal testes when injected into other animals have been shown to have estrogenic activity. Because of the difficulty inherent in obtaining sufficient human testicular tissue for assay, most investigators have turned to semen for such studies. In 1954, Diczfalusy[17] demonstrated by solvent partition, countercurrent distribution, and fluorometric analysis that whole

human semen contained the three natural estrogens, estradiol-17-β, estrone, and estriol. That these hormones are elaborated by the tubular elements of the testes seems likely, since seminiferous tubule destruction results in increased amounts of urinary follicle-stimulating hormone (FSH). Urinary assays for FSH thus become important in the evaluation of the state of the testicular tubules, in the absence of direct evidence from biopsy.

Gonadotropin assays are not difficult to perform, but have two major drawbacks that are: (1) the length of time involved (usually two weeks) to complete the test, and (2) the need for space to house animals for the bioassay.

Seminal estrogen determinations are neither practical nor feasible for the average laboratory to undertake.

The seminal plasma also contains the constituents contributed to it by the prostate gland. These are: citric acid, acid phosphatase, amino acids, fibrinogen (found before liquefaction but not after), fibrokinase, fibrinolysin (necessary for liquefaction), proteolytic enzymes, a bacteriostatic substance, zinc, and vitamins C and B.[18,19]

Citric acid. The reason for the high concentration of citric acid in semen is obscure; however, it is known that it disappears from seminal fluid after castration, and reappears with androgen therapy. Thus it is hormone-dependent and therefore a secondary sex characteristic. Its deficiency or absence in semen should lead one to a further evaluation of testicular Leydig cell function.

Acid phosphatase. Acid phosphatase, found in abundance in prostatic fluid, is known to function in at least two ways. It acts to liberate fructose from 6-phosphofructose by splitting off phosphoric acid thus making utilizable sugar available to the spermatozoa. In addition, when prostatic fluid is mixed with vesicular secretion this enzyme dephosphorylates phosphorylcholine, which is derived from the seminal vesicles, to liberate free choline. The concentration of choline gradually increases as semen is allowed to stand after ejaculation.

Both citric acid and acid phosphatase content of semen are readily determined in the laboratory. Since little is known of the function of citric acid in semen at this time, estimation of its level is only of value clinically as a reflection of androgen level. On the other hand, it would be of considerable value to know that acid phosphatase was present in sufficient quantities to assure adequate concentrations of fructose for sperm utilization.

The bulk of the seminal fluid is derived from the seminal vesicles. This fluid contributes the flavins, which give semen its yellow color, adds potassium in excess of sodium, and furnishes bicarbonate to act as a buffer.

Fructose. Fructose from the seminal vesicles is the main reducing substance of semen, and is the sugar normally utilized by sperm. It is derived from blood glucose which is converted to fructose through the action of phosphohexose isomerase and alkaline phosphatase present in male accessory gland tissue. Mann[10] calls the rate of fructose utilization the "index of fructalysis" and has found that this rate is

closely related to sperm density and motility. Fructose concentration is hormone-dependent, and can be used as an index of testicular androgen secretion. Adding to the total reducing power of semen are ascorbic acid, glutathione, and ergothionene from the seminal vesicles. The latter two contribute to this reducing power through their sulfhydryl groups, which may act to protect these groups within the spermatozoa.[20]

Metabolism of sperm. The presence of a complete cytochrome system in human spermatozoa was first demonstrated by Mann.[21] Despite this mechanism for aerobiosis, spermatozoa are anaerobic, and produce lactic acid as an end product of their metabolism whether or not they are in an atmosphere of oxygen.[22] Exposure of the cells to oxygen or air rapidly causes senescence, and cessation of motility. Additional proof that they are anaerobes lies in the fact that respiratory poisons have no effect on their motility.[23]

Since semen is exceedingly rich in reducing substances (fructose and ascorbic acid) and oxygen-binding substances (ergothionene and glutathione), oxygen can be diverted from cytochrome, permitting a reductive environment to prevail so that metabolism will proceed along glycolytic lines. It has been suggested that the more rapid oxidative metabolic pathways are reserved for the moment of fertilization, when a final burst of explosive energy may be of paramount importance.[23]

Immunologic properties of semen. The antigenicity of spermatozoa has long been recognized. More recently it has been shown that distinct antigens are also present in the seminal plasma.[24] Thus, the potential for infertility is introduced in two ways: (1) autoimmunity in the male, and (2) antigen-antibody response in the female.

Autoantibody production in the male is believed to occur in response to the release of antigens from injured or infected testes and/or their ducts. These antigens, carried by macrophage cells to plasma cells and lymphocytes, induce antibody production. This, in turn, causes agglutination and even cytolysis of sperm. Complete destruction of testicular tubules can occur if antigen combines with hapten, which results in an even greater antibody response. The result of this process is reduced fertility or complete sterility due to an autoimmune reaction.[24,25]

An autoimmune phenomenon can sometimes be detected by microscopic examination of a drop of semen wherein clumping of the sperm may be seen. Probably a more accurate test for this would be to mix the patient's blood serum with his own washed spermatozoa, if autoantibodies are present agglutination of the sperm should occur.

In the reverse situation, infertility of the marriage partners may result from antibody formation in the female against the male's sperm or seminal plasma antigens. It is postulated that after copulation the residual sperm that are not washed out immediately are phagocytized in the female genital tract or are carried via the fallopian tubes to the peritoneal cavity to be phagocytized there. In either

event antigens are set free to which the female responds by antibody production. Repeated copulations maintain the cycle of antigen release and antibody production which, within the female genital tract causes agglutination and cytolysis of sperm, as well as produces uterine contraction.[25]

If the antibodies in the wife are circulating, her blood serum should agglutinate her husband's washed spermatozoa. If they are tissue antibodies, a subdermal injection of a suspension of the husband's sperm should cause wheal formation. This test is quite simple to do, and yet it might point to the source of infertility in what otherwise appears to be a normal couple.

References

1. Ward, E. B.: Evaluation of infertile couple. Cleveland Clin. Quart. **28:** 189-196, 1961.
2. Hotchkiss, R. S.: Etiology and Diagnosis in Treatment of Infertility in Men, 1st ed. Pub. No. 53, American Lecture Series, Thompson, W. D., editor. Springfield, Illinois: Charles C Thomas, 1952, 73 p.; p. 30-42.
3. Kolmer, J. A.: Clinical Diagnosis by Laboratory Examinations, 3d ed. New York: Appleton-Century-Crofts, Inc., 1961, 543 p.; p. 265-269.
4. Miller, S. E., editor: A Textbook of Clinical Pathology, 5th ed. Baltimore: Williams & Wilkins Co., 1955, 1208 p.; p. 1100-1104.
5. Frank, I. N.; Benjamin, J. A., and Segerson, J. E.: Cytologic examination of semen. Fertil. & Steril. **5:** 217-226, 1954.
6. Van Duijn, C.: VIII. Cytomicrochemistry of human spermatozoa. J. Roy. Microscop. Soc. **74** (pt. 2): 69-107, 1954.
7. Leuchtenberger, C.; Schrader, F.; Weir, D. R., and Gentile, D. P.: Desoxyribosenucleic acid (DNA) content of spermatozoa of fertile and infertile human males. Chromosoma **6:** 61-78, 1953.
8. Leuchtenberger, C.; Weir, D. R.; Schrader, F., and Leuchtenberger, R.: Abnormal amounts of desoxyribosenucleic acid (DNA) in germ cells of human males with suspected infertility. Excerpta Medica **8:** 418, 1954.
9. Ferguson-Smith, M. A.: Chromosome abnormalities as cause of human infertility. Fertil. & Steril. **13:** 34-46, 1962.
10. Mann, T.: Chemical methods in analysis and evaluation of semen. Proc. Soc. Study Fertil. **3:** 50-55, 1951.
11. McClean, D., and Rowlands, I. W.: Role of hyaluronidase in fertilization. Nature **150:** 627-628, 1942.
12. Kurzrok, R.; Leonard, S. L., and Conrad, H.: Role of hyaluronidase in human infertility. Am. J. Med. **1:** 491-506, 1946.
13. Chang, M. C.: Mammalian fertilization and possibilities of its control. Acta endocrinol. suppl. **28:** 121-131, 1956.
14. Schubert, G., and Wohlzogen, F. X.: Fermentative Steurung der Ovulation. (Enzymatic control of ovulation.) Wien. med. Wchnschr. **109:** 267, 1959.

15. Yamane, J.: Primary significance of testicular hyaluronidase for fertilization in mammals, p. 70-72, *in* Sec. 1, Proc. Internat. Cong. Animal Reproduction, 3d Cambridge, England, 1956.

16. Tolksdorf, S.: In vitro determination of hyaluronidase, p. 425-457, *in* Glick, D., editor: Methods of Biochemical Analysis, vol. 1. New York: Interscience Publishers, Inc., 1954, 521 p.

17. Diczfalusy, E.: Characterization of the oestrogens in human semen. Acta endocrinol. **15:** 317-324, 1954.

18. Williams, W. W.: Biochemistry of semen. Internat. J. Fertil. **4:** 29-37, 1959.

19. MacLeod, J.: Current reviews: Human semen. Fertil. & Steril. **7:** 368-386, 1956.

20. Mann, T.: Biochemical aspects of semen, p. 1-8, *in* Wolstenholme, G.E.W., editor: Mammalian Germ Cells; Ciba Foundation Symposium. Boston: Little, Brown & Co. 1953, 302 p.

21. Mann, T.: Studies on metabolism of semen; cytochrome in human spermatozoa. Biochem. J. **48:** 386-388, 1951.

22. MacLeod, J.: Relation between metabolism and motility of human spermatozoa. Human Fertil. **7:** 129-141, 1942.

23. Gross, M.: Biochemical changes in reproductive cycle. Fertil. & Steril. **12:** 245-262, 1961.

24. Weil, A. J.: Antigens of adnexal glands of male genital tract. Fertil. & Steril. **12:** 538-543, 1961.

25. Katsh, S.: Pharmacology and immunology of human ejaculate. Internat. J. Fertil. **6:** 53-66, 1961.