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Protein Contributions of the Male Accessory Organs to the Composition of Human Seminal Plasma as Determined by High Resolution Two-Dimensional Electrophoresis

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PROTEIN CONTRIBUTIONS OF THE MALE ACCESSORY ORGANS TO THE COMPOSITION OF HUMAN SEMINAL PLASMA AS DETERMINED BY HIGH RESOLUTION TWO-DIMENSIONAL ELECTROPHORESIS

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ABSTRACT

PROTEIN CONTRIBUTIONS OF THE MALE ACCESSORY ORGANS TO THE COMPOSITION OF HUMAN SEMINAL PLASMA AS DETERMINED BY HIGH RESOLUTION TWO-DIMENSIONAL ELECTROPHORESIS

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Human Seminal Plasma was evaluated using a High Resolution Two-Dimensional Electrophoresis technique adapted by this laboratory. Seminal plasma from healthy volunteers with recently proven fertility were characterized using this technique to establish "normal" protein distribution patterns in the two-dimensional polyacrylamide gel slab. In addition, time studies were performed on selected specimens to determine the effects of liquefaction on seminal plasma protein composition within the first several hours after collection. Split ejaculates were collected for identification and elaboration of accessory organ components in the whole seminal plasma specimen. A more direct approach to accessory organ component analysis involved electrophoresis of semen from patients with known fertility disorders (i.e. reproductive organ dysgenesis or dysfunction), patients who have undergone surgical procedures (varicocele repair, vasectomy, trans-urethral

resection etc.), or specimens from patients who have produced secretions as a result of prostatic or seminal vesicle massage in the course of urological examinations. From these results, patterns of protein "spots" in the two-dimensional gel were associated with a particular accessory organ. Finally, seminal plasma from infertile individuals suffering from (idiopathic) polyzoospermia, hyperspermia, oligozoospermia, or azoospermia were evaluated.

DEDICATION

I would like to dedicate this work to my wife

Carol Marie

as well as to three other individuals who have had a great influence on my academic career:

Mr. Robert L. Householder
who started me on this journey,

Dr. John R. Vercellotti
who gave me guidance along the way, and

Dr. James H. Yuan
my advisor for the past six years.

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Dr. James Yuan has been my instructor, advisor, director, and councelor as well as a friend for the past six years. I'd like to thank him for the privilege of being his student, and for all of the support he has given me in the course of my studies and research.

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direction.

Others who have contributed to this effort include Sheri Coble from the Urology Associates' laboratory, Kathy Flagge and Rosita Acosta from the ODU Andrology Lab, and the many "nameless" patients and donors who provided specimens for this research.

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I. INTRODUCTION

> GOD created man in his image; in his image he created him; male and female he created them. God blessed them saying "Be fruitful and multiply..." That is why a man leaves his mother and father and clings to his wife, and the two of them become one body.
> (Genesis 1:27-28, 2:24)

Child bearing and midwifery in most ancient societies was a task generally left to women. Over the years however, these early practices gradually became formalized as a branch of medicine known as obstetrics. Women who had difficulty in conceiving, or who remained barren in their marriages, began consulting these specialized physicians in their desire to discover the reason for their "problem". Out of this grew the discipline of gynecology - that branch of medicine dealing with the bodily functions and diseases peculiar to women. Often, after finding no significant abnormalities on thorough examination of the woman, and ascertaining that her husband was producing "viable" semen based on rudimentary semen analysis, she was pronounced infertile for idiopathic reasons.

Today, in the United States, there are a surprisingly large number of couples desiring to "be fruitful and multiply", but who have remained childless because of fertility problems. It is now known however, that between

30% and 40% of the time it is the husband who is at fault (women still account for 50% of the problem, with the remaining portion being undiagnosed or attributed to both partners) (1,2).

The diagnosis of decreased male fertility has, until recently, been performed by a variety of medical specialists including gynecologists, urologists, endocrinologists, and even psychiatrists. As more and more is learned about the complexities of male reproduction, clinicians are becoming more dependent on a relatively new type of specialist: the Andrologist, or one who specializes in male fertility disorders.

A. The Male Reproductive System.

1. Developmental Aspects : The human male reproductive system consists of both the primary and secondary (or accessory) sex organs along with the external genitalia. Phenotypic development of an XY individual *in utero* is dependent upon proper hormonal stimulus of the bipotential gonad. Placental chorionic gonadotrophin (hCG) along with maternal lutinizing hormone (LH) cause mesenchymal cells (or the interstitial cells of Leydig) in the medullary region of the primitive gonad to begin producing testosterone which, along with its metabolites (primarily 5-alpha-dehydro-testosterone) are regionally concentrated by an androgen binding protein (ABP). This substance is produced by the adjacent somatic or Sertoli cells under the influence of

maternal follicle stimulating hormone (FSH). These developments normally occur at 6-8 weeks gestation. Simultaneous with the above events, gonocytes in the surrounding germinal epithelium begin proliferation and differentiation into spermatogonia.

Increased androgen levels cause further masculinization by insuring development of the Wolffian duct into efferent ductules, the epididymides, vasa deferentia and seminal vesicles. Similarly, FSH stimulates production of an inhibition factor which causes degeneration of the Mullerian duct system which would have developed into the uterus and oviducts in a genotypic (XX) female.

The development of male external sex organs also is influenced by testosterone. The enzyme 5-a-reductase present in genital skin converts testosterone to 5-a-dihydrotestosterone (DHT) which is responsible for mid-line fusion of the primoidial urethral folds and labio-scrotal swellings to form the penis (and associated urethral glands), the scrotum, and the prostate. Absence or deficiency of this enzyme results in defects of genital development (3,4).

Leydig cell activity is quiescent during childhood development, becoming active again at puberty with the development of secondary sexual characteristics and the onset of the final stages of spermatogenesis resulting in fertility. Defects at any stage of male development can result in subfertile individuals as can environmental factors and/or physical trauma in later life. Other reasons

for male subfertility will be discussed in subsequent paragraphs.

2. Male Sex Organs and Semen Production : The testes are located outside of the body cavity in the scrotal sac so that countercurrent heat exchange in the internal spermatic or cremasteric vein will allow the heat sensitive germinal epithelia to experience temperatures that are two to three degrees (Celsius) lower than the normal body temperature. This location does leave them more susceptible to mechanical injury and environmental radiation - both of which can severely hamper sperm production.

Developing spermatozoa are released into the coiled seminiferous tubules and conducted to the rete testes by the convergent tubuli recti (see Figure 1). Testicular seminal plasma (5,6) is initially formed in the seminiferous tubules and is probably a product of Sertoli cell and Leydig cell activity, as well as of the secretory epithelia of tubuli recti, rete testes, and possibly the efferent ducts. Testicular plasma is an isoosmotic fluid with the major solutes being the electrolytes sodium and chloride. The protein concentration is low: less than 20 g/dl in the bull (7). The major non-serum proteins in testicular plasma are thought to be Androgen Binding Protein (ABP) and Transferrin (TF). The Blood-Testes barrier was postulated in rats by Setchell et. al. in 1969 (8), and later observed in humans by Koskimies (9). This barrier is thought to be responsible for the fluid concentration of serum proteins in and around the seminiferous tubules. Conversely, this same barrier

FIGURE 1. The Male Reproductive System: a. Ureters; b. Ampulla; c. Seminal Vesicles; d. Prostate; e. Vas Deferens; f. Varicocele; g. Bulbo-Urethral (Cowper's) Gland; h. Caput Epididymis; i. Rete Testis; j. Cauda Epididymis; k. Testicle; l. Seminiferous Tubules; m. Urethral (Littre's) Glands; n. Urethra; o. Bladder. (From Glasser [1]).

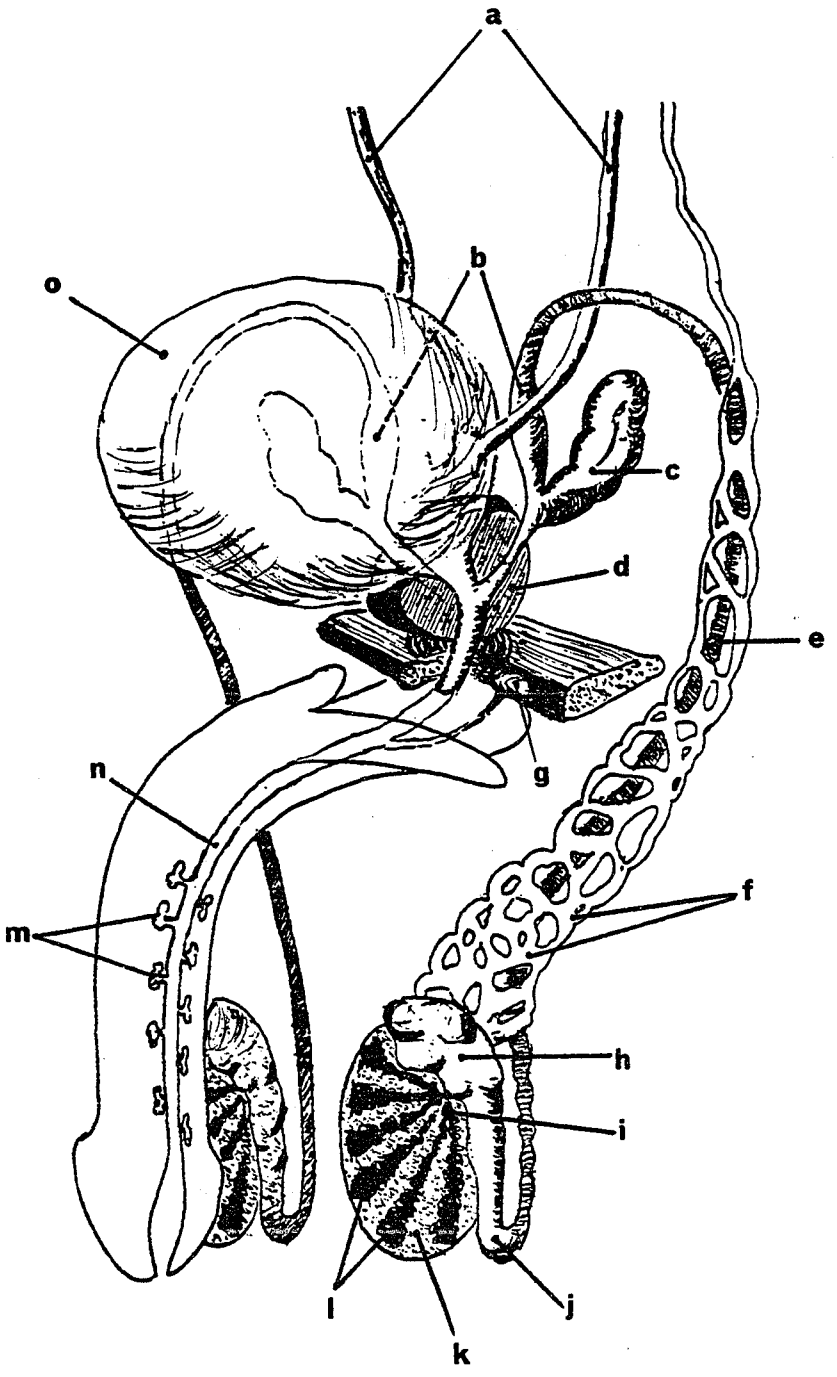

FIGURE 1. The Male Reproductive System.

prevents testicular components from coming in contact with the blood stream causing antibody formation.

Spermatozoa in testicular plasma are immature, nonmotile, and incapable of fertilization. Kinoplasmic droplets are observed at the junction of the head and neck region of these inmature forms. This kinoplasmic droplet contains the testes specific LDH-X; a sixth isozyme of lactate dehydrogenase not found in serum consisting of a "C4 homotetramer" as described by Music and Rossmann (5, p.103).

Upon passing from the ductuli efferentes into the caput epididymis, testicular plasma is rapidly concentrated by the absorption of fluids from the epididymal lumen. The human epididymides are coiled tubes approximetely 5 meters long and are generally divided into three regions: the head (caput), the body (corpus), and the tail (cauda). The resorptive capacity of the epididymis varies with location, but approximately 99% of the fluid leaving the testes is absorbed. Sperm maturation also takes place in the epididymis under the influence of testicular androgens produced by the interstitual (or Leydig) cells in the seminiferous tubules. These androgens are concentrated and transported into the caput by ABP. The epididymis itself is also dependent on testicular androgen for maintenance of structural integrity and physiological function.

Part of the spermatozoal maturation process in the epididymides includes the deposition of epididymis specific glycoproteins on the surface of the sperm cell (10,11). Spermatozoal maturation is also highly dependent on the

androgen controlled levels of sialomucoproteins and lipoproteins produced by the epididymides, thus explaining the high levels of glycosidases and other lysosomal proteins found in this region.

Another function of the epididymides is the removal of dead, dying, or otherwise defective spermatozoa primarily by phagocytic activity, but also by the action of lysosomal enzymes such as acid phosphatase, deoxyribonuclease, and proteases (12). Normal sperm are protected from these activities by their mucoprotein coat and also due to the presence of enzyme inhibitors. All of these degratory activities contribute to the cellular debris observed in ejaculated seminal plasma.

As spermatozoa progress through the corpus of the epididymis to the cauda, they shed their kinoplasmic or cytoplasmic droplets and become progressively more motile. Degeneration of these droplets by phagocytic or lysosomal activity results in the release of LDH-X and other cytoplasmic contents into the lumen of the epididymis thus leading to their possible detection in the ejaculated plasma (6, 13, 14).

Upon leaving the cauda of the epididymides, the semen enters the vasa deferentia which serve primarily as "conduits" from the testes up through the inguinal canal and on to the terminal dilitation known as the ampullae (located anterior to and slightly lower than the bladder in the vicinity of the prostate). The vasa deferentia also have limited secretory and resorptive capabilities: these

activities also being androgen dependent. Very little, if any, degratory activities occur in these organs.

During embryonic development, the terminal portion of each vas deferens becomes dilated to form the ampullary glands. At 13 weeks development, a "diverticlum" forms on each ampulla which develop into the seminal vesicles (SV)(3, 15). By the end of the second trimester, vas deferens and seminal vesicle structural development is essentially complete. By the end of the seventh fetal month, the seminal vesicles take on their adult form and begin secretory activities which continue until the child is between one and two years old. SV secretions then subside until the onset of puberty.

The ampulla serve as the primary storage recepticles for spermatozoa prior to ejaculation and not the seminal vesicles as originally believed. Although both glands may exhibit secretory activity, the seminal vesicles are the primary organs of secretion in the male reproductive tract (5, 16, 17). The bulk of the seminal plasma constituents produced at ejaculation are secreted by the seminal vesicles. Major HSP components assimilated by the seminal vesicles include fructose, and enzymes associated with semen coagulation as well as the prostaglandins (which were originally thought to be primarily products of the prostate). Secretory activity in the seminal vesicles is under strict androgenic control. Any abnormality in testosterone output seriously affects SV secretion.

Since the seminal vesicles and the vasa

deferentia/ampullae are of mesonephric duct origin, congenital disturbances may result in non-development of both of these organ systems. Failure to palpate the vasa deferentia on clinical examination of azoospermatic individuals may indicate a lack of the seminal vesicles as well. This can be verified by testing for fructose in the ejaculated semen. Since fructose is produced exclusively in these organs its absence is diagnostically significant.

The point at which each seminal vesicle stems from their respective ampullary gland gives rise to the contralateral ejaculatory ducts which converge in the prostate and open into the prostatic urethra at the verumontanum.

The prostate is probably the most well studied of the accessory organs because of its propensity for causing problems in later life. The prostate is the largest of the accessory glands, situated behind the pubic symphysis and surrounding the urethra as it leaves the base of the bladder. Embryonically, the prostate arises primarily from endodermal tissue in the urogenital sinus, but includes tissue arising from both the Wolffian and Mullerian ducts as well (18). Whereas seminal vesicle and other Wolffian duct tissue development is under the control of testosterone, prostatic endoderm does not begin development until testosterone is converted to 5-a-DHT by the emergence of 5-a-reductase activity in the urogenital tract. This differentiation is usually observed around 11-12 weeks gestational age.

McNeal (19) has described four major subdivisions of the adult prostate: 1) the non-glandular stroma, 2) the peripheral zone (surrounding the urethra), 3) the central zone (between the ejaculatory ducts and the proximal urethra), and 4) the pre-prostatic or transitional segment. Of these four divisions, the central zone is believed to be of Wolffian mesodermal origin and, as such, is least affected by the action of the reduced androgen DHT. The transitional area, on the other hand, represents a mixture of bladder and urogenital sinus germ layers and (like other transitional epithelia) is subject to dysplasias (e.g. benign prostatic hyperplasia - BPH) in later life as androgen/estrogen ratios change.

The peripheral zone is the principal site of prostatic carcinoma formation. Prostatic carcinogenesis, once developed, is androgen-dependent, with the cancer tissues taking up considerably more testosterone than the surrounding normal tissue (18). Merchant et al. (20), as well as others (21), have shown that the prostatic utricle - an estrogen sensitive Mullerian duct vestige - is on very rare occasions subject to a form of endometrial carcinoma emphasizing the significance of the embryonic origin of the various adult structures.

The prostate is an exocrine gland producing both apocrine and merocrine type secretions. Apocrine secretions occur when the apical portion of the glandular epithelia break down and release part of the cellular contents into the ductal lumen. This type of secretion is responsible for

the relatively high concentration of what are generally regarded as intracellular constituents present in the emitted seminal plasma.

The bulbo-urethral (or Cowper's) glands are situated in the urogenital diaphragm just below the prostate. Excretory ducts from these paired glands empty into the bulbous urethra. These glands are the male counterpart of the female Bartholin's glands (22). Since there are so few dysfunctions or other pathological conditions associated with these glands, they have not been well studied, and thus very little is known about their physiological role in male reproduction.

If little is known about Cowper's glands, even less is known about the urethral glands even though they were discovered by Alexis Littre in 1700! These numerous glands are found throughout the corpus spongiosum and empty into the cavernous urethra. Their development is not observed until late in the second trimester. The secretions of these glands, as well as the bulbo-urethral glands, may serve to buffer and lubricate the distal urethra prior to ejaculation, and in lower animals have been shown to exhibit pheromonal properties as well (5, p. 9).

3. The Production of Semen : As mentioned earlier, male fertility is not initiated until the onset of puberty. At that time, increased androgen levels signal the testes to begin the final stages of spermatogenesis and the accessory glands to once again begin secretion of seminal plasma.

As spermatozoa are released from the germinal cells in

the testes, they are carried to the epididymis by androgen-dependent neuromuscular contractions of the ductuli efferentes. It is here that they shed their kinoplasmic droplets and gradually become more motile and capable of fertilization as they approach the cauda. The testicular plasma in which these events take place is almost entirely reabsorbed by the epididymis leaving a high concentration of spermatozoa in an iso-osmotic fluid in which most of the osmotic force is provided by organic solutes such as glycerylphosphorylcholine (as opposed to the normal inorganic ions).

Neuromuscular contractions of the cauda expel the epididymal semen into the vasa deferentia which then transport the semen outward by weak contractions of the muscularis during periods of sexual inactivity or by strong paristaltic actions mediated by hypothalamic hormones and the sympathetic nervous system during sexual arousal. Spermatozoa are stored in the ampullae prior to ejaculation but may be detected in the urine with extended periods of continence.

Neurological mechanisms controlling ejaculation are quite complex. Kedia and Markland (23) have described three phases of the process; seminal emission, antegrade ejaculation, and projectile ejaculation.

Cortical response to afferent somatic input from the genitalia (via the pudental nerve) results in an increase in efferent sympathetic activity from the thoracolumbar spinal ganglia and hypogastric nerve. This increased neural output

causes smooth muscle contractions in the various accessory organs resulting in the secretion of their contents into the ductal lumina (seminal emission) followed by expression into the posterior urethra with partial bladder neck closure (antegrade ejaculation). Parasympathetic outflow (pelvic nerve) results in clonic striated muscle contractions of the various cavernosus muscles, as well as those in the pelvic floor and lower body. With complete closure of the bladder neck, the semen is rapidly propelled outwards in rhythmic projectile contractions.

Ejaculation is a sequential event with the various organs contributing their contents to the ejaculatory stream (generally) in a specific order. The urethral glands (Cowper's and Littre's) being in the distal portion of the urethra, release their mucilaginous secretions during the excitatory phase, and may be emitted (in part) as a pre-ejaculatory secretion prior to orgasm. These scant secretions may make up only 3-5% of the total semen volume.

Prostatic secretions constitute the majority of the first portion of the ejaculatory stream, comprising 15-20% of the total volume. The initial portion of the ejaculate is also high in spermatozoa along with their attendant epididymal and ampullary secretions. These constitute 7-10% of the total semen volume. The seminal vesicle secretions are emitted last and make up a majority of the total seminal plasma volume - approximately 70-80%.

Numerous researchers (24-31) over the years have made use of the "split ejaculate" technique in which semen is

collected in several discrete fractions. This technique has proven to be useful for evaluating the components of the various fractions and their attributed organ systems. First fractions, being rich in spermatozoa , have even been successfully used in insemination procedures - especially when an individual suffers from oligozoospermia as a result of hyperspermia (24). Figure 2 shows a typical distribution of three semen components in a split ejaculate.

Amelar and Hotchkiss (24) found that in approximately 6% of the individuals they sampled (5 out of 86) there was a reversal in the order in which spermatozoa were released, with the terminal portions of the ejaculate being richer in sperm than the initial portions. In two cases, pregnancy resulted when later fractions were used in AIH procedures. Mann et al. (32), has noted that ejaculate reversal is a cause of infertility in some animal species.

4. Semen Biochemistry : Probably the two most significant events mediated by the secretions of the various accessory glands are the coagulation and liquefaction of the specimen after ejaculation. Seminal coagulation is not observed in males suffering from congenital bilateral aplasia of the vasa deferentia and seminal vesicles (33). Observations of split ejaculates has shown that the first portion of the ejaculated stream does not coagulate at all, or, if it does, it liquefies immediately. Terminal portions of the stream, on the other hand, do coagulate immediately and require much longer periods to liquefy than a normal whole specimen. Addition of a portion of the first fraction

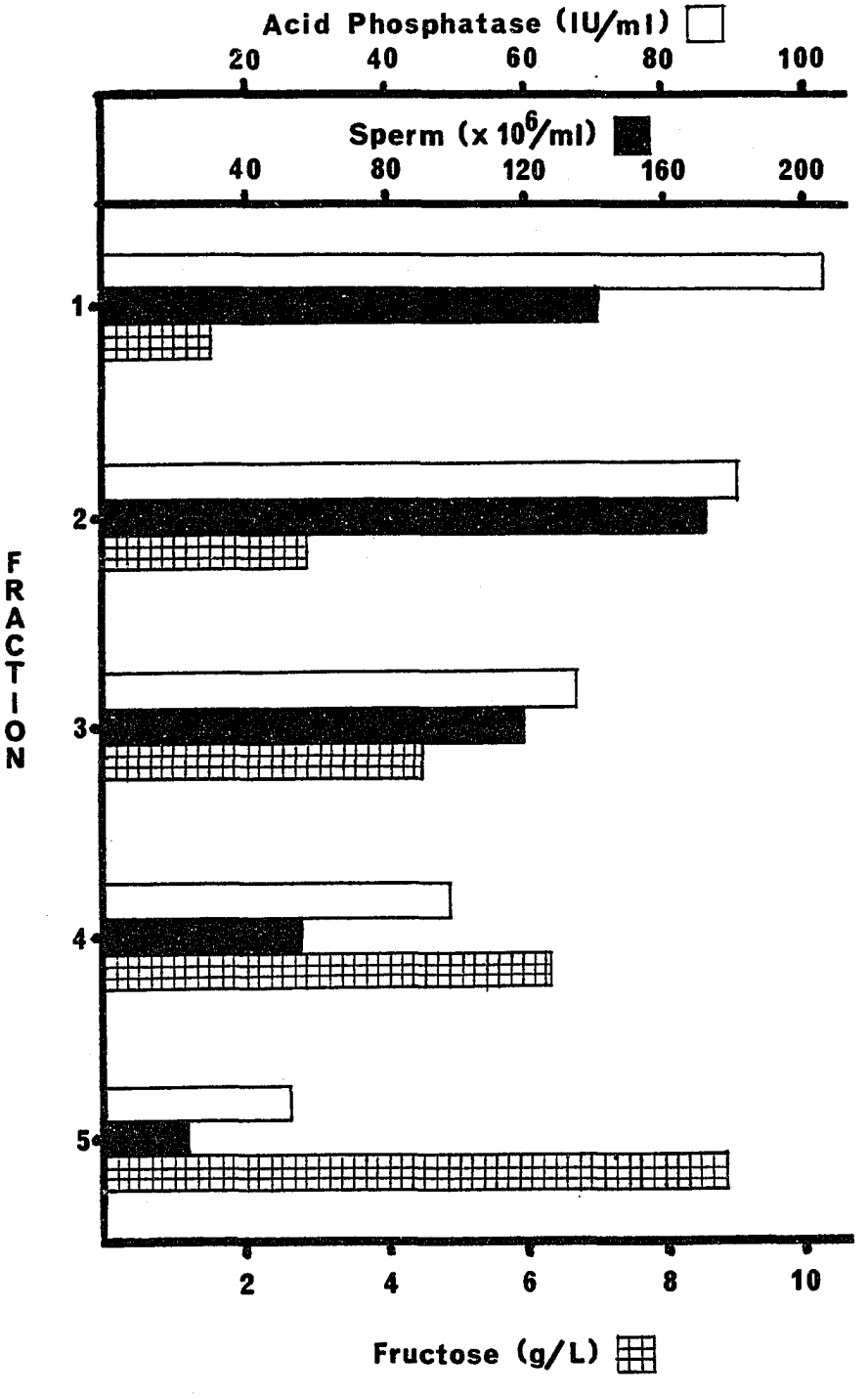

FIGURE 2. Distribution of Three Semen Components in the Split Ejaculate

to a terminal portion of the specimen results in rapid liquefaction. It is believed that the protein substrate and enzymes for coagulation are provided by the seminal vesicles while the prostate provides the liquefying agent(s).

The process of liquefaction was initially believed to be analogous to the fibrolytic activity of blood (34). However, recent reports show that this is not the case (although some blood components are found in HSP) (28, 30, 35-37). Koren and Lukac (36) found that a collagenase-like protein (CLP) may be responsible for the initial phase of liquefaction with further seminal "clot" liquefaction attributed to a seminal proteinase.

Table 1 lists some of the common components found in semen in association with their probable gland of origin. Mann and Lutwak-Mann have noted (5, 17) that some of the components of seminal plasma are generally considered by biochemists to be associated with plants rather than animals, particularly fructose, citrate, and glycerylphosphorylcholine (which is especially abundant in potatoes!). In addition, some of the proteins found in seminal plasma are associated with intracellular contents as opposed to extracellular material. This can be explained (as mentioned earlier) by the fact that apocrine type secretions occur in both the prostate and the seminal vesicles. This action can also account for the relatively large amount of insoluable particulate matter and cellular debris observed during microscopic examination of semen.

A great variety of compounds have been identified in

GLAND	Vol %	PROTEINS	NON-PROTEINS
Littre's and Cowper's	3-5	Sialo-Muco Protein Secretory IgA (SC, J piece?)	
Prostate	15-20	Acid Phosphatase Creatinine Kinase γ-Glutamyl transferase Lysozyme Prostate Binding Protein Liquefaction Enzymes	Citric Acid Zinc Polyamines
Testes, Epididymis, Vasa Deferentia, and Ampulla	7-10	Spermatozoa Androgen Binding Protein LDH-X	Transferrin Phosphorlyglyceryl Choline Androgens
Seminal Vesicles	70-80	Coagulation Enzymes Lactoferrin	Fructose Prostaglandins

TABLE 1. Common Semen Components and Their Probable Accessory Gland of Origin

semen - the listing in Table 1 is far from complete (see also 26, 38, 39). For the purposes of this discussion, however, emphasis will be given to some of the major protein constituents which may be visualized by electrophoretic techniques.

Human prostatic acid phosphatase (E.C. 3.1.3.2) is probably the most widely known and studied enzyme present in semen. It was discovered in 1935 by Kutcher and Wolberg (5, p. 285) in prostatic secretions and has since been shown to be "one of the most important secretory, non-lysosomal products of the prostate gland" (40). Secretion of this enzyme is androgen-dependent and its appearance in serum in increased amounts is indicative of prostatic carcinoma. Unfortunately, detection of increased serum levels is not apparent until the malignancy is well established (41).

Human prostatic acid phosphatase (PAP) is a dimeric sialoprotein with a molecular weight of 100,000 Daltons. The subunits are identical (MW 50,000 Daltons) and have carbohydrate compositions similar to other secreted glycoproteins. The isoelectric point (pI) varies between 4.1 and 5.2, with this microheterogeneity being abolished upon treatment with neuraminidase to yield a single protein with a pI of 6.0 (40, 42). PAP is also a metallo-enzyme requiring zinc for activity.

Dermer, Silverman, and Chapman (43) identified a series of protein components in human prostatic fluid with a molecular mass of around 40,000 Daltons and an acidic pI. They have tentatively identified these proteins as the BB

isozymes of creatine kinase (CK-BB). CK-BB is present in relatively high levels (50,000 ug/L) in prostatic fluid.

Tauber et al. (30) reported that lysozyme is an enzyme present in initial fractions of a split ejaculate and thus might be prostatic in origin. Lysozyme is a monomeric protein with four intra-chain disulfide linkages. It has a molecular weight of around 14000 Daltons, and in its native state is positively charged with an isoelectric point of 11.

Rosalki and Rowe (44, 45) described a protein present in seminal plasma with a distribution similar to that of prostatic fluid proteins. This enzyme was determined to be gamma-Glutamyltransferase (gGT). They reported that this enzyme was present in seminal plasma in levels almost 300 times greater than are found in blood serum. The molecular weight of this enzyme is approximately 80000 Daltons.

Another protein found in the prostate with a lower binding affinity for androgens has been variously called Prostate Binding Protein (PBP) or Prostatein (46, 47). Carter and Resnic (48) have recently shown that a component of human prostatic fluid closely resembles that of PBP identified in rat prostatic fluid. This protein has a molecular weight of approximately 17,000 Daltons and a pI of about 5.8.

Human serum transferrin (hTf) is monomeric protein capable of binding two atoms of ferric iron. It consists of 678 amino acids with a molecular weight of 79,500 Daltons, and consists of two homologous domains - one associated with each metal binding site. There are approximately seven

disulfide linkages and two asparagine-linked glycans in the molecule (49). Transferrin mediates iron exchange in the body and is probably one of the major secretory products of the Sertoli cells in the testes (50-55).

It has been proposed that, since the so called "blood testes barrier" (the manifestation of Sertoli cell tight junctions) prevents movement of macromolecules such as hTf from serum to the tubular lumen, testicular transferrin is secreted by the Sertoli cells to serve as a "source of iron for the heme proteins or for non-heme metalloproteins in developing germinal cells" (53).

There has been much discussion about whether or not transferrin detected in seminal plasma originates in the testes, or other organs like the prostate, or both. If the detected Tf is primarily of testicular origin, it may well serve as a marker of Sertoli cell function (50, 55).

Androgen Binding Protein (ABP) is another important protein secreted by the Sertoli cells. ABP is a glycoprotein with a molecular weight of approximately 90,000 Daltons. Its production by Sertoli cells in culture is stimulated by FSH (56). ABP (also known as Sex Hormone Binding Globulin - SHBG) selectively binds testosterone, and to a greater extent, DHT, allowing these androgens to be accumulated in the seminiferous epithelium and in the vicinity of differentiating germinal cells (5, p. 94). Sertoli cell ABP is also responsible for transport of testosterone and DHT to the epididymis (5, p. 143).

ABP is also found in the prostate as well as the other

accessory organs. Again, this protein allows for the accumulation of androgen in these tissues so that the appropriate steroid hormone/tissue receptor complexes may be formed allowing for the regulation of mRNA production and thus protein synthesis.

As previously mentioned, LDH-X is closely associated with developing spermatozoa. It is primarily a mitochondrial enzyme with broad substrate specificity. It is a tetrameric protein made up of four identical testes-specific C subunits. LDH-X is a basic protein with an overall molecular weight of 140000 Daltons and a subunit weight of between 31 and 38 kDal (13).

Along with providing the bulk of the secretions in the ejaculated semen, the seminal vesicles secrete both the substrate and enzymes responsible for semen coagulation. Tauber et al. (30) reported that lactoferrin (Lf) shows a seminal vesicular distribution as well. This glycoprotein has a molecular weight of 70000 Daltons, and, like hTf, can bind two atoms of iron. Unlike hTf however, the Lf-Fe complex is more stable at acidic pH's. The lactoferrin concentration in semen is similar to that of human milk. These two forms are not identical however (5, p. 280).

B. Semen Analysis.

Andrological evaluation of human semen is often used to determine the quality of the specimen produced by a given individual so that an assesment can be made about his ability to impregnate his wife. This assesment is generally made after repeated or prolonged attempts at conception have

resulted in a barren union. The goal of semen analysis in this instance then, is to determine whether the inability to procreate lies with the husband, the wife, or (as is often the case) with both.

1. Historical Aspects : Leeuwenhoek did the first microscopic analysis of semen in 1677, when he observed numerous "seminal animalcules" swimming around along with crystals of what later was determined to be spermine. Over a century later, Vauquelin published a treatis on the uniqueness of semen in 1791. In the interim, anatomists were defining the various reproductive organs in the body: William Cowper described the bulbo-urethral glands in 1698; Alexis Littre described the urethral glands (1700); and John Hunter studied the effects of castration on the seminal vesicles and prostate in 1786 (5, pp. 1-10).

The methods of semen analysis have improved significantly from these early observations, but progress has been slow - due possibly to the nature of the specimen and the manner in which it is collected. Biblical injunctions against "spilling of the seed" (Gn 38:11), as well as social customs and religious repression no doubt have had an influence on the development of assay procedures over the centuries, especially in earlier times (57). Masturbation is, of course, the method of choice for the collection of semen since latex condoms contain agents which adversely affect sperm viability, and coitus interuptus (withdrawal) is prone to contamination from vaginal secretions as well as loss of a portion of the specimen

(29).

2. Traditional Semen Analysis : In the past, semen analysis has relied on sperm count, motility and morphological studies, and microbial analysis to evaluate one's fertility status. Abnormalities found with any of these factors may provide the physician with a probable cause for infertility. Often, no overt abnormality can be found, however.

Sperm count has long been used as an indication of fertility. An individual is generally considered "fertile" if his sperm count falls within a certain range (usually 20-50 million/milliliter as a lower limit with 250 million/milliliter as an upper limit). Oligozoospermic individuals are those whose counts fall below the lower limits of normal , while those with counts above that range are termed polyzoospermic. Polyzoospermia has been correlated with significant increases in miscarriages and other abnormalities in pregnancy (5, p. 71).

Although sperm numbers are important, even Leeuwoenhouk noted in 1685, that they must have sufficient viability to survive in the womb (5, p. 1). Sperm motility and rate of forward progression are important parameters in semen evaluation. Large numbers of non-motile or poorly motile sperm are hardly conducive to pregnancy. Many factors may contribute to poor sperm motility: sperm immaturity, incomplete seminal plasma liquefaction, high post-liquefaction viscosity, and immunological agglutination, just to name a few.

Semen volume is also as important as sperm numbers. Most andrologists consider sperm concentration to be more important than total sperm numbers (1). The fact that the spermatozoa are released early in the ejaculatory process (as mentioned previously) has led to the successful use of initial portions of the ejaculate for insemination procedures. These hyperspermic individuals may suffer from decreased fertility because of sperm dilution or "washout" by terminal portions of the ejaculatory stream. Hypospermia - decreased seminal plasma volume - can also contribute to subfertility by failing to provide sufficient matrix for the spermatozoa to attain adequate vaginal placement.

Next to the physiological adequacy of the spermatozoa after successful spermatogenesis, the seminal plasma is probably the most important factor in determining male fertility. It not only serves as a fluid matrix to the spermatozoa for physical transport from the male's reproductive system to the female's, but also serves to provide nutrients, protection in a hostile environment, final maturation and/or capacitation factors, buffering action, etc. Thus, much information can be gained from biochemical analysis of the plasma as well as from the more traditional cellular studies performed on the sperm.

3. Biochemical Analysis : Biochemical analysis of seminal plasma has come into greater use in recent years as more and more information is gained about the complexities of human reproduction. Clinicians have known about the value of both qualitative and quantitative determination of

such components as fructose, citric acid, and acid phosphatase for more than half a century. More recently, new and improved methodologies have allowed for the determination of a wide variety of substances in ever decreasing amounts (c.f. Table 1). Immunological assays not only allow for the determination of antibodies present in seminal plasma and blood (such as the Franklin-Dukes and Isojima procedures for detecting microagglutination and sperm immobilization antibodies [58]), but provide a sensitive means of analyzing for other components as well using specific antibodies coupled with enzyme probes, fluorescent markers, or radioisotope labels as a means of detection.

Although such procedures have proven to be of great clinical value, they are generally time consuming, require much specimen, and each assay only provides information about the analyte in question. If the physician desires information about several different components, a separate procedure is usually required for each result requested.

4. High Resolution Two-Dimensional Electrophoresis (HR2DE) as a Technique for the Analysis of Seminal Plasma Protein Constituents : HR2DE is a technique whereby a small volume of seminal plasma (50-100 µl) can provide both qualitative and semi-quantitative (for the time being at least) information on all proteins present in seminal plasma at a concentration of at least a nanogram per milliliter of specimen. This technique can routinly resolve over several hundred HSP proteins on a single analysis.

HR2DE is a relatively new technique, being first used by O'Ferrell in 1975 (59). Proteins are first separated by their isoelectric point in an ampholyte gradient polyacrylamide gel under denaturing and dissociating conditions. The proteins are denatured to their primary amino acid configuration by the actions of 8 Molar urea and anionic detergents. Disruption of inter- and intra-chain disulfide bonds is accomplished by the addition of the reducing substance 2-mercaptoethanol. The pH gradient is established in the cylindrical polyacrylamide gel by the addition of ampholytes - polyamino sulfonic acids of varying chain lengths. The seminal plasma proteins migrate through the pH gradient until they reach a point where they are electrically neutral, at which point they cease to move and form a tight protein band.

After the first dimension separation, the cylindrical gel containing the separated HSP proteins are then mounted orthagonally to SDS-polyacrylamide concentration gradient "slab" gels for a second separation based on protein size. The anionic detergent sodium dodecylsulphate (SDS) causes the denatured protein to be surrounded with a uniform negative charge so that the primary separating force in the second dimension is the sieving action of the gradient gel rather than electromotive attraction. The resulting two-dimensional gel - or electropherogram - is then stained by various techniques for visualization of the separated proteins. In 1977, and 1978, Anderson et al. published further refinements to this technique (dubbing it the

ISODALT technique) and proposed its use as a method suitable for the ambitious task of mapping the entire human genome (60-63)!

To date, slight variations to this technique have been used by numerous researchers to analyze the protein constituents in complex biological specimens such as blood serum (60), urine (64-66), cerebrospinal fluid (67), cell lysates (68, 69), and tissue homogenates (70). Our laboratory adapted this technique for the analysis of human seminal plasma between 1979 and 1980 (71, 72). Edwards, Tollaksen and Anderson (73) used their ISODALT procedure along with the BASODALT procedure of Willard et al. (74) to look at the effects of liquefaction on seminal plasma composition in 1981. Their ISODALT procedure produced seminal plasma protein distributions very similar to ours. In their report they described a series of basic proteins which they could observe in the ISODALT gels but were better resolved with the BASODALT technique. These proteins were observed to disappear with time and it was proposed that they were associated with semen liquefaction. In addition, they described another group of proteins ("Group A") that they believed were associated with testicular secretions, since they appeared to be absent in the semen of a limited series of post-vasectomy donors when compared with "normal" seminal plasma..

Carter and Resnic (48) as well as Dermer et al. (43) used two-dimensional electrophoresis to investigate the protein content of prostatic fluid in 1982, with the

identification of some of the proteins mentioned previously (e.g. PBP, CK-BB, etc.).

A recent search of the available literature has found few others who are doing work in the area of whole seminal plasma electrophoresis. Thus, the field is ripe for continued work in this area.

II. STATEMENT OF PURPOSE

It has been the purpose of this project to provide a method of seminal plasma analysis providing comprehensive biochemical information about the protein components in human semen which may possibly impact on patient fertility. Existing techniques currently only provide information about individual seminal plasma constituents - generally with a separate analysis required for each component requested. On a single analysis however, High Resolution Two-Dimensional Electrophoresis (HR2DE) is capable of simultaneously resolving over several hundred seminal plasma components.

The goals of this study have been to a.) establish a "normal" seminal plasma protein distribution pattern in the resulting two-dimensional electropherogram, b.) to observe the effects of semen coagulum liquefaction on the distribution of proteins in the seminal plasma electropherograms with respect to time, c.) to differentiate the proteins in the electropherogram according to their accessory organ of origin, and d.) to observe protein differences associated with several classes of subfertile or infertile individuals.

Hopefully, the information presented here will allow the Andrologist and other clinicians to gain a better insight into the pathophysiology associated with reduced

fertility which, when properly managed, may ultimately lead to improved rates of conception in a given individual.

III. EXPERIMENTAL TECHNIQUES

A. Materials

Acrylamide, N,N'-methylene-bis-acrylamide, Coomassie Brilliant Blue (CBB) R-250, glycine, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), and urea were obtained from Sigma as were purified human transferrin and lactoferrin. Electrophoresis grade agarose and BioLyte (3/10, 7/9, and 5/7 range ampholytes) as well as the High and Low Molecular Weight Protein Standards were obtained from BioRad. Eastman Kodak was the source of N,N,N',N'-tetramethylethylenediamine (TEMED) and the tracking dye bromophenol blue (BPB). Nonidet P-40 (NP-40) was purchased from Particle Data Labs, Ltd.. Purified IgA and IgM were obtained from Cappel Laboratories, Inc.. Purified mouse LDH-X was a gift of Dr. C. Y. Lee. All other reagents were analytical grade quality.

B. Equipment

Sample centrifugations were accomplished using a IEC clinical centrifuge for low-speed semen centrifugations or an Eppendorf 5414 Microfuge for high-speed separations. A Scientific Products Temp Blok Module Heater was used for heat denaturation of seminal plasma specimens.

First dimension IEF separations were carried out in

locally cut 4 mm ID borosilicate glass tubing in a Hoefer Scientific Instruments Electrophoresis Chamber, Model DE-102. Second dimension separations were accomplished in chambers that were locally fabricated according to specifications given in Appendix A. This design offered no significant advantages over commercially produced or otherwise available models except that they were less expensive to produce.

Acrylamide gradients were cast with the use of a Pharmacia GM-1 gradient mixer and a Technicon Auto Analyzer Proportioning Pump fitted with 1.0 ml/min pump tubing (green-green) set up as shown in Appendix A2.

An E-C Apparatus Corporation Model E-C 400 Power Supply was used for all electrophoretic separations.

C. Human Subjects

The majority of the semen specimens used during the course of this study were considered "excess specimens" in that they were produced under orders of a physician for other diagnostic purposes (usually a basic semen analysis). A normal ejaculate has a volume of between three and five milliliters. A basic semen analysis generally requires less than one milliliter to complete: the remaining specimen is generally discarded. Instructions were given to the technologists in the laboratories cooperating with this study on how to treat any semen remaining after the requested analysis had been completed.

Permission was solicited (75) and obtained (76) from the Old Dominion University Institutional Review Board for the Protection of Human Subjects to use both volunteer and paid donors to obtain semen from proven fertile individuals. These donors provided the semen used to establish normal electropherograms and for the specialized collection techniques (i.e. timed specimens and split ejaculates) (proposal). These individuals were advised of the nature of the project as well as the method of semen collection, and were required to sign an informed consent form and complete a short questionnaire (see Appendix B) before a specimen was collected. All information on these individuals was kept confidential.

D. Methods

1. Sample Collection.

a. Whole Semen: Semen was collected by masturbation into a 50 mL screw-top, polypropylene, conical centrifuge tube. These tubes were acid washed with 5% nitric acid (if subsequent trace metal analysis was to be performed) or sterile (if microbial determinations were indicated). Explicit collection instructions were given in Appendix B5. After collection, individuals were instructed to deliver the specimen to the laboratory within one hour. The individuals were also instructed to accurately note the time of collection, whether or not any semen was spilled, and the length of time since the last ejaculation.

b. Time Study Experiments: Individuals participating in time study experiments were instructed to collect the specimen as noted above in the Andrology Lab collection room so that it could be delivered to the lab within five minutes of collection. The donor started a stopwatch at ejaculation so that accurate time intervals for denaturation could be maintained.

c. Split Ejaculates: Several methods of collecting split ejaculates were evaluated. The first few specimens were collected by masturbating into a large (345 mm x 225 mm x 50 mm) Pyrex dish that had been divided into sections so that individual segments of the ejaculated stream (i.e. "spurts") could be sampled for sequential analysis. Subsequent specimens were collected into an acid washed polystyrene six-well Costar Tissue Culture Cluster (microtiter plate), Cambridge, MA. These containers were not only more convenient, but had integral covers to prevent evaporation loss and contamination.

d. Urethral Gland Secretions: In certain individuals, it was possible to collect droplets of pre-ejaculatory fluid (i.e. urethral gland secretions) after sufficient sexual arousal. These secretions were collected in a 1.5 mL microcentrifuge tube and delivered to the lab with the whole semen specimen.

e. Prostatic Fluid: Occasionally, it was possible to obtain prostatic fluid in the course of urological examinations. These secretions were collected by a urologist in a 1.5 mL screw-cap microcentrifuge tube and

frozen immediatly.

f. Epididymal Plasma: In the course of vas deferens transection (during vasectomy) several attempts were made to collect small amounts of testicular fluid from the vaso-lumen either proximal or distal to the cut using sterile 50 ul capillary tubes. This technique was unsuccessful however.

2. Semen Analysis : Once received in the laboratory, patient specimens were subjected to standard semen analysis procedures as described elsewhere (29, Appendix C). These procedures included noting physical characteristics (volume, color, state of liquefaction, pH), sperm count, motility studies, live-dead ratio, microbial analysis (where indicated), and morphology studies. Approximately 90 minutes after the noted collection time, 300 µL of the specimen were removed (using metal-free pipet tips) and transferred into an acid-washed 1.5 mL microcentrifuge tube. This aliqout was centrifuged at low speed (less than 300xg) for five minutes to separate sperm cells and debris from the seminal plasma. One hundred microliters of seminal plasma were removed and placed in a microcentrifuge tube containing lyophilized denaturing reagent (described in next section). After the reagent dissolved, the specimen was stored at 4° C for subsequent analysis. The remainder of the specimen was used for other research projects.

Timed donor specimens were denatured at five minute intervals after the noted collection time by placing the specimen directly into the microcentrifuge tube containing

the lyophilized urea/SDS mixture without first separating the spermatozoa from the seminal plasma. After denaturation, the specimens were subjected to high-speed centrifugation (15000 x g) for two minutes and the supernatant seminal plasma withdrawn for electrophoresis.

The volumes of the individual portions of the split ejaculates were measured along with pH and, when possible, 100 ul were removed from each fraction for trace metal analysis as well as a portion for sperm count. One hundred microliters were removed from each fraction for denaturation and electrophoresis.

3. Seminal Plasma Denaturation : A denaturing solution was prepared containing 8 mol/L urea and 2% (w/v) sodium dodecyl sulfate (SDS). This solution was dispensed in 125 uL aliquots into individual 1.5 mL screw-cap microcentrifuge tubes. These tubes were frozen at -70° C for several hours and then lyophilized. After lyophilization, the tubes were stored at 4° C until use. To reconstitute, 100 µL of seminal plasma were added with vortexing to dissolve the pellet. The specimen could again be stored at 4° C or subjected to subsequent procedures. Just prior to electrophoresis of the specimen, 20 µL of 10% Nonidet P-40 (NP-40) and 5 µL of 2-mercaptoethanol (BME) were added to the sample followed by heating at 95° C for five minutes. After cooling, the specimen was ready for application to the isoelectric focusing gel.

4. Isoelectric Focusing (IEF) : IEF gels were prepared in 135 mm x 4 mm ID glass tubing sealed at one end with

Parafilm. The gel solution was prepared to contain 8 mol/L urea, 2% (v/v) NP-40, 2% w/v ampholytes (60% Biolyte 5-7:20% Biolyte 7-9: 20% Biolyte 3-10), and 40 g/L acrylamide monomers (38.0 g/L acrylamide and 2.0 g/L N,N'-methylene-bis-acrylamide). Polymerization was initiated by adding 7 mg of ammonium persulfate (APS) and 2.5 µL of N,N,N',N'-tetramethylethylenediamine (TEMED) per gram of total acrylamide monomer. After casting the gels, they were overlayered with water saturated n-butanol to minimize air contact and surface distortion.

Once the gels polymerized, they were assembled into the electrophoresis chamber and each cylinder overlayered with 8M urea/2% SDS. The gels were then prefocused for 15 minutes at 200 v (constant voltage), 15 minutes at 300 v, and 30 minutes at 400 v. The cathode solution was 0.02 mol/L NaOH with 0.01 mol/L phosphoric acid at the anode.

After prefocusing, the urea/SDS solution was removed from the gel tubes and 40 µL of denatured specimen were applied. The gels were then electrophoresed for approximately 11,000 volt-hours with the final hour being at 950 v to sharpen the focus. After focusing, the gels were extruded from their tubes for subsequent procedures.

5. IEF Gel Equilibration : After removal from the focusing tubes, the IEF gels were placed in glass sample tubes with approximately 2 mL of equilibrium buffer (10% w/v glycerol, 5 % v/v BME, 2% w/v SDS, and 0.0625 mol/L Tris chloride, pH 6.8) for approximately five minutes. After equilibration, the gels were removed from the buffer,

blotted with lint-free filter paper and affixed to the SDS slab gel as described in paragraph III.C.8.

6. pH Profile : One gel per IEF run was left blank for pH profiling. After electrophoresis, this gel was removed from its tube and cut into 10 mm segments. Each segment was added to 2.0 mL of deionized water and allowed to equilibrate for six hours at room temperature. After this time, the pH of each segment in solution was determined using a standard pH meter. This data was plotted with pH versus length.

7. The Second Dimension SDS Slab Gel : Linear gradient slab gels were cast with acrylamide concentrations varying from 170 to 70 g/L. These acrylamide solutions were prepared from a stock 300 g/L acrylamide solution (285 g/L acrylamide and 15 g/L bis-acrylamide) and made up to contain 1.0 g/L SDS and 0.375 mol/L Tris (pH 8.8). A 1.5 cm stacking gel containing 4.0 g/L acrylamide, 1.0 g/L SDS and 0.125 mol/L Tris (pH 6.8) were immediatly overlayered on the unpolymerized gradient gel. This allowed both gels to polymerize simultaneously preventing separation of the stacking gel from the resolving gel. Polymerization of both gels was initiated by the addition of 1.1 mg APS and 1.75 µL of TEMED per gram of total acrylamide monomer in solution. After casting, the gels were again overlayered with water saturated n-butanol. After polymerization (within two hours), the butanol was rinsed from the top of the gel and replaced with stacking gel buffer diluted 1:4 to remain overnight (while the first dimension separation was being

accomplished).

8. Preparation for the Second Dimension Separation : The slab gel chambers were prepared for electrophoresis by removal of the sealing gaskets, and rinsing both the upper and lower gel surfaces with deionized water. The upper surface was then blotted dry and covered with molten agarose (1% in equilibrium buffer) to prepare a bed to receive the equilibrated IEF gel. The IEF gel was then placed on the agarose bed (origin to the right) and sealed in place with additional hot agarose. Molecular weight markers were prepared by adding commercially produced standard protein mixtures to the molten agarose (0.2 g/L). This solution was drawn up in a 100 μL capilary and allowed to solidify. After cooling, the mixture was extruded from the capilary and 5 mm segments were sealed in place on the slab to the right of the IEF gel.

9. Slab Gel Electrophoresis : Chilled electrophoresis buffer (0.025 mol/L Tris, 0.192 mol/L glycine, and 0.1% w/v SDS) was added to both the upper and lower electrophoresis chambers. Separations were carried out at 4 C between 20 and 25 mA constant current per gel. Electrophoretic progress was monitored by the addition of 0.05% w/v bromophenol blue to the cathode buffer compartment. Separations were terminated when the tracking dye reached the bottom edge of the slab gels (usually within 6.5 hours).

10. Protein Visualization : After electrophoresis, the slab gels were removed from the glass plates and fixed in staining solvent (50% v/v methanol, 10% v/v acetic acid) for

3-15 hours. The gels were then stained for two hours in 2.0 g/L Coomassie Brilliant Blue R-250 (CBB R-250) in staining solvent, destained for 2-3 hours in full strength staining solvent, and then again in half-strength staining solvent until the desired background color was obtained. The gels were then stored in deionized water in Zip-Lock freezer bags.

11. Protein Pattern Interpretation : The stained two-dimensional gel slab, or electropherogram, can be read like a cartesian graph with the vertical axis representing increasing molecular weight (in Daltons) and the horizontal axis representing increasing pH. Selected protein components were identified by comparison with previously published data (43, 48, 52, 60, 71, 73) or by co-electrophoresis of purified proteins (see Appendix D-79). Each gel was traced and photographed for comparison with other seminal plasma specimens. A composite map was constructed from seminal plasma maps of those individuals who were known to be healthy and fertile. Pattern alignment enabled detection of variations in protein locations (relative to albumin and acid phosphatase, for example) between seminal plasma specimens. Comparison of maps from the different patient types, or seminal plasma fractions, allowed for the determination of which groups of components in the total protein map were contributed by the various accessory organs.

12. Photography : All electropherograms were photographed using Kodak Plus X 35 mm black and white film

(ASA 125) through a Tiffen green #1 filter. Exposures were generally taken with backlighting provided by 20 W Westinghouse Agro-Lite fluorescent lamps at f 11 at 1/15 second. Gel tracings were photographed in sunlight using high contrast Kodalith film or reproduced on 3M reversed image transparency film.

IV. RESULTS

A. Time Course Studies.

Initial studies were carried out to investigate the changes in HSP proteins with respect to time so that an optimal sampling time could be established allowing for a consistant comparison between patient specimens. Since the majority of the specimens used in this study were produced for purposes other than ours, it was not possible to collect the specimen into a solution which would inhibit semen liquefaction or proteolytic activity thus allowing for a more carefully controlled analysis of seminal plasma proteins.

Evaluation of seminal plasma using the HR2DE technique showed that very few changes could be observed in the specimen after about 60-90 minutes. This is consistent with the work of others (35-37), and the fact that gross liquefaction is essentially complete after 15-20 minutes. Thus, it was decided that all patient specimens would be denatured at approximately 90 minutes after collection. This allowed for ample transportation time to the laboratory (patients were instructed to deliver the specimen to the lab within one hour of collection) and for preparation of the sample (i. e. centrifugation) prior to denaturation. This depended heavily on the patient's honesty in accurately reporting the time of collection.

In volunteer donor studies, it was possible to accurately control the time of collection since these individuals were willing to cooperate more closely with the needs of the program. Collection of the specimen in a room in the immediate vicinity of the laboratory allowed for receipt of the specimen within two to three minutes of ejaculation. By denaturing aliquots of the specimen at 2.5-5 minute intervals after collection with subsequent two-dimensional analysis, it was possible to observe the effects of liquefaction on the seminal plasma protein constituents.

Figure 3 shows the effects of time on the distribution of proteins in a representative specimen (SE02). It can be seen that there are several series of basic proteins along the right gel margins with molecular weights ranging from 45-65 kilo-Daltons (kDal) that rapidly disappear as liquefaction progresses. These proteins are essentially absent after about 20 minutes, but in one specimen (SE07) were gone in less than ten minutes. This rapid disappearance of proteins was also reported by Edwards, et al., (73) using their non-equilibrium BASODALT technique. These basic "coagulation" proteins correspond well with those proteins emitted in terminal portions of the ejaculate that are associated with seminal vesicle secretions (as discussed in subsequent paragraphs). As time progresses, the higher molecular weight proteins beginning with a protein

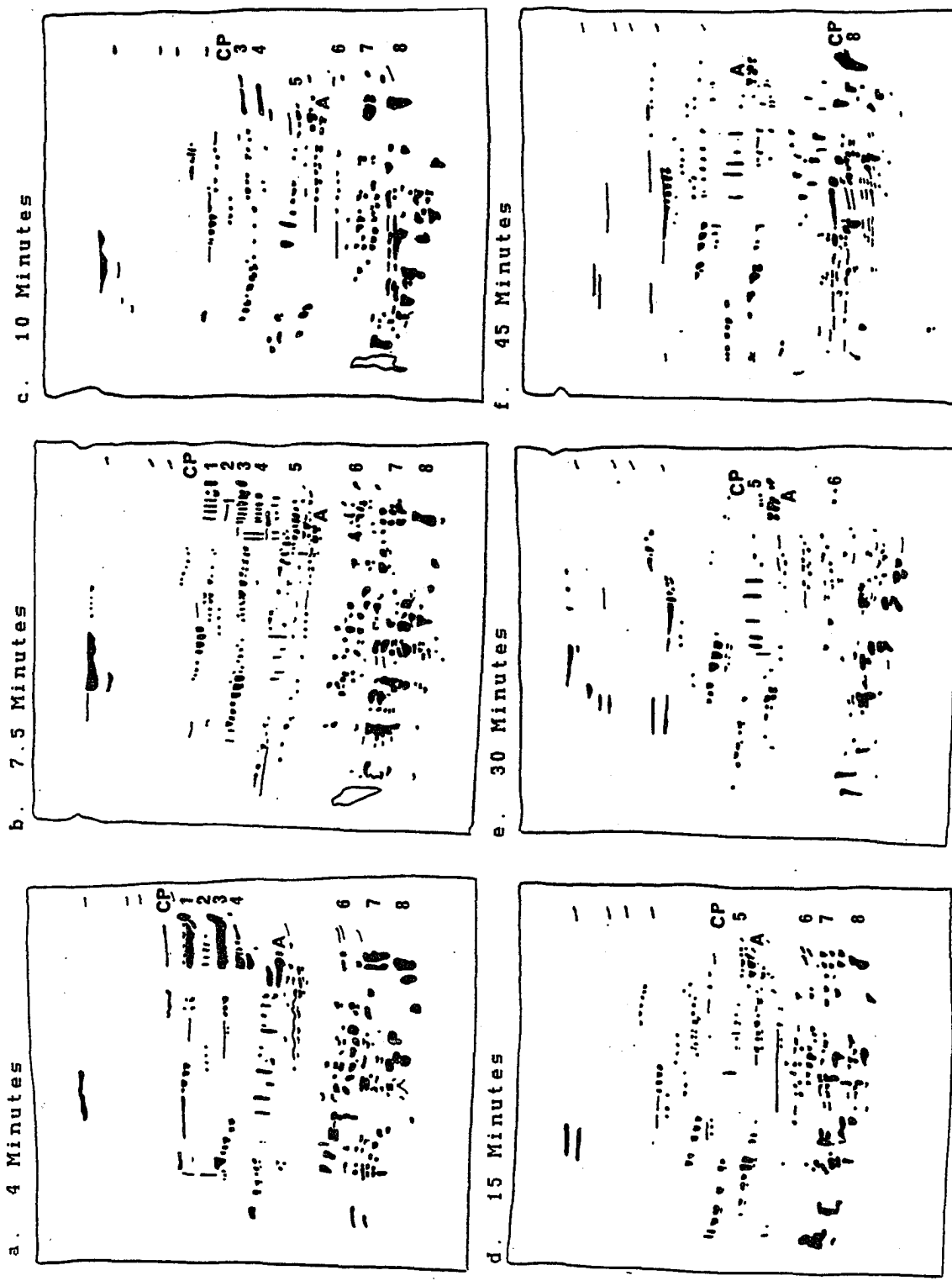

FIGURE 3. The Effects of Time on the Distribution of Seminal Plasma Proteins.

designated CP 1 (M. W. 63-65 kDal) give way to a series of progressively smaller fragments differing by about 5 kDal (CP 2 = 57.5 kDal, CP 3 = 52.5 kDal, and CP 4 = 47.3 kDal). Series CP 5 and 6 (37-40 and 18.1 kDal) do not disappear as rapidly as the others, but are essentially absent after 45 minutes. Series A is not associated with the liquefaction process since it appears in relatively constant amounts throughout the time observed and can be observed in most specimens after periods of 90 minutes or more. Series CP 7 and CP 8 (15.3 and 14.5 kDal respectively) appear to increase slightly with time, but the magnitude of increase does not necessarily correlate well with the disappearence of the other proteins.

B. Split-Ejaculate Studies.

Much information was gained from this series of experiments. The donors, on a whole, experienced little difficulty in using the six-well Costar culture plates for specimen collection: only one individual was unable to produce more than three fractions - all others were able to distribute their ejaculates into six or more fractions. Table 2 gives an example how the seminal plasma volume and spermatozoa were distributed between the various fractions as well as the pH of each fraction (measured with Hydrion Paper), and distribution of zinc (77). These data correlate well with the findings of others (24-30) in that the bulk of the spermatozoa are emitted in the initial portion of the ejaculate. In addition, the high levels of zinc and other

Specimen SE03

FRAC	pH	VOL (ml)	% VOL	COUNT ml	% of Tot COUNT	Zn (ppm)
1	7.0	0.55	12.2	320	63.0	164
2	7.5	0.81	18.1	90.1	23.6	149
3	8.0	0.77	17.1	5.2	1.4	98
4	8.0	1.24	27.7	18.2	4.8	51
5	8.0	0.54	12.1	4.1	1.1	24
6	8.0	0.57	12.7	1.9	0.5	14
Total or Ave		4.48		85.0		82

TABLE 2. Comparison of Various Parameters in a Split Ejaculate.

components such as acid phosphatase in the initial fractions confirms that these secretions originate in the prostate as previously reported (39).

Comparison of electropherograms of first fraction components with prostatic fluid (collected by prostatic massage) shows that there is a good correlation of proteins suggesting that the very first portion of the ejaculate is predominately prostatic in origin (see Figure 4). Of particular interest is the presence of the well-known prostatic marker protein acid phosphatase (P1), which can be seen to quantitatively decline in subsequent fractions (see Figure 5). This protein was previously identified by our laboratory (71) by co-electrophoresis of the purified enzyme. Several other proteins are observed to be primarily prostatic in origin as well. These are designated P2-P4 in Figures 4 and 5. The P2 series shows the exact same relationship to albumin and acid phosphatase as the protein proposed to represent the BB-isozyme of creatine kinase by Dermer et al. (43). These proteins decrease slightly in quantity as the ejaculation sequence progresses, but are still present in significant quantities in terminal portions of the ejaculate. Proteins P3 and P4 exhibit a similar prostatic distribution. These proteins (M. W. 16.2 and 17.8 kDal) were observed by Carter and Resnic (48). They determined that P4 was analogous to Prostatic Binding Protein from the rat ventral prostate (46). They also observed that P3 diminished in quantity with increasing age.

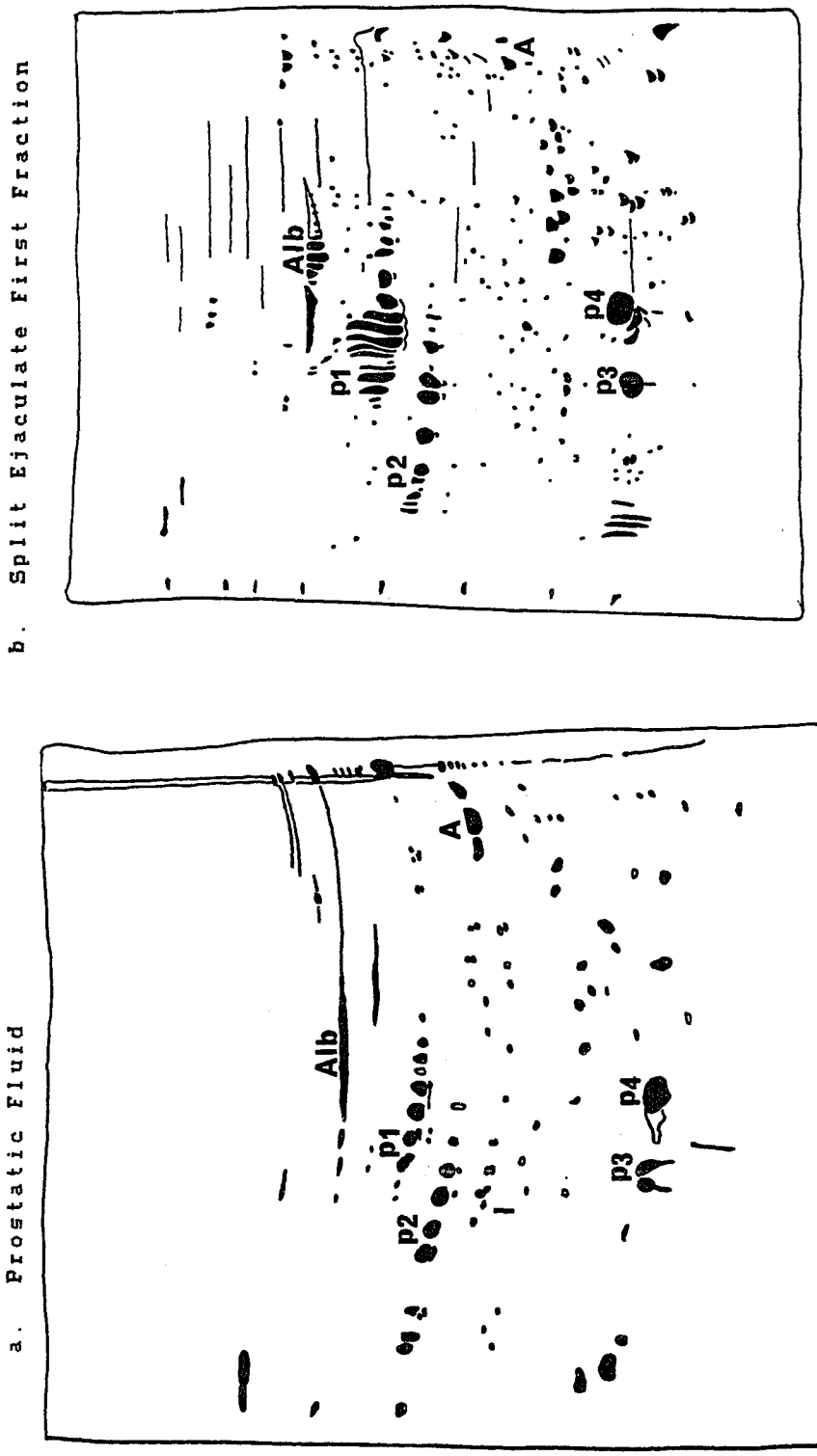

FIGURE 4. Comparison of Electropherograms from Prostatic Fluid and a Split Ejaculate First Fraction.

A comparison of the electropherograms of seminal plasma proteins from the various fractions of a representative split ejaculate is given in Figure 5. One can readily observe considerable differences in the distribution of proteins between the initial and terminal portions of the ejaculate. Terminal fractions can be associated with seminal vesicle secretions. As previously discussed, the seminal vesicle secretions are responsible for liquefaction of coagulated semen. As previously shown in Figure 3, these basic proteins (designated CP1-8) rapidly disappear in conjunction with the dissolution of the seminal clot in the whole specimen. Terminal split ejaculate fractions do not rapidly liquify however, since they are isolated from the initial portions of the ejaculate containing the proteolytic enzymes contributed by the prostate. Fractions 5 and 6 have been observed to remain coagulated for up to two hours. Subsequent addition of 100 µl of semen from fraction 1 causes rapid liquefaction (within ten minutes) of these terminal fractions.

As can be observed from Figure 5, there is a continual increase in the lower molecular weight proteins ranging from 10-25 kDal as the ejaculation sequence progresses. In the initial fraction there are only three to four major proteins (i. e. P3 and P4) present as compared with almost 30 protein spots of the same approximate spot intensity in the same area of the terminal fraction.

A heterogeneous series of proteins designated "L" are

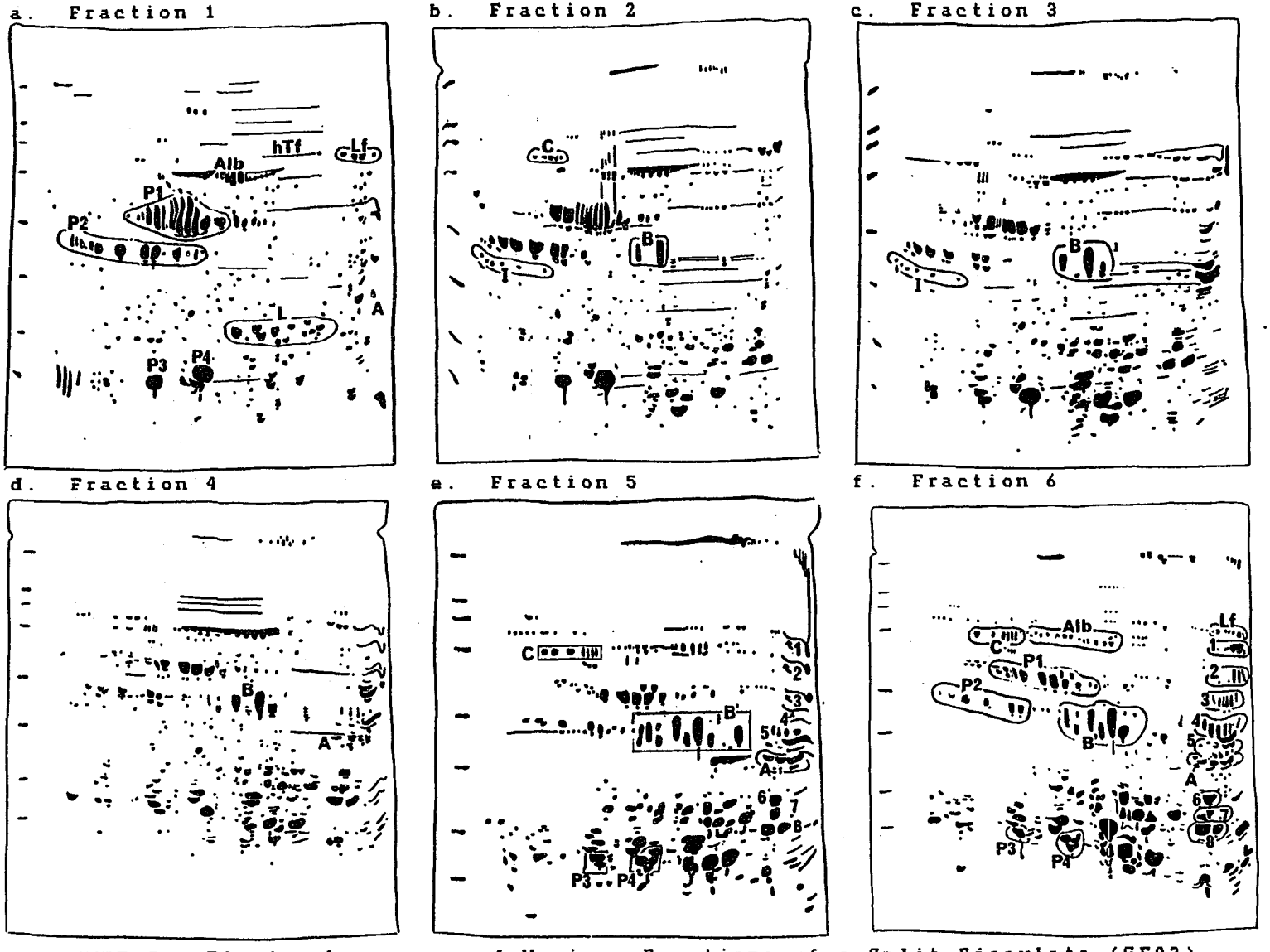

FIGURE 5. Electropherograms of Various Fractions of a Split Ejaculate (SE02).

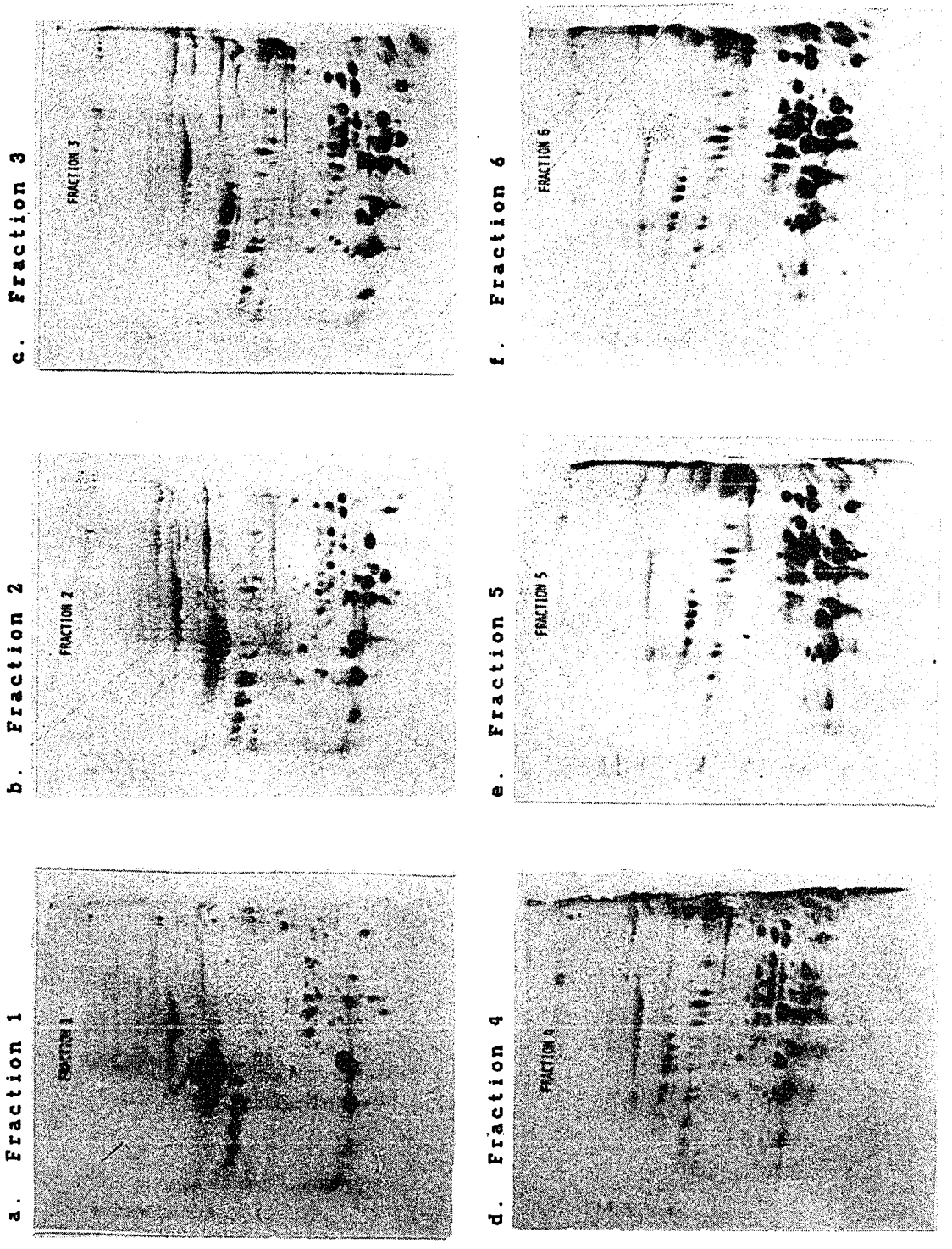

FIGURE 5. Electropherograms of Various Fractions of a Split Ejaculate (SE02).

present in all fractions of the ejaculate with an apparently slightly greater concentration in the terminal fractions. These proteins fall in an area identified as the immunoglobulin light chains (κ and λ) by co-electrophoresis of the purified proteins (see Appendix D-79A and D-79B). In addition, a protein band labeled "γ" is observed primarily in fractions 1-5; these being most predominant in fraction 2, and only slightly visible in fractions 6-8. These proteins may represent the γ-chains of IgG (see Appendix D-79B). An anomaly that can not be explained is the presence of increased amounts of heavy chains in the terminal fractions of the ejaculate without a concomittant increase in light chains. Other split ejaculate series (Appendix D-67 to D-72) exhibit varying amounts of the heavy chain with relatively constant amounts of protein in the light chain area.

A series of three to four basic proteins labeled "A" have an average molecular weight of 27,000 Daltons. These proteins migrate in the same area as the C monomers of purified LDH-X when run alone and co-electrophoresed with HSP (see Appendix D-79E and [13, 14]). Another set of proteins present in all but the initial fraction, in this case, are the two prominant proteins in the 34-40 kDal molecular weight range labeled "B". These two proteins are joined by a whole series of like-weight proteins arranged in vertical columns or palisades as the ejaculate sequence progresses (series B' in Fraction 8). Series C, an acidic group of proteins with an average molecular weight of 80.3

kDal, is also observed in all fractions, but in increased amounts in terminal portions. A protein previously identified (71) as albumin (Alb) is also present in the initial or prostatic secretions, with concentrations decreasing significantly as the ejaculation sequence progresses. Transferrin (hTf) appears to increase in fractions 1-3 and then decrease in fractions 4 and 5, with little observed in fractions 6-8. This correlates well with the premise that hTf is produced primarily by the Sertoli cells and thus its detection is in conjunction with distribution of testicular secretions and spermatozoa from the ampullary glands. Lactoferrin (Lf) was reported by Tauber et al. (28) to follow a seminal vesicle distribution. One can observe that in this sequence, Lf is present in all fractions with increased amounts in the terminal portions of the ejaculate. The locations of transferrin and lactoferrin were identified by electrophoresis of purified proteins (Appendix D-79C and D-79D).

C. Other Seminal Fractions.

As mentioned in Chapter III, isolation of testicular/epididymal secretions from the vas deferens during vasoligation procedures was unsuccessful. Seminal plasma differences between "normal" and vasectomized individuals will be discussed in subsequent paragraphs.

Numerous researchers (c.f. 26, 37) have reported the presence of large "sago-" or "tapioca-like" globules upon macroscopic observation of freshly ejaculated semen. These

globules gradually disappear as liquefaction proceeds, but persist if liquefaction is incomplete or delayed. They are also observed to be present in greater quantities in terminal portions of the split ejaculates, but in some cases (e. g. SE03), were present in initial portions as well. Some of these globules were isolated from less than five minute old semen and from incompletely liquified specimens and were washed in several changes of normal saline to remove seminal plasma contaminants prior to denaturation. Microscopic observation of these globules revealed that they were not membrane bound, but resembled the fiberous coagulum observed by Tauber and Zaneveld (37). Analysis by HR2DE gives rise to electropherograms as shown in Figure 6a. When comparing this electropherogram with those representing terminal fractions of the split ejaculate (Figure 6b), one can see that these globules most likely represent "packets" of seminal vesicle proteins which have not yet been invaded by proteolytic enzymes from prostatic (or first fraction) secretions.

HR2DE analysis of Pre-Ejaculatory Fluid (PEF) yielded results as shown in Figure 7. The relatively high molecular weight, acidic distribution of these proteins is strikingly different from that of whole seminal plasma. This distribution is characteristic of mucoproteins containing large numbers of sialic acid residues (59, 64). Series D on the acidic margin of the gel (pI 4.1-4.5) has a molecular weight ranging from 43.6-45.5 kDal and may correspond to acid glycoprotein as identified by Anderson (60).

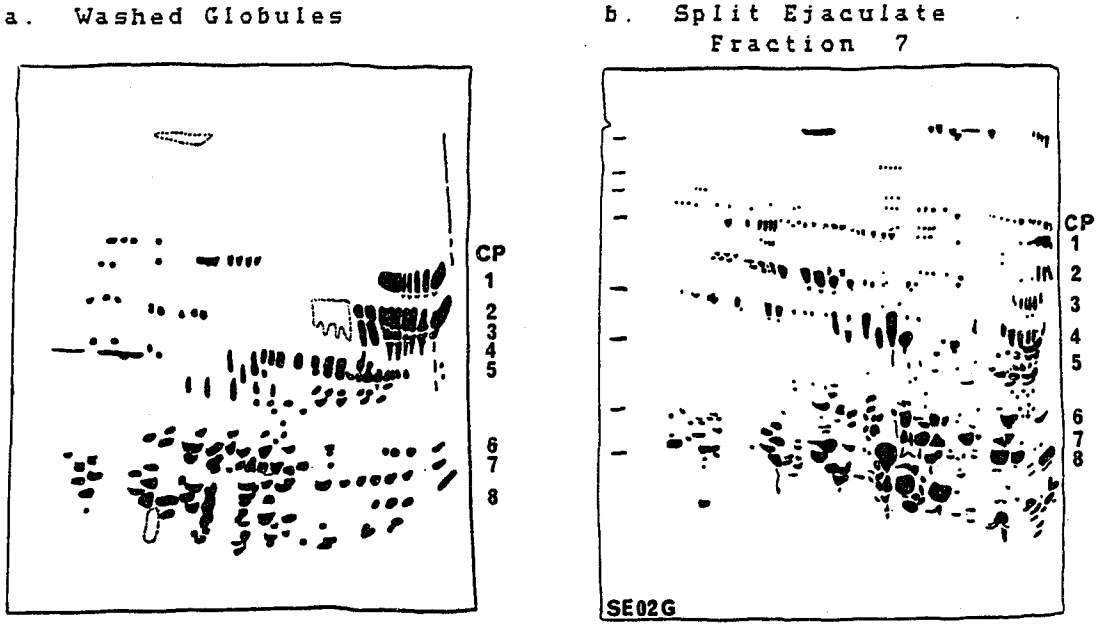

FIGURE 6. Comparison of Washed "Globules" from Non-Liquified Semen and a Terminal Fraction of a Split Ejaculate.

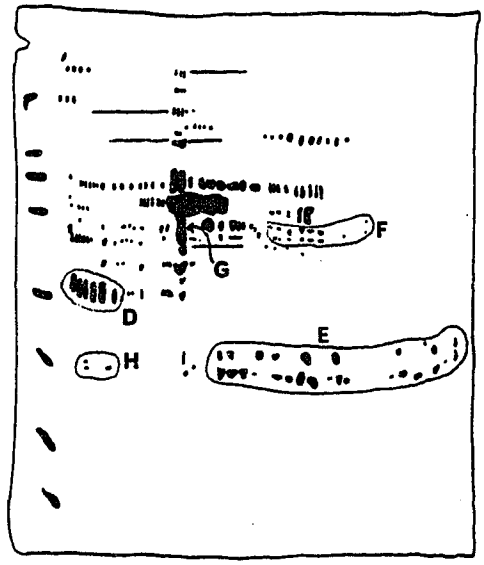

FIGURE 7. Electropherogram of "Pre-Ejaculatory Fluid.

a. Washed Globules

b. Split Ejaculate Fraction 6

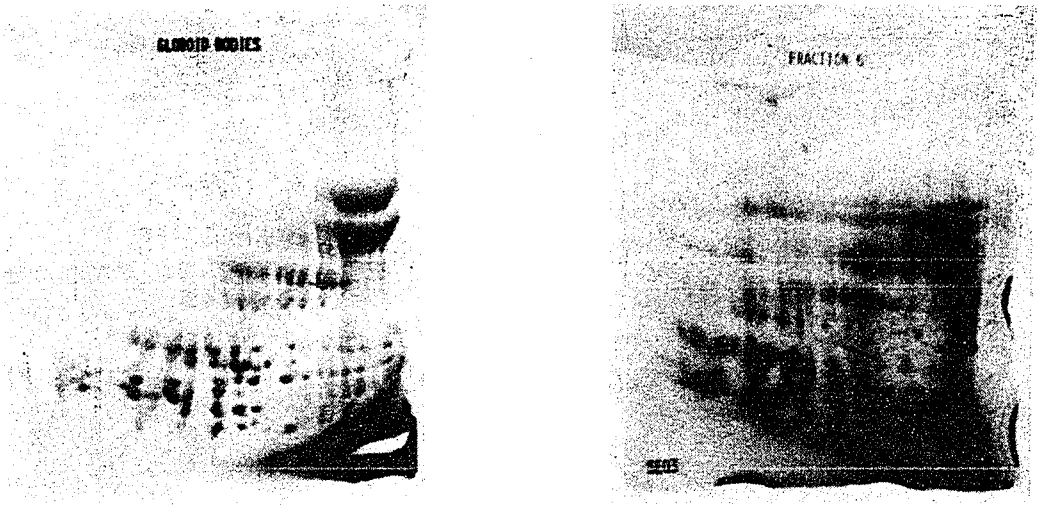

FIGURE 6. Comparison of Washed "Globules" from Non-Liquified Semen and a Terminal Fraction of a Split Ejaculate.

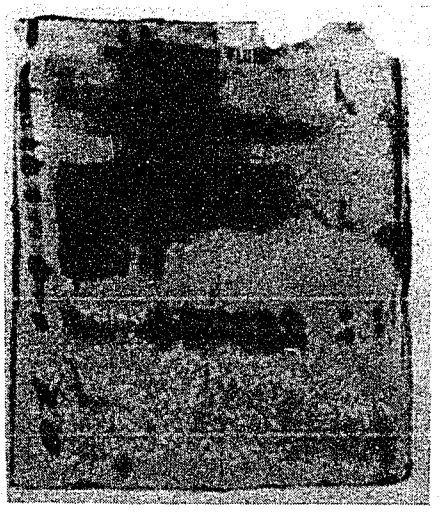

FIGURE 7. Electropherogram of "Pre-Ejaculatory Fluid.

Series E corresponds with the immunoglobulin light chains and , while Series F (molecular weight 58.9-61.7 kDal) corresponds with the heavy chain (see also Appendix D-62). The area marked "G" represents a high concentration of a single protein ranging in molecular weight from 58.9-64.5 kDal. Series H are negatively charged proteins with a molecular weight of around 30,000 Daltons.

D. "Normal" Volunteer Studies.

Figure 8 represents a composite map of post-liquefaction proteins common to the HSP produced by individuals with recently proven fertility. Proteins other than those designated with roman numerals are labeled as identified in previous discussions.

Series I has a molecular weight of 35,000 Daltons and is present in most normal electropherograms observed with notable exceptions. Series II proteins are acidic, low molecular weight (10-20 kDal) proteins which differ in amount and location from individual to individual and may be primarily prostatic in origin. This group contains the proteins P3 and P4 previously discussed.

Series III proteins form very characteristic constellations of spots in most specimens. These proteins are arranged in vertical stacks with the lower portions skewed to the acidic edge of the gel forming characteristic "J" shaped patterns in the gel. Series IV contains another very heterogeneous set of proteins contributed primarily by the seminal vesicles, again differing both in quantity and

FIGURE 8. Composite Map of Post-Liquifaction Seminal Plasma Proteins from Individuals with Recently Proven Fertility: Alb = Albumin; hTf = Transferrin; Lf = Lactoferrin; aAT = alpha-1-Antitrypsin; CP 1-8 = Coagulation Proteins (from seminal vesicles); A = Lactate Dehydrogenase isozymes (including LDH-X); B' = Seminal Vesicle Proteins; C = gamma-Glutaryltransferase (gGT); = IgG Heavy ( ) Chain; L = Immunoglobulin Light Chains (and); P1 = Acid Phosphatase Isozymes; P2 = Creatine Phosphatase-BB; P3 = Lysozyme (?); P4 = Prostate Binding Protein (PBP); I = Testicular Protein; II = Variable Region; III = Characteristic "J"-shaped Proteins (from Seminal Vesicles); IV = Variable Region (from Seminal Vesicles); V = Variable Region containing Subset L; VI = Variable Region containing Subset ; VII = High Molecular Weight Proteins associated with Sperm Count.

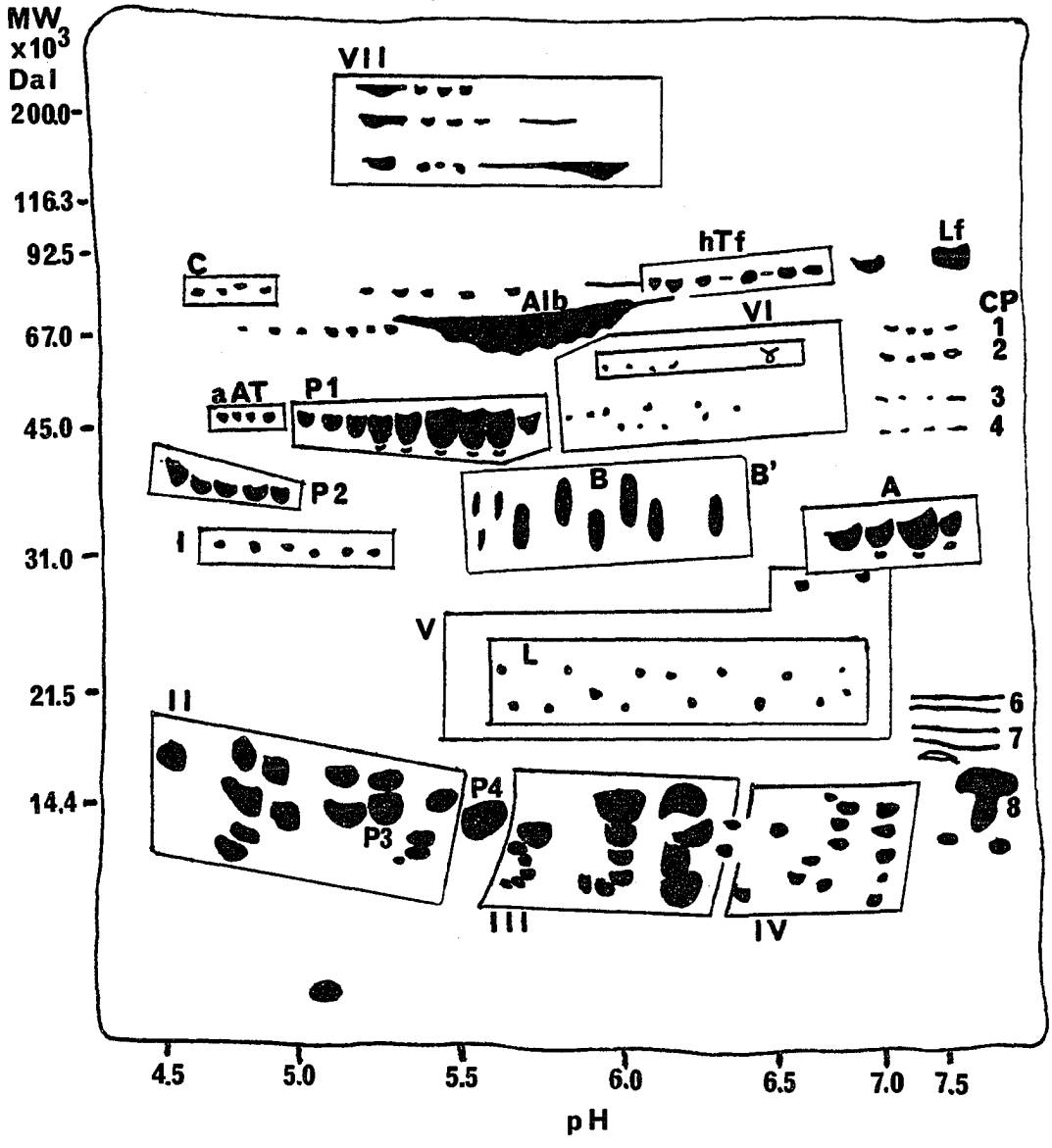

FIGURE 8. Composite Map of Post - Liquifaction Seminal Plasma Proteins from Individuals with Recently Proven Fertility.

location from individual to individual. Series V proteins contain the subset L, or immunoglobulin light chain proteins as well as other proteins contributed by both the prostate and seminal vesicles. Series VI are neutral to basic proteins with molecular weights ranging from 45-6 kDal. This group also shows a great deal of variation in both quantity and location of proteins from specimen to specimen.

Series VII are acidic, high molecular weight proteins ranging from 100- to over 200 kDal in size. The location of these proteins with respect to albumin and PAP is fairly reproducible. They are heterogeneous and usually only present in trace amounts except as noted below.

E. Patient Studies.

1. Azoospermic Patients : Figure 9 shows a composite of electropherograms from three different azoospermic patients. The specimen producing the gel represented in Figure 9a (RS012), came from an individual suffering from pan-testicular failure as a result of radiation treatment (10 days) and Bleomycin therapy (11 months) for Hodgkin's Disease. Seminal plasma analysis presents a fairly normal distribution of proteins in the gel. This is to be expected since the aforementioned therapies generally only affect germinal tissue function resulting in a lack of spermatozoa production, with most other reproductive functions remaining normal (78). A notable protein difference is the lack of the series I proteins mentioned above.

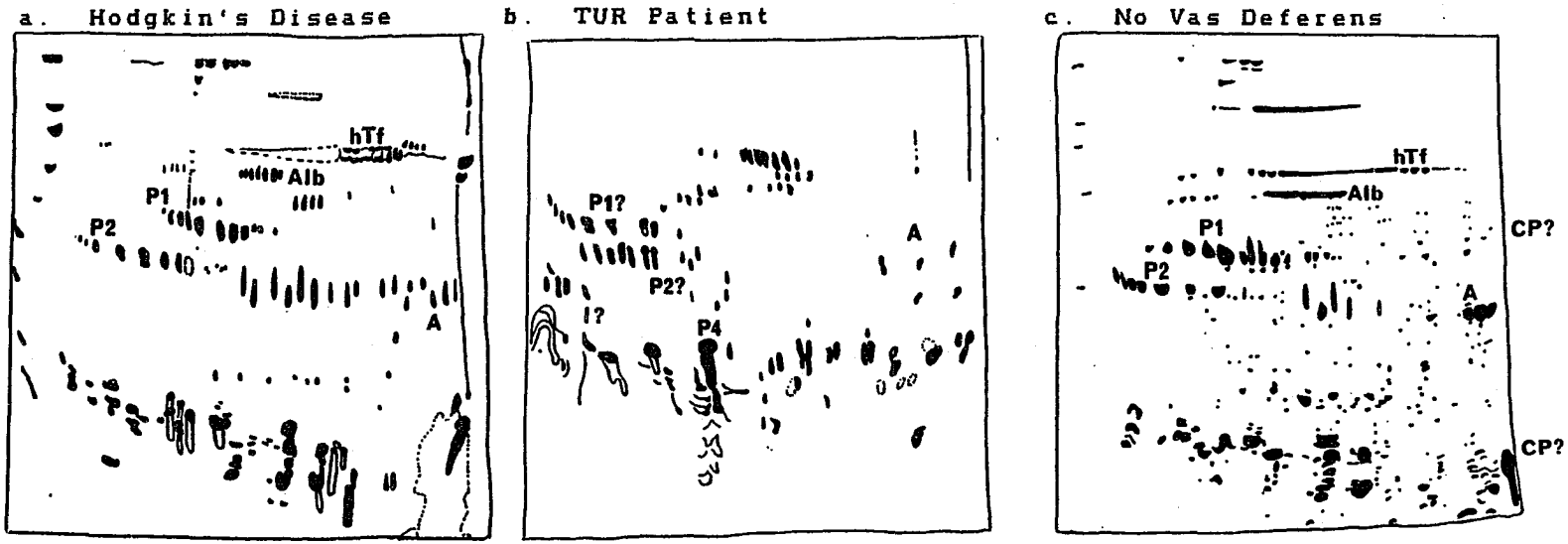

FIGURE 9. Electropherograms from Azoospermic Patients

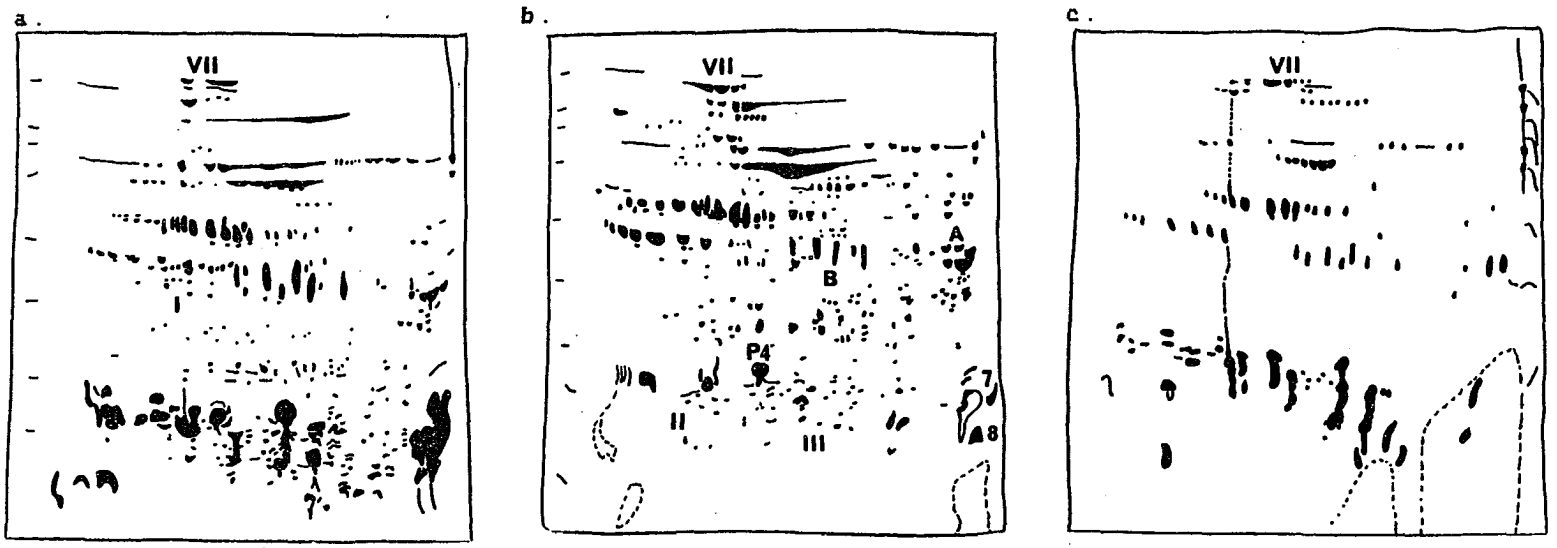

FIGURE 10. Electropherograms from Post-Vasectomy Patients.

a. Hodgkin's Disease b. TUR Patient c. No Vas Deferens

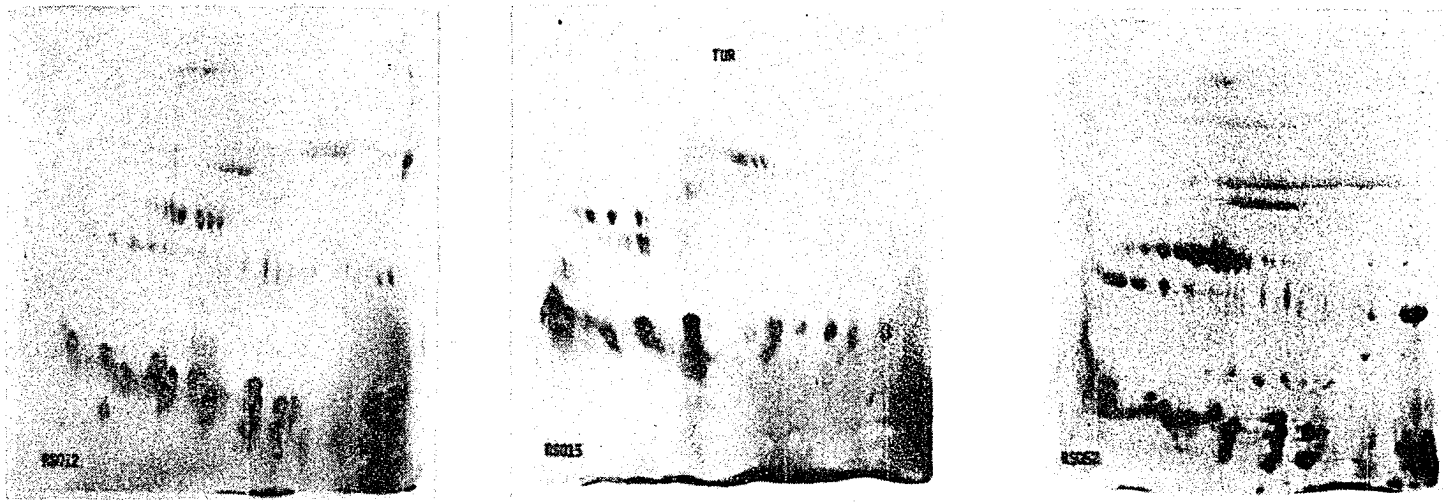

FIGURE 9. Electropherograms from Azoospermic Patients

a. b. c.

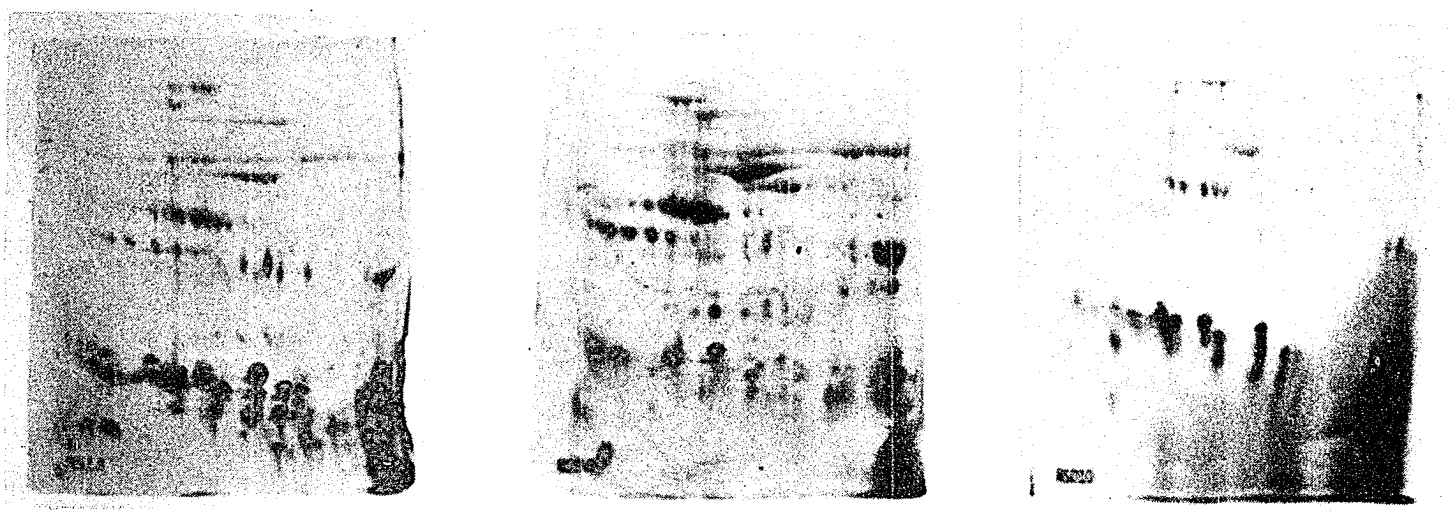

FIGURE 10. Electropherograms from Post-Vasectomy Patients.

Figure 9b was produced from seminal plasma collected from an individual (RS015) who had undergone a Trans-Urethral Resection (TUR) procedure for a congenitaly blocked ejaculatory duct. This pattern is grossly abnormal when compared to Figure 8, but is reasonably comparable to the prostatic fluid map given in Figure 4. This is to be expected since all secretions proximal to the ejaculatory ducts are blocked. The abnormalities observed in the locations of P1 and P2 are not due to overloading or distortions in either of the acrylamide gels since this pattern was reproduced in two different separations run on separate occasions. P4 (Rosalki and Rowe's Prostatic Binding Protein) is present in greatly increased amounts. Missing from the pattern is transferrin - a Sertoli cell product.

Specimen RS052 in Figure 9c, was produced by a patient in whom no vasa deferentia could be palpated on scrotal examination. As in Figure 9a, this pattern is relatively normal. Transferrin appears to be present in the sample, as well as some evidence of coagulation proteins along the right-hand margin of the gel.

2. Post Vasectomy Patients : Specimen RS053 was produced by an individual who had undergone elective sterilization via vasectomy several months prior to this analysis. This individual had previously fathered two children. A relatively normal distribution of HSP proteins can be seen in Figure 10a. Series VII proteins appear to be more pronounced in this and other post-vasectomy specimens

analyzed as compared to normal individuals. Series I appears to be present in this specimen.

Figure 10b shows a map of seminal plasma from an individual (RS049) with Peyronie's Disease who had undergone a bilateral vasectomy in conjunction with a right epididymectomy in November of 1981. This was necessitated by the formation of an epididymal abcess subsequent to a bout of epididymitis. A small right hydrocele developed around the right testicle after surgery and was aspirated in December, 1982. The specimen was collected five months later in May, 1983.

One can observe a striking absence of the low molecular weight series II and series III proteins yielding a pattern similar to that of prostatic fluid. The series B vesicular proteins are present, however, as are CP7 and 8. The series A proteins appear to be present in multimeric forms or in conjunction with a series of proteins with similar electrophoretic characteristics (CP5 ?). A band of proteins in the series VI region is greatly enhanced as are proteins in series VII. Series I is not observed to be present.

A third patient in this series (RS010) had undergone a vaso-vasostomy (vasectomy reversal) three weeks prior to the collection of this sample in an attempt to regain his fertility. The procedure was evidently successful, since spermatozoa were observed in the specimen (18 million/ml). The specimen was moderately viscous however, with non-specific clumping observed. The electropherogram is not of the best quality, but never the less shows a relatively

normal distribution of proteins with the exception that series VII is enhanced. It is not possible to determine if series I is present or not due to the quality of the gel.

3. Varicocele Patients : Results from varicocele studies are difficult to interpret in that these individuals presented a whole spectrum of results ranging from small left varicoceles with normal semen parameters to severe oligozoospermia (less than 5 million sperm/ml), or varicoceles with additional complications such as epididymitis or pyuria. In addition, there was another group of patients who had undergone varicocelectomy (spermatic vein ligation) anywhere from two to ten months priot to analysis.

Figure 11 gives a representation of seminal plasma from a small left varicocele patient with normal sperm parameters (11a); a patient with a small left varicocele and severe oligozoospermia (11b); a patient with a large left varicocele (complicated with left testicular atrophy) and severe oligozoospermia (11c); a five month post-varicocelectomy patient with moderate oligozoo- spermia (11d); a seven month post-operative patient with severe oligozoospermia (11e); and a post- bilateral varicocelectomy patient whose wife subsequently became pregnant (11f).

Series VII proteins appear to be increased in practically all of these patients - especially in the severely oligozoospremic patients (11b, c, e, and d). Series I proteins were not observed in any of the varicocele specimens except RS018 and RS030 (11a and d) which were only

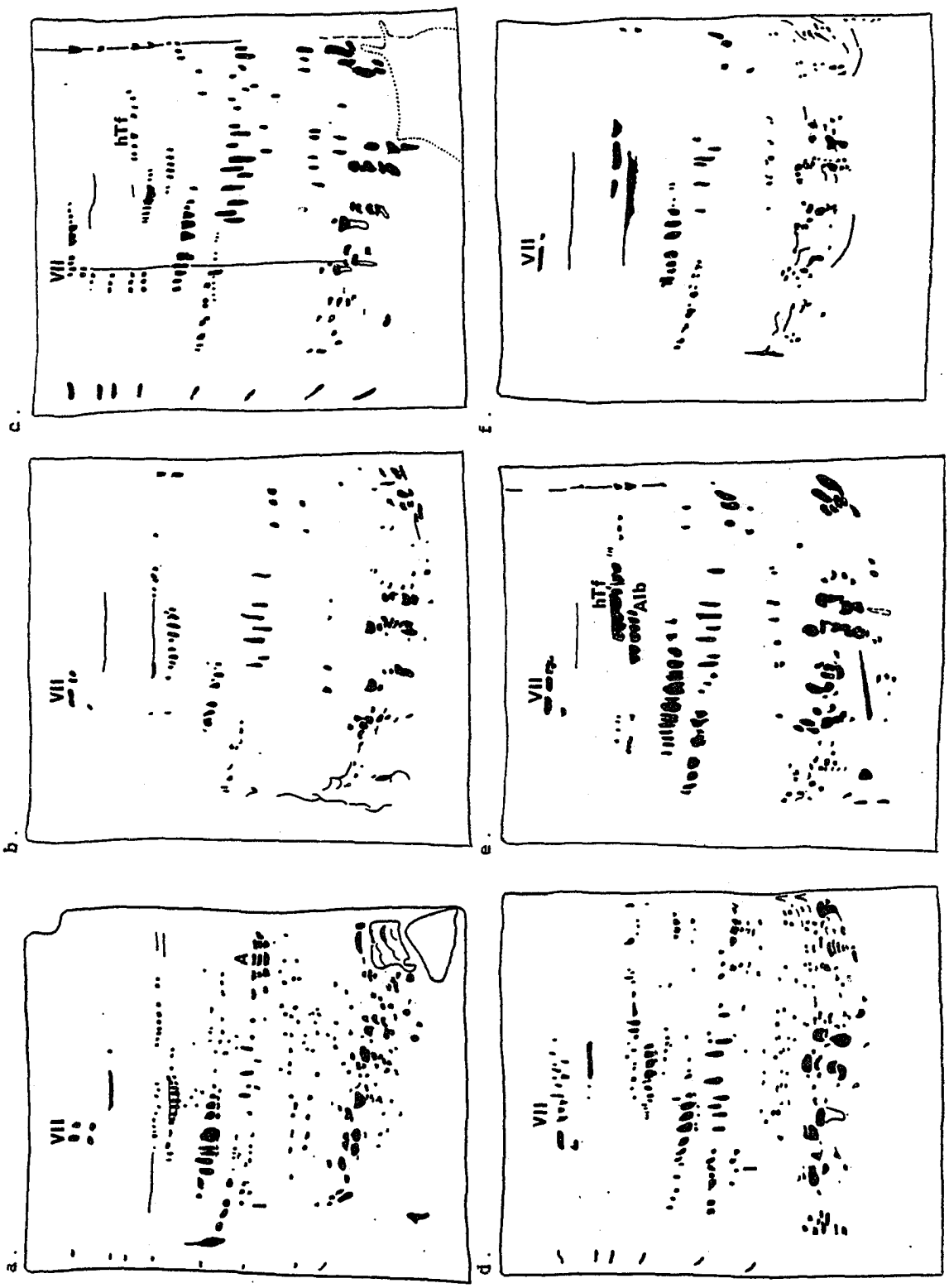

FIGURE 11. Electropherograms of Seminal Plasma from Varicocele Patients.

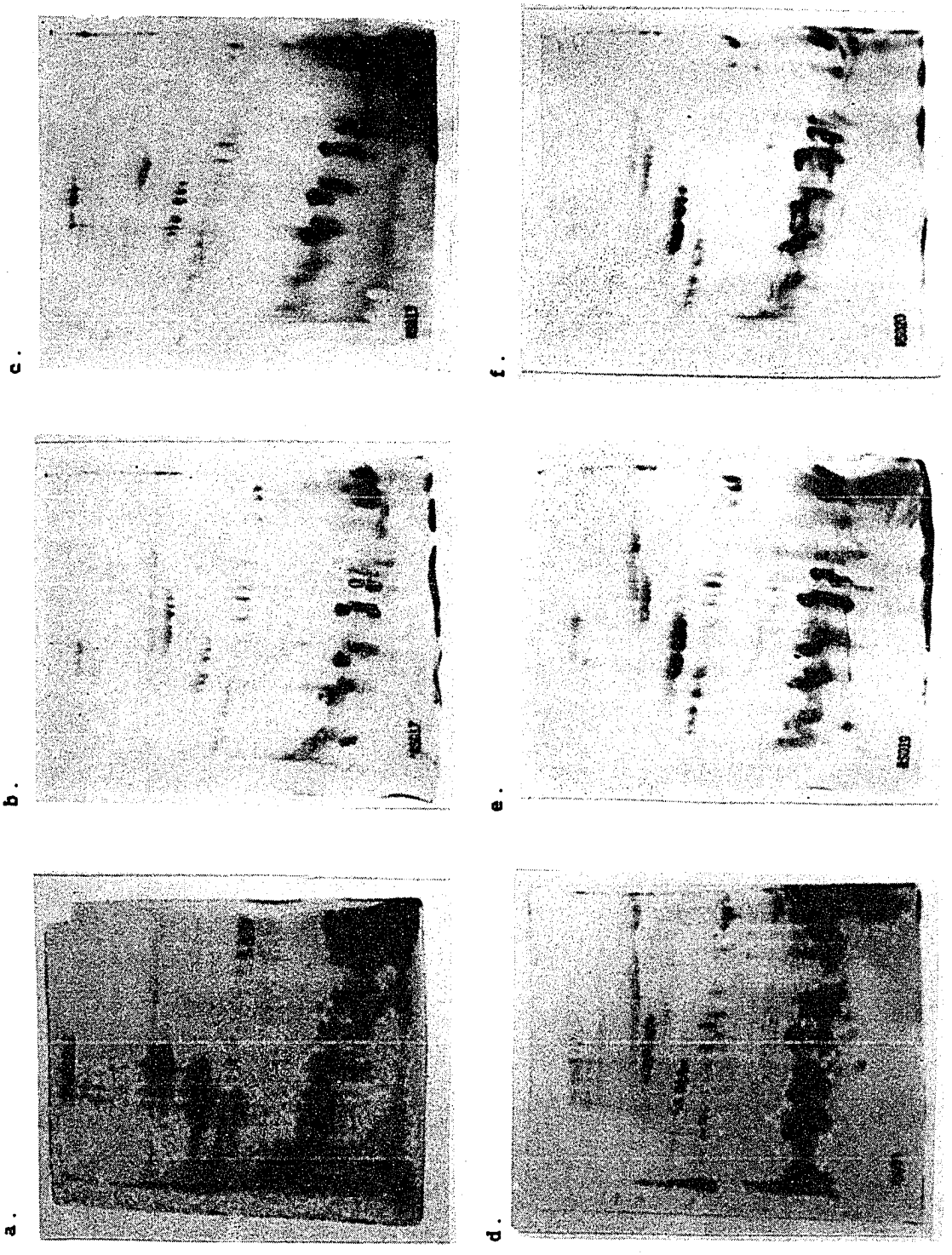

FIGURE 11. Electropherograms of Seminal Plasma from Varicocele Patients.

moderately oligozoospermic.

Specimen RS018 (11a) demonstrated a remarkable heterogeneity in the series A proteins (although the higher molecular weight bands may represent incomplete liquefaction of CP5). The series VII proteins are present in quantities similar to those in normal individuals (i. e. only slightly visible - as opposed to the discrete spots observed in 11c).

Specimen RS019 (11e), shows increased amounts of transferrin, whereas hTf is only faintly visible in 11c where the patient is suffering from testicular atrophy. This specimen demonstrates a large number of discrete high molecular weight proteins (80-200 kDal) which are not generally observed in seminal plasma, while the low molecular weight components are somewhat reduced.

4. Other Patients with Unique Seminal Plasma Characteristics : Specimen RS034 (Figure 12a) was produced by a patient exhibiting severe oligozoospermia as well as hyperspermia. He had also suffered from epididymitis three months prior to the collection of this specimen. As seen in the severely oligozoospermic varicocele patients discussed previously, the series VII proteins are present in increased amounts. There is very faint evidence of the presence of series I proteins . There are six distinct hTf spots as opposed to a normal heterogeneous band. There is also considerable vertical streaking between the acid phosphatase isomers and the series VII proteins which is only observed in a few other specimens.

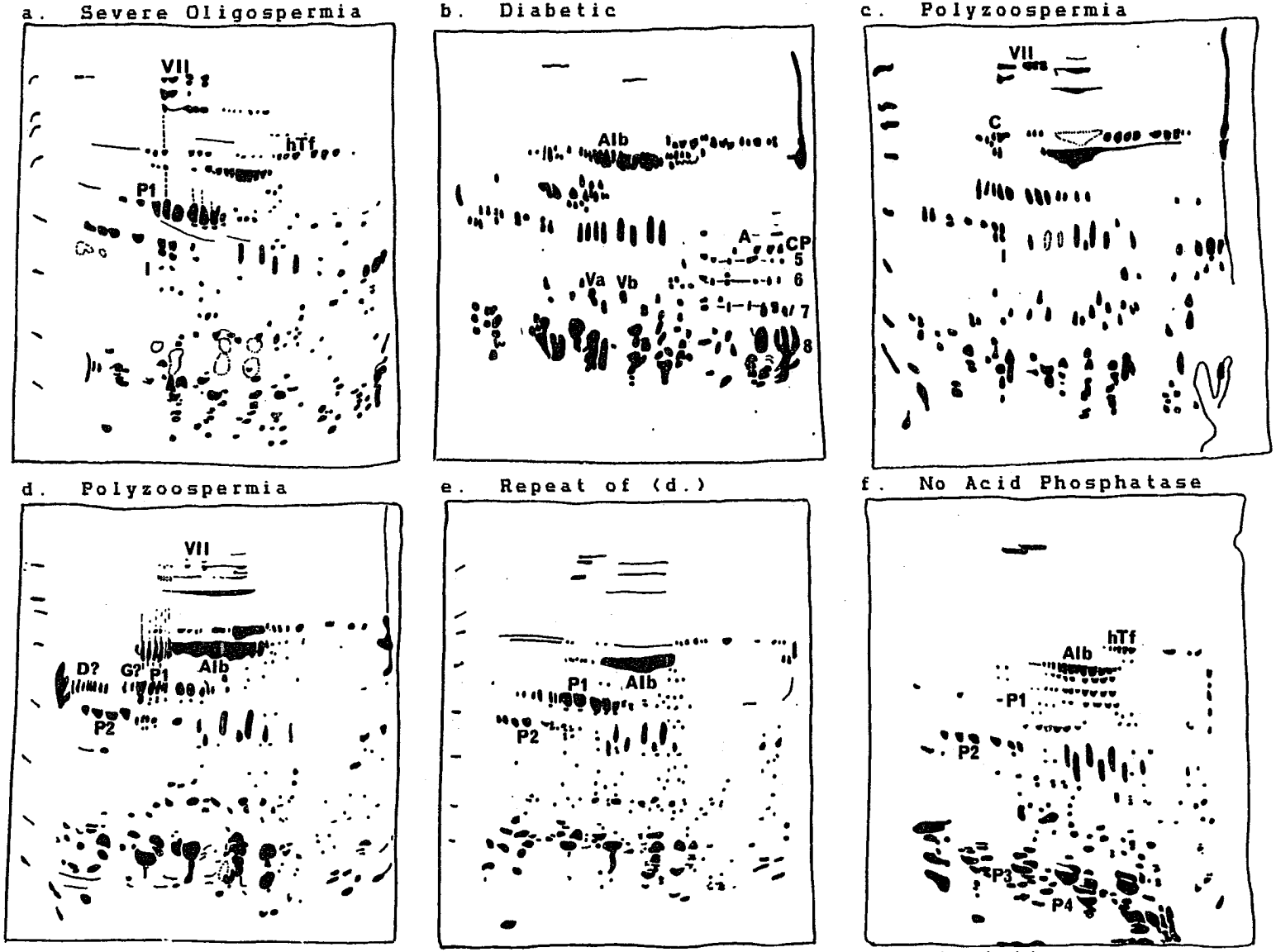

FIGURE 12. Seminal Plasma with Other Unique Characteristics.

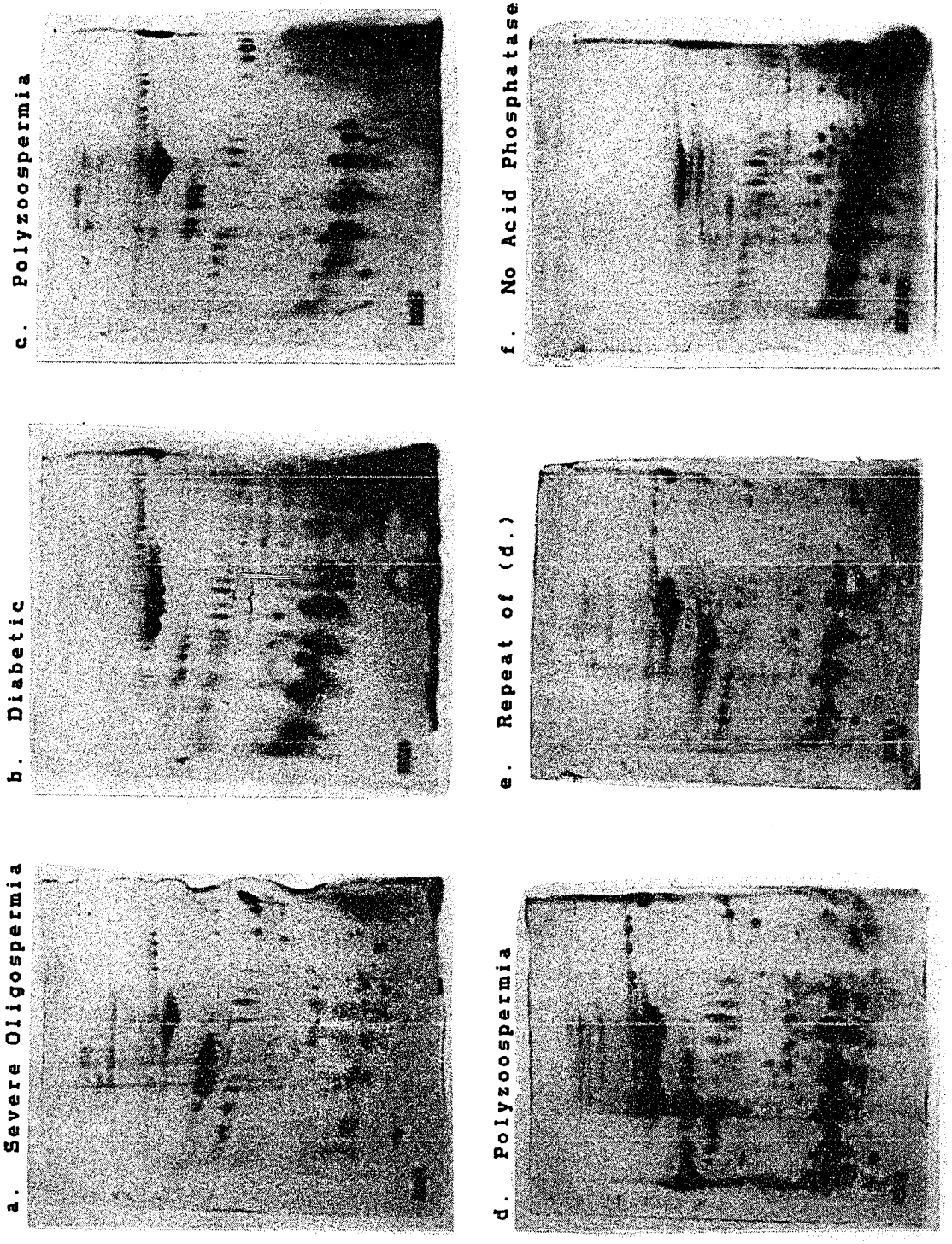

FIGURE 12. Seminal Plasma with Other Unique Characteristics.

Patient RS014 (Figure 12b) is a 23 year old diabetic. His diabetic classification and level of control are unknown. He was hypospermic (semen volume 0.7 ml) as well as asthenozoospermic (total mobility 11%). The sperm count was within the normal range, but approximately one third of the sperm were dead. The most striking observation in this electropherogram is the heterogeneity and increased levels of albumin due possibly to non-enzymatic glycosylation. This gel also shows signs of incomplete liquefaction as shown by the presence of faint bands of protein in the CP5-8 regions. Two prominent proteins are also observed in the V or L series area. These have been designated Va and Vb.

Several individuals presented with polyzoospermia - or sperm counts greater than 250 million/ml. RS006 had undergone a varicocelectomy 4 months prior to this analysis. His count was over 400 million/ml (total count = 1.2 billion!) with about 30% of these being dead. Motility was about 50%. Series VII proteins were enhanced as well as those in area C. Series I proteins are visible. Other areas appear to be normal.

RS031 had a count of 225 million/ml with about 12% dead and 75% motility. The electropherogram shows some very obvious differences from normal. When compared with Figure 7, however, one can see proteins similar to those found in pre-ejaculatory fluid superimposed on an otherwise typical HSP protein distribution. Re-analysis of this same specimen one week later gave rise to a relatively normal protein distribution (see Appendix D-31).

HSP 2414 was produced by an individual whose wife was undergoing evaluation for admission to the EVMS Vital Inception of Pregnancy (VIP) Program. This individual was a 34 year old white male who had undergone varicocelectomy in November of 1982 (eight months prior). He and his wife had been attempting to conceive for almost two years. Hormone analysis revealed slightly increased testosterone levels (1200 ng/ml) and decreased levels of FSH (11 mIU/ml). Trace metal analysis revealed low levels of zinc (45000 ug/L). In observing Figure 12e, one can see three repeating patterns of spots in the vicinity of albumin differing in molecular weight by about 5-6 kDal. Acid phosphatase (P1) is completely lacking from the pattern: the small series of spots in this region represents a series of proteins which migrate just ahead of ACP and which are observed in most normal specimens. Decreased or absent ACP levels correlate well with the low zinc levels observed in this specimen since acid phosphatase is a zinc-containing enzyme. Other prostatic secretions are present in this specimen however, as indicated by the presence of P2-P4 proteins. Series A proteins are also present, but in reduced amounts.

Appendix D gives a complete listing of HR2DE gel tracings from most of the patients and donors evaluated in this project. These data are offered so that a retrospective analysis of specimens can be made as new information from future seminal plasma studies becomes available.

V. DISCUSSION

Initial time course studies were oriented towards determining a consistent time to sample the seminal plasma after ejaculation. Optimally, it would be desirable to sample the specimen as soon after collection as possible. However, it was generally not feasible to have the patient collect the specimen at the laboratory (especially at the Leigh office or for the Lamaze volunteers) because of the lack of privacy and psychological factors associated with masturbation in "public". These psychological factors can lead to inhibition of arousal resulting in difficulty in producing a specimen, or the production of a poor quality specimen (as compared with semen emitted under the influence of high levels of neuro- and hormonal stimulation associated with actual intercourse).

An alternative would be to provide the patient with a collection container containing a preservative solution of some sort to prevent any changes from taking place prior to analysis (analogous to adding heparin or EDTA to whole blood to prevent clot formation). However, since these specimens were usually collected under the direction of a physician to obtain the diagnostic information normally associated with a "routine" semen analysis, this was not possible either. Therefore, since (as previously discussed) few changes could

be observed in the electropherograms after about 90 minutes, it was decided that this would make a good "standard" denaturation time for the patient studies at least. This provided ample time for the patient to deliver the specimen to the laboratory and to do the initial work-up necessary (see Apendix C) on the specimen prior to denaturation. Use of this 90 minute timespan as a standard for denaturation depends on the patient's honesty in accurately reporting the time of collection.

After these initial studies, it was subsequently decided that a closely controlled analysis of the initial events associated with liquefaction should be studied. Analysis of a non-liquefied specimen presented technical problems, however. Accurate pipetting of a "gel" was difficult and was resolved in part by cutting the ends off of the plastic pipet tips so that the coagulum could pass through the resulting wider bore. In addititon, there was some concern about the possibility of contamination of the seminal plasma with sperm proteins since it was not possible to remove them by centrifugation prior to denaturation. It has been shown by others (79) that considerable concentration of sperm protein extracts is necessary before spermatozoal proteins can be visualized. Even then, it is necessary to use silver-staining techniques to observe any significant amounts of protein. As additional proof that spermatozoal contamination of HSP was not a problem in gels stained with Coomassie Brilliant Blue (CBB), liquefied seminal plasma

from a donor was denatured both with and without centrifugation to remove sperm and subsequently analyzed by HR2DE with no observable differences found (results not shown).

The observation that the coagulation proteins could be visualized as well as their subsequent diminution with time demonstrates that semen additives are not necessary to carry out this type of analysis. It does however, require patient/donor cooperation in providing a specimen "on demand" to the laboratory as soon as possible after collection (within five minutes).

Continued use of the "standard" 90 minute denaturation time probably still represents a realistic approach for future routine analysis however: especially in specimens where there is no reason to suspect that liquefaction is a problem (i. e. the specimen has liquefied). Caution must be given however, to differentiate between incomplete liquefaction and seminal plasma with a high viscosity. Very viscous specimens are difficult to handle both for routine semen analysis and for analysis by HR2DE. The few very viscous specimens that were analyzed by this technique either provided poor quality gels, or little in the way of meaningful results. The technical problems associated with this classification of specimens still need to be addressed.

In its original conception, it was invisioned that the bulk of the useful information gathered in the course of this research would come from the evaluation of seminal plasma from different classes of individuals with previously

diagnosed abnormalities thus allowing us to make comparisons between specimens based on known differences etc.. However, due to the rarity of certain disorders, the lack of patient availability, and, in some cases, patient cooperation, it was possible to obtain only a few specimens of this type providing any useful information. Considering the availability of specimens from the Andrology Laboratory and a renowned local urology practice - both dealing with fertility disorders and artificial inception of pregnancies etc., this seems to be a bit surprising. However, even with the large volume of research that has been accomplished over the years dealing with male fertility, "idiopathic oligozoospermia" is all too often still a common diagnosis. In other cases where a "diagnosis" is more certain, an explanation of "why" certain semen parameters are present is often not possible.

The split ejaculate technique has often been considered a "crude" means of obtaining fractionated semen although the "partitioned" nature of semen has been known since the late eighteenth century (5, p. 56). Consideration had been given to using this technique early on in this study. However, it was not until the end of the project that funds were made available to provide remuneration for the services of a group of donors specifically solicited for this purpose.

The data collected in these split ejaculate studies provided a great deal of information about the protein contributions of the various accessory organs and their distribution on HR2DE separation. Comparison of these

various fractions with electropherograms of whole semen then allowed for a retrospective evaluation of the almost one hundred patient specimens previously analyzed. It then became possible to look at some of these results which had been classified as abnormal, artifactual, or, as in one case (RS031), thought to be due to contamination, and re-evaluate them in terms of specific accessory organ (dys-)function.

Obtaining pure prostatic fluid from healthy individuals is a difficult task. Prostatic palpation on rectal examination is an uncomfortable procedure - not one that men usually submit to willingly (especially after the initial experience!). Healthy individuals do not normally produce any significant quantity of fluid which is able to be collected for analysis. As men age however, and prostatic hypertrophy becomes more apparent, fluid is more likely to be expressed. The question is, however, does this represent a normal specimen?

Comparison of split ejaculate first fractions with prostatic fluid has allowed us to differentiate which components in a map of whole seminal plasma are contributed specifically by the prostrate (see Figure 8). Other studies have made use of prostatic fluid expressed from glands which had undergone the trauma of surgical removal or autopsy prior to analysis (see for example Dermer et al. [43]). The split ejaculate technique, on the other hand, provides a convenient method of analysis resulting in no patient discomfort, or introduction of artifacts by use of pathological or surgically-removed material.

Use of the split ejaculates in conjunction with HR2DE in this project has also given further evidence in support of the sequential release of accessory organ components during the ejaculatory process. In addition, there is some evidence of the heterogeneity of the prostate gland itself in that different portions of the gland (as described by McNeal for example [19, 82]) may be functioning in concert to produce a composite fluid consisting of the secretions of several functionally different glands. This would offer a basis of explanation for the seminal plasma results observed in specimen HSP 2414 shown in Figure 12. In this specimen, the acid phosphatase isomers are completely lacking while other prostatic components are present. If the gland were functioning as a unified organ, it would seem that all of the components would be altered as a result of a given abnormality. In light of the fact that the gland is being influenced by both hormonal and neural stimuli, especially during arousal, it is highly probable that different anatomical areas would respond differently to both of these types of stimuli and that this would be reflected in the composition of the fluid as a whole as it is being released. Thus, one could explain why certain prostatic components decrease more rapidly in concentration than others when observed in serial split ejaculate fractions such as was shown in Figure 5, or how some components can be present in a specimen such as HSP 2414 and others not. Of course, the absence of components in a specimen such as this might also represent the observation of a genetic deficiency or a

post-translational defect resulting in the absence or relocation of certain protein constituents.

The protein P3 in Figure 4 is tentatively identified as lysozyme based on its similarities of physical characteristics and migrational properties with the low molecular weight standard marker protein lysozyme. The presence of lysozyme in high concentrations in prostatic fluid (as reported by Tauber [30]) and the fact that there are only two major proteins observed to be migrating in the 15,000 dalton molecular weight range of both first fractions and pure prostatic fluid make P3 a possible candidate - especially since P4 was previously identified by Carter and Resnic (53) as Prostatic Binding protein. The fact that no other protein spots are showing up in this low molecular weight region (or any other region) does not necessarily mean that they are not present in the specimen however. Proteins with isoelectric points below about 4, or above 7.5 will either migrate off of the first dimension gel or not enter it because of the limited pH gradient, and thus would not be visualized in the second dimension electropherogram. Additional confirmation of the location of lysozyme is needed.

Both the time course studies and the split ejaculate analysis have demonstrated a class of very basic proteins dubbed "coagulation proteins" along the right-hand, or basic, gel margin from ejaculates denatured very soon after collection of the specimen. That these series of proteins rapidly disappear as semen liquefaction progresses

implicates their involvement with the formation and dissolution of the seminal clot. Observation of these proteins in terminal fractions of the ejaculate stream further indicates that they are produced by the seminal vesicles, which are known to be associated with semen coagulation. The enzymes responsible for semen liquefaction are assumed to be produced by the prostate. Identification of those components in first or prostatic fractions responsible for initiating this activity has not been made however.

As observed here and by others (73), the cleavage products of the liquefaction process do not appear to be present in the gel once liquefaction is completed. That is, there appears to be no quantitative increase in any given protein spot in the gel with time. Our initial assumptions were that some of the low molecular weight components (in the range of 10-15 kDal) might possibly represent some of these cleavage products, but these misconceptions were dispelled when we observed that most of these components were present in whole semen almost immediatly after ejaculation. The observation of a regular decrease in molecular weight of the successive coagulation proteins (CP 1-8) would seem to indicate that small peptide fragments (5 kDal or less) are being cleaved off of CP-1 and traveling with the salt front in the second dimension gel. Often times there is observed a non-specific staining of the lower right corner of the slab gel which may represent a homogenious staining of these peptide products.

The existence of macroscopic seminal globules in some fresh semen specimens is commonly acknowledged, but they are generally not present in a majority of specimens observed. Their presence may possibly be associated with some pathological condition which affects semen liquefaction. The fact that these globules were shown to be "packets" of seminal vesicular secretions due to the similarity with terminal split ejaculate fractions would tend to confirm these observations. Little has been reported in the literature about these globules other than their observed presence.

Donors providing the scant urethral gland secretions, or "pre-ejaculator fluid" (PEF) used in this study reported that these secretions were difficult to produce "on demand" using purely mechanical stimulation (i. e. masturbation). One individual found it necessary to elicit the assistance of a female partner to provide the stimulus necessary for production of this secretion. This would lead one to believe that their incidence of detection would be low in specimens collected by relatively unstimulated masturbation, due to their low volume of secretion (3-5% of the total ejaculate) and their low protein concentration. Indeed, visualization of the protein spots in Figure 7 was only accomplished by restaining the gel using a silver stain technique after initially staining it with CBB R-250. The initial CBB stain showed only very faint spots in the vicinity of series D and G whereas silver staining gave the results shown in Figure 7 (see also Appendix D-77B).

Analysis of specimen RS031 revealed a constellation of spots superimposed on a normal HSP protein distribution. This superimposed pattern had a remarkable resemblance to the pattern in the analyzed PEF specimens. The circumstances surrounding the collection of this particular specimen are unknown, other than the fact that it was produced at home for a routine semen analysis by an individual who had an elevated sperm count (225 million/ml).

Re-analysis of the same specimen one week later showed the disappearance of these proteins leading us to believe that the initial analysis was contaminated by some unknown source. Retrospective analysis almost six months later after carrying out the PEF studies showed that the source of this "contamination" might have been urethral gland secretions which subsequently disappeared with standing, or in association with freezing and thawing of the specimen for re-analysis.

Mucous membranes guarding the entrances to the body are rich in immunoglobulin A (IgA); a secretory dimer of IgA slightly different in composition from circulating serum IgA in the bloodstream. This component maintains vigilance at these body orifices providing immunological defense against the invasion of potentially harmful substances. Secretory IgA consists of two IgA monomers which are complexed with what has been described by Tomasi and Bienenstock (80) as a secretory component (SC) protein, and a "J-Piece" protein described by Koshland (81). SC was reported to have a molecular weight of 58000 Daltons, while the "J-piece" is a

negatively charged protein with a molecular weight of 15000 Daltons (as determined by gel filtration). There are several proteins in the pre-ejaculatory fluid represented in Figure 7 that may correspond to these secretory components. Proteins in Series E and F have similar migrational characteristics to the light and heavy chains of IgA respectively. Protein G is a very prominent spot with a molecular weight ranging from 59-65 kDal. Proteins in area H are acidic (pI 4.5-5) and have a molecular weight of 30000 Daltons.

Detection of IgA in secretions from the mucosa of the distal or cavernous urethra is in accordance with findings in other mucosal membranes. The findings of Tauber et al. in 1974 (28) showed that IgA could be immunologically detected in initial portions of the split ejaculate, with decreasing concentrations in subsequent fractions. IgA was not detected in 15% of their specimens, however. This may be due to the level of stimulation at the time of collection.

Another interesting observation in PEF is the increased presence of the high molecular weight series VII proteins noted previously. This series of proteins was also observed to be increased in many of the oligozoospermic patients (sperm count of less than 50 million.ml) we observed, and in even greater amounts in most of the severely oligozoospermic patients (count less than 5 million/ml). The significance of this observation is not known.

In their paper on the elecrophoresis of seminal plasma

(73), Edwards et al. described a group of proteins ("Group A") that were observed to be present in "fertile" individuals, but not in post vasectomy patients. They proposed that these proteins may be of testicular origin. These observations were based on a comparison of three post-vasectomy patients with five "normal" specimens. We have observed a group of proteins (designated Series I) which have similar migrational characteristics (relative to P2) to Dewards' Group A. Observation of over 200 seminal plasma electropherograms has shown that our series I is not always present in "intact" individuals, and does appear to be present in at least one post-vasectomy seminal plasma evaluated (RS053). The length of time after the vasectomies was not given by Edwards (73). Most of our post-vasectomy patients were submitting their semen for a post-operative verification of the absence of sperm. Detection of these proteins in recent vasectomy specimens may represent residual secretions since it is known that sperm may be present up to several months after vasoligation. On the other hand, absence of this protein in long term vasectomy patients may represent the loss some accessory gland secretion that was closely associated with testes-specific factors.

The absence of this series in "intact" patients can also be associated with subfertility or some pathological condition such as varicoceles. The absence of this group of proteins in most oligozoospermic varicocele patients evaluated may indicate that it is associated with testicular

function. Its observed presence in two only slightly oligozoospermic patients (RS018 and RS030) may indicate that the particular lesion produced may only be associated with prolonged venous incompetence, and that early surgical intervention might prevent the disappearance of this substance. This is an area that demands further study.

A final comment is added to discuss the absence or presence of proteins observed in seminal plasma. All of the proteins observed in seminal plasma electropherograms are not necessarily specific to the male reproductive system. Some of these proteins may represent transudates from serum (73) or may be present as a result of infectious processes. The presence of significant levels of pathogenic organisms as well as the body's immune response to these organisms may contribute observable levels of protein to the seminal plasma electropherogram. One of our prospective donors with a history of trichomoniasis was observed to have a high level of unidentified organisms in his semen. Electropherograms of his seminal plasma (Appendix D-63) showed a general increase in the numbers of proteins observed throughout the gel. Additional work in this area may prove to be fruitful.

VI. SUMMARY

In this work, we found that it is not necessary to add proteolytic inhibitors to seminal plasma in order to observe proteins associated with liquefaction if the specimen is analyzed immediatly after collection. For studies of proteins other than those associated with coagulation/liquefaction, we found that 90 minutes represents a good "standard" denaturation time with few observable changes in the HSP electropherograms after this time.

The split ejaculate technique provides a very good means for studying accessory organ secretions, and for the identification of protein constituents associated with these glands. As the ejaculation sequence progresses, one can observe definite quantitative changes of various protein components associated with both the prostate and the seminal vesicles.

Our prostatic fluid electropherograms are almost identical to the distribution of proteins in initial split ejaculate fractions, and compare favorably with the work of Dermer et al. (43) and Carter and Resnick (48). We suggest that additional work be carried out to see if protein P3 represents Lysozyme.

Analysis of macroscopic globules found in whole semen

has shown that these substances represent "packets" of unliquefied seminal vesicle secretions. The distribution of proteins in electropherograms of these secretions match those found in terminal fractions of split ejaculates.

Pre-ejaculatory fluid has been analyzed for the first time by HR2DE, and shows a distribution of proteins characteristic of acidic sialomucoproteins. We suggest that confirmation of the presence of secretory IgA in these secretions be made by co-electrophoresis of sIgA and by immunological methods.

Several series of proteins appear to be associated with male fertility status. Series I proteins may, or may not, be identical to the Group A proteins of Edwards (73). These proteins were observed to generally be absent in post-vasectomy patients , and in severely oligozoospermic patients, but were observed in at least one post-vasectomy patient, contrary to Edwards' results. Series VII proteins are also associated with severe oligozoospermia. The significance of these results are not known. It is suggested that additional studies be carried out in these areas.

In closing, the almost constant association with this project for the past five years has provided the opportunity to spend many sometimes exciting moments and often-times arduous hours immersed in the basic research of human male fertility. The chosen technique - High Resolution Two-Dimensional Electrophoresis - was a relatively new

technique when we first began to use it in 1978. It has since come into great popularity as a very powerful analytical tool for the analysis of complex biological specimens. We have shown that it is particularly well suited for the analysis of human seminal plasma, and, when coupled with the split ejaculate technique, provides a very simple means for determining which components of whole seminal plasma are contributed by the various accessory sex organs associated with male reproduction.

Application of these results to patient data has provided, and should continue to provide, additional insight into some of the possible reasons for reduced male fertility. As a result, it is hoped that ultimately some form of medical treatment may be provided so that these individuals may go forth and

"...be fruitful and multiply..."

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APPENDIX A-1

Locally Fabricated Vertical Slab Gel Electrophoresis Chamber

(see Figure A1)

a. Upper Buffer Chamber: 145 x 70 x 50 mm.

b. Faceplate: 1/2" white Plexiglass cut 215 x 175 mm (notch 135 x 40 mm).

c. Faceplate Gasket: 5/16" OD x 3/16" ID x 1/16" wall Tygon tubing.

d. Notched plate: 1/8" DSB glass plate cut to pattern 190 x 165 mm (notch 20 x 135 mm).

e. Spacer: 190 x 10 x 1.5 mm Teflon.

f. Sealing Gasket: 3/32" OD x 1/32" ID x 1/32" wall Tygon tubing.

g. Front Plate: 1/4" plate cut 190 x 165 mm.

h. Clamp: Cut from 1/2 inch aluminum

i. Lower Buffer Chamber: 190 x 190 x 55 mm.

j. Platinum Wire: 26 gauge x 150 mm.

All Plexiglass except the Faceplate is cut from 1/4" stock and milled to size. Pieces were glued together with Plexiglass Solvent. Chambers were sealed with silicone caulk to prevent leakage.

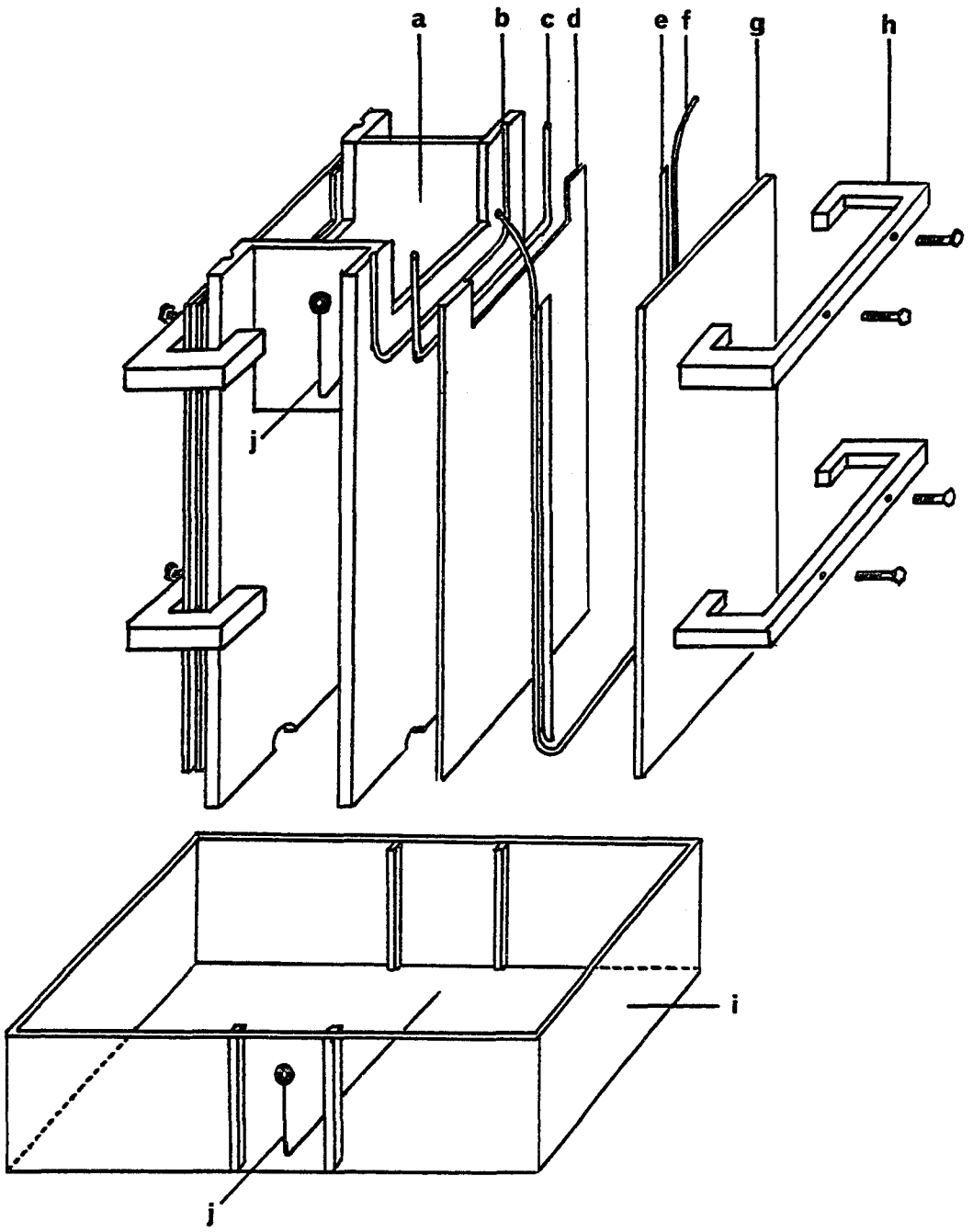
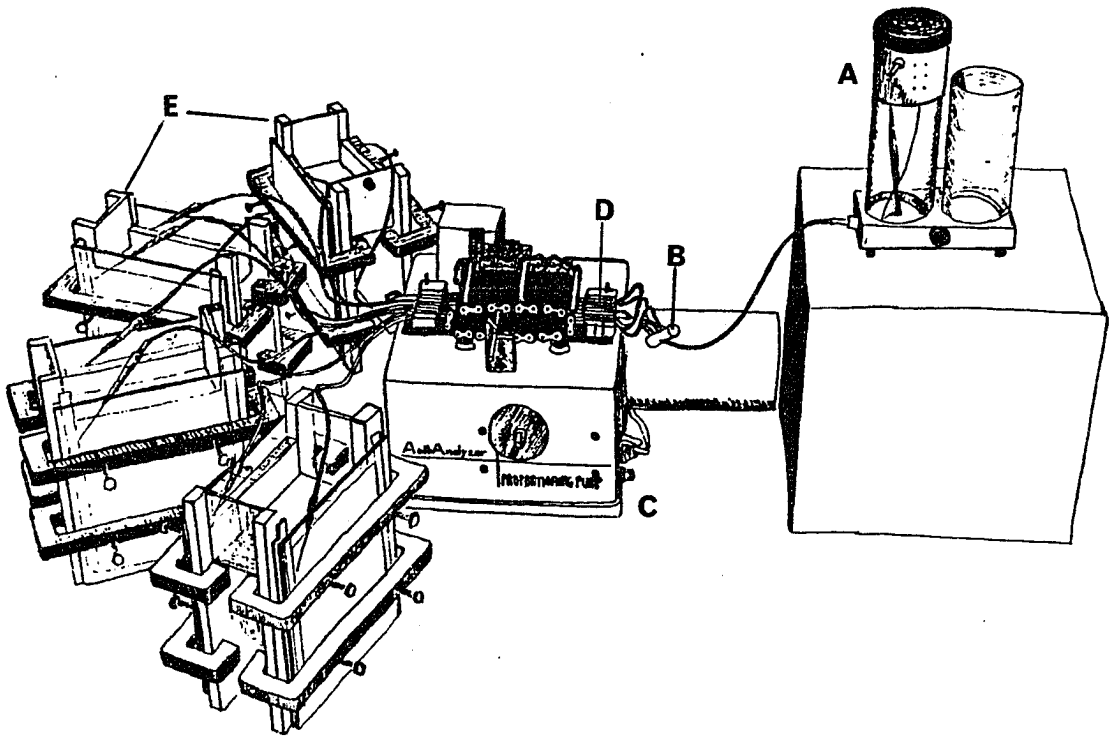


FIGURE A-1 Vertical Slab Gel Electrophoresis Chamber

APPENDIX A2

Acrylamide Gradient Casting Apparatus



A. Gradient Mixer (Pharmacia GM-1)

B. Manifold: Constructed from a piece of 1/2" OD, 1/4" ID plexiglass tubing capped at both ends with holes drilled to accept nine tubing nipples (Technicon part number 116-0061P02) which were sealed in place with epoxy cement.

C. Technicon Auto-Analyzer Proportioning Pump

D. Pump Tubing: (Technicon part number 116-0532P14) color coded Green-Green; flow rate 1.0 ml/min.

E. Upper Chamber portion of the Vertical Slab Gel Electrophoresis Chamber with assembled glass plates.

Approximate Gel Volume: 30 ml/gel (total volume = 250 ml)

Casting Time: Approximately 15 minutes.

APPENDIX B

Solicitation of Human Subjects

The following forms were submitted to, and approved by, the Old Dominion University Institutional Review Board for the Protection of Human Subjects for use in soliciting semen donors for this project.

Individuals with recently proven fertility were sought from area Lamaze/Parenthood training classes with seven individuals responding. Semen donors for the specialized collection projects were solicited from the Andrology Laboratory donor pool and through limited on-campus (both ODU and EVMS) advertising.

The Solicitation Form (Appendix B1) was handed out by Lamaze instructors as a first approach to those individuals. On a subsequent class meeting, the primary investigator for this project met with interested individuals and presented a brief over-view of the project's goals (Appendix B2). Those choosing to participate filled out the Informed Consent Form (Appendix B3) and the Questionaire (Appendix B4). They were given a copy of the Collection Instructions (Appendix B5) along with a container and asked to complete the Collection Data form (Appendix B6) at the time of collection.

Similar instructions were given to the other donors except that the Solicitation Form was not used and collection instructions were as given in para. D.1.b and c.

APPENDIX B1

Solicitation Form

Several area scientists are investigating some of the causes of male infertility in conjunction with the ODU Andrology Laboratory, the ODU Clinical Chemistry Program, and the Biomedical Sciences Program at Eastern Virginia Medical School. In support of these research projects, volunteers are needed with recently proven fertility to donate semen specimens to be used solely for biochemical, immunological, and trace metal analysis (absolutely NO specimens will be used for any type of insemination experiments). All data will be held strictly confidential. If you would be interested in participating in these projects, please notify your instructor during the break or after class. There will be a short meeting with some of the scientists involved during the break at next week's class to give out additional information.

APPENDIX B

Solicitation of Human Subjects

The following forms were submitted to, and approved by, the Old Dominion University Institutional Review Board for the Protection of Human Subjects for use in soliciting semen donors for this project.

Individuals with recently proven fertility were sought from area Lamaze/Parenthood training classes with seven individuals responding. Semen donors for the specialized collection projects were solicited from the Andrology Laboratory donor pool and through limited on-campus (both ODU and EVMS) advertising.

The Solicitation Form (Appendix B1) was handed out by Lamaze instructors as a first approach to those individuals. On a subsequent class meeting, the primary investigator for this project met with interested individuals and presented a brief over-view of the project's goals (Appendix B2). Those choosing to participate filled out the Informed Consent Form (Appendix B3) and the Questionaire (Appendix B4). They were given a copy of the Collection Instructions (Appendix B5) along with a container and asked to complete the Collection Data form (Appendix B6) at the time of collection.

Similar instructions were given to the other donors except that the Solicitation Form was not used and collection instructions were as given in para. D.1.b and c.

fertilty status. We have developed a technique (High Resolution Two-Dimensional Electrophoresis) that enables us to simultaneously evaluate several hundred protein constituents in a given semen specimen. We believe that these complex protein patterns are related to an individual's fertility status, and have used this technique to evaluate the seminal plasma of a number of infertile patients. It is necessary at this point however, to compare these results with specimens contributed by individuals who have recently proven that they are fertile.

Trace metal analysis of semen is another area of investigation that may provide the physician with information aiding in his diagnosis and possible treatment of infertility problems. Certain trace elements (or minerals) such as iron, copper, zinc, and selenium are essential for proper protein function and metabolism. Conversely, elements such as cadmium or lead are associated with toxic exposures and can seriously affect fertility. We have looked at these metals and others in seminal plasma from infertile males and it is now necessary to again, compare these results with data from proven fertile individuals.

Masturbation is the method of choice for collection of semen for the above analysis. Other collection procedures are not acceptable because they may lead to contamination of the specimen from various outside sources. There are no known medical risks associated with semen collection via this method. The benefits that may be derived from

participation in this project include the satisfaction of knowing that you have contributed to the basic understanding of male reproductive function, and that this information may ultimately help others to conceive who otherwise might have remained infertile.

APPENDIX B3

Informed Consent Form

Project Name: Human Semen Evaluation

This is to certify that I, _____, hereby agree to participate as a volunteer in a scientific investigation as a part of the educational and research program of Old Dominion University under the supervision of Drs. James H. Yuan, Patricia A. Pleban and Edward E. Gaunt.

The investigation and the nature of my participation have been described and explained to me, and I understand the explanation. I further understand that I may withdraw from the project at any time, without penalty or prejudice.

I have been afforded the opportunity to ask questions concerning the purpose of this project and all such questions have been answered to my satisfaction. I understand that should I have additional questions in the future about this project or the manner in which it is conducted, I may contact one of the above named individuals at 440-4078.

I understand that I am free to withhold any answer to specific items or questions in any questionnaire submitted to me for this project. I understand that any data or answers to questions will remain confidential with regard to my identity. I further understand that no data will be identified with me nor will any information be released to persons outside of the research team without the team first obtaining my written permission.

I acknowledge that I was informed that there are no known risks which can be incurred as a result of participating in this experiment. Therefore, I understand that no medical or psychological assistance will be made available to me by either Old Dominion University or any member of the research team as a result of any physical or emotional harm I may experience as a result of participating in this project.

I have been informed that I have the right to contact the Old Dominion University Institutional Review Board for the Protection of Human Subjects should I wish to express any opinions regarding the conduct of this study.

_____ Date: _____

Signature of Volunteer

_____ Date: _____

Signature of Witness

APPENDIX B4

D O N O R I N F O R M A T I O N

Specimen #

Age	Area of Residence
Weight	Occupation
Height	Race

1. How many children have you conceived?

2. Have you had any major illnesses in the last six months (i.e. flu, bad colds, fever, allergies)?

3. Are you taking any drugs or medications (please list)?

4. Do you take vitamins? If so, what kind? How often do you take them?

5. Have you ever had any type of reproductive organ problems such as prostatitis, epididymitis, tortion, varicocele, etc? If so, how was this treated?

6. Have you ever had the mumps? Venereal Disease?

7. Have you ever had any types of rheumatoid diseases (arthritis, etc)?

8. Do you now smoke, or have you smoked in the past year? How much, on an average, per day? _____packs/day

9. Are you exposed to any type of toxic substances on your job such as metal fumes (from welding, etc.), solvent vapors, radiation, etc.? Please describe.

APPENDIX B5

C O L L E C T I O N I N S T R U C T I O N S

Realizing that semen collection by masturbation is a "touchy" subject for most men, we would like to make this as easy for you as possible. You have been provided with a specimen container in which the semen is to be collected and transported, and some information sheets to be filled out. Please fill them out as completely as possible. All information will be held in strictest confidence.

In order for you to provide a specimen that is scientifically useful, it is necessary that you follow certain collection procedures. An abstinance period of between three and five days is required in which no ejaculations occur. If a specimen is collected less than 72 hours from a previous emission, the semen may not have had enough time to be replenished with sperm and other semen components resulting in a biased analysis. Similar problems occur if it has been longer than five days since your last emission. Additionally, it is absolutely necessary that the entire ejaculate be caught in the collection container. If any of the specimen is lost it will seriously affect the results. To avoid contamination, please do not remove the blue cap from the collection container until just prior to collection. Ensure that your hands and genital areas are clean, and that no pubic hairs fall into the container.

Once you have successfully collected the specimen, accurately note the time it was collected and other information requested on the reverse side of this form. Time of collection is important. It is necessary that the sample be delivered to the lab within ONE HOUR of this time. Also, please do not subject the specimen to heat or cold--ideally it should be kept at body temperature until delivered.

Thank you for your cooperation. If you have any questions please call 440-4104.

APPENDIX B6

C O L L E C T I O N D A T A

Specimen #_____ Date_____

Time Collected:_____

(Please deliver to the lab
within ONE HOUR of this time)

Was any specimen lost during collection?

Date of last intercourse or ejaculation:

THANK YOU FOR YOUR COOPERATION

APPENDIX C

SEMEN ANALYSIS PROCEDURES

The following procedures are used to evaluate semen received from patients or donors.

Receipt of Specimen

1. Upon receipt of the specimen, ensure that the time of collection is accurately noted. Obtain this information from the patient along with all other information not recorded.
2. Note state of liquifaction and specimen color (yellowish color may indicate a high count).
3. Measure the semen volume (after complete liquifaction) with a wide bore 10 mL graduated serological pipet. (If specimen is to be cultured, use a sterile pipet.)
4. Approximately 90 minutes after the noted collection time, remove 500 uL of well mixed specimen using a metal free pipet tip and centrifuge at low speed (less than 300 x g) for five minutes in an acid washed 1.5 mL microcentrifuge tube. Afer centrifugation, remove 100 uL of the seminal plasma for denaturation and the remainder of the seminal plasma for trace metal analysis.

Motility Procedures

1. Prewarm glass microscope slides and eosin stain (0.5%

w/v in distilled water) to 37 C.

2. Dispense 50 uL of well mixed semen onto a prewarmed labeled slide being careful not to form bubbles on the surface of the pool. Cover with a 24x40 mm coverslip and allow to settle for 5-10 minutes on the slide warming tray.

3. On another prewarmed slide dispense 50 uL of semen and 15 uL of warmed eosin stain . Mix well with the pipet tip and coverslip. Allow the slide to settle while doing the motility count.

4. Count a minimum of ten microscope fields for each classification of motility (i.e. quick progressive, sluggish, and non-motile) using the oil immersion objective.

5. To determine the live/dead ratio, count a minimum of ten fields on the eosin stained slide, differentiating between live non-motile sperm (non-staining) and dead sperm (pink colored) using the oil immersion objective. %dead = (# pink staining sperm) / (total non-motile sperm) x 100.

Morphology Procedures

While doing the motility counts, one should be on the look-out for abnormal sperm types. A table of commonly encountered sperm types is given by Zaneveld and Polakoski in (29). The particular type of abnormality observed along with its prevalence should be recorded.

Sperm Counting Procedures

1. Based on sperm populations observed during motility

studies, make up two of the following dilutions (i.e. if there are a large number of sperm in a high power field make up a greater dilution than if only a few are observed.).

1:25 Dilution (Dilution Factor = 25)

Pipet 2.40 mL of Sperm Diluting Solution (0.37% v/v formalin in 0.6 mol/L bicarbonate buffer) into a clean 10 mL vial and add 100uL of well mixed semen. Cover the vial and invert several times to mix.

1:50 Dilution (Dilution Factor = 50)

Pipet 4.90 mL of Sperm Diluting Solution into a 10 mL vial and add 100 uL of well mixed semen. Cover the tube and invert several times to mix.

1:75 Dilution (Dilution Factor = 74.96)

Pipet 5.00 mL of Sperm Diluting Solution into a 10mL vial and add 66.7 uL of well mixed semen. Cover the vial and invert several times to mix.

1:101 Dilution (Dilution Factor = 101)

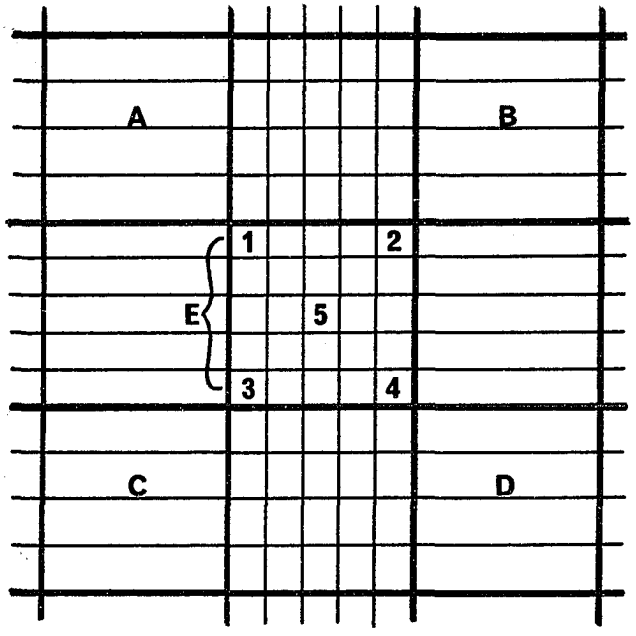
Pipet 5.00 mL of Sperm Diluting Solution into a 10 mL vial and add 50 uL of well mixed semen. Cover the vial and invert several times to mix.

2. Allow dilutions to set for 10-15 minutes to ensure complete sperm kill.

3. Apply the specimen to a dry hemocytometer and allow sperm to settle to the counting plane in a humidified Petrie dish for 5-10 minutes.

4. Count the number of whole sperm/sperm heads in each of

the four corner grids (E1, E2, E3, and E4 in the diagram) and the center grid (E5). Include those sperm touching the left and lower boundaries of the grid but not the right or upper boundaries. Count both halves of the chamber.



5. If sperm concentration is less than 20 million/mL the count must be made using the four outer corner grids (marked A-D above) as well as the entire center grid (marked E).

5. Count the second dilution as above and calculate the results as follows:

Multiplication Factor (MF) =

2000 (Grids A, B, C, D, and E)

50000 (Small grids E1, E2, E3, E4, and E5)

SPERM CONCENTRATION = MF X Dilution Factor (DF) X # counted

TOTAL SPERM COUNT = Sperm Concentration X Semen Volume

7. Comparison of the counts resulting from the two different dilutions should agree within ± 10 %:

$$\left[\frac{(\text{Count A} - \text{Count B})}{\text{Count A}}\right] \times 100 = \% \text{ Error}$$

If the counts do not agree, the dilutions must be remade and counted again.

APPENDIX D

The data in the following appendix represent a 50% reduction of actual gel tracings. These tracings were produced by placing the gel on a "light box" to provide fluorescent backlighting and then tracing the observed pattern of spots in the gel onto a 8"X 8"X 1/16" glass plate placed on top of the gel. Tracings were accomplished by using a fine tip permanant ink marker. The tracings were then transferred to a piece of paper in a similar manner.

The information in these tracings is a two-dimensional representation of three-dimensional information. That is, one should not assume any quantitaive information from the size of the spot: size in no way represents staining density and thus protein concentration in the original gel. These tracings are provided to convey protein positional information only.

The molecular weight marker proteins were generally added to most second dimension separations. These proteins can usually be observed along either the right or left hand margin of the gel. The proteins used and their approximate molecular weights are given as follows:

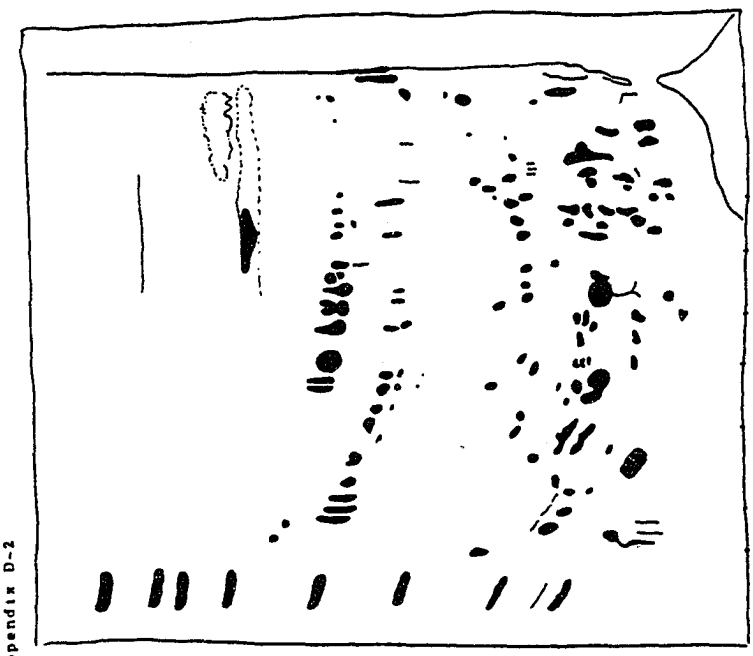
Myosin	200000 Daltons
β-Galactosidase	116300
Phosphorylase B	92500
Bovine Serum Albumin	67000
Ovalbumin	45000
Carbonic Anhydrase	31000
Soy Trypsin Inhibitor	21500
Lysozyme	14400

The pH gradient along the bottom of the gel ranged from 4-4.5 along the left margin to 7.5-8 along the right margin.

Figure 8 is included again in this appendix for easy reference.

FIGURE 8. Composite Map of Post - Liquifaction Seminal Plasma Proteins from Individuals with Recently Proven Fertility.

Appendix D-2



SPEC # RS002 RUN # P021 DATE 1/17/83 Abstinence ?

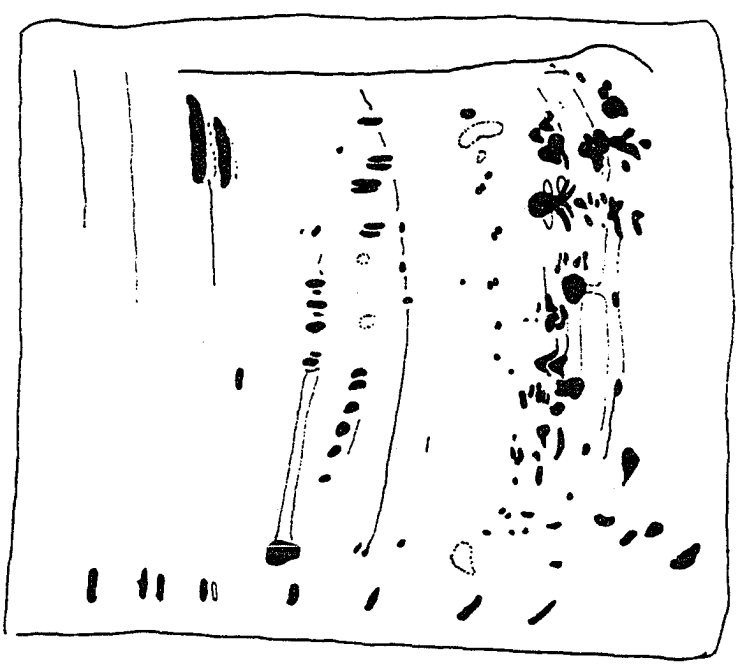
Count 162.5 M/ml Volume 1.5 ml. pH 8

Mobility: 84% OP, 4% S, 12% NM, 52% Dead.

Morphology:

Diagnosis: Left varicocele

Appendix D-1



SPEC # RS001 RUN # P023 DATE 1/17/83 Abstinence ?

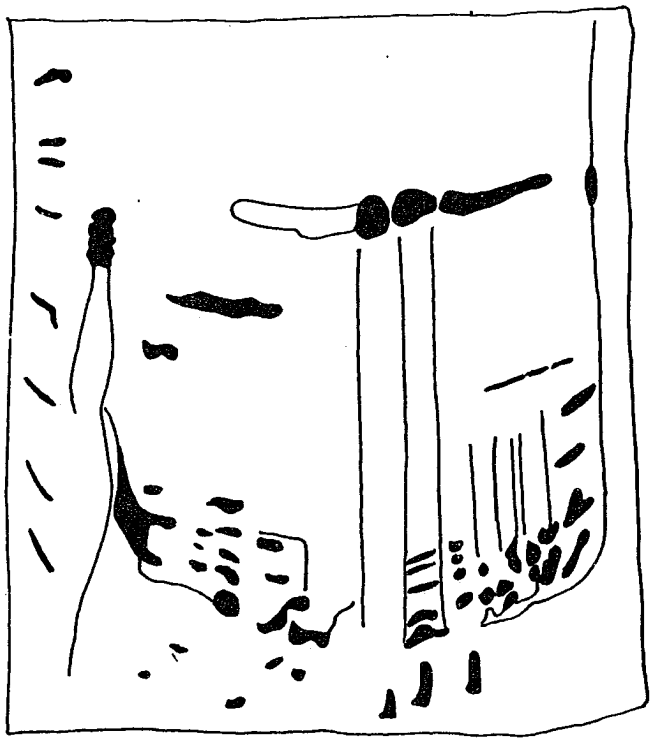
Count 87.5 M/ml Volume 3.5 ml. pH 7.5

Mobility: 30% OP, 4% S, 66% NM, 52% Dead.

Morphology: normal

Diagnosis: Left varicocele

Appendix D-3



SPEC # RS003 RUN # P023 DATE 1/11/83 Abstinence 4

Count 25 M/ml Volume 1.0 ml, pH 8

Mobility: 18% QP, 30% S, 52% NM, 25% Dead.

Morphology:

Diagnosis: Normal exam

Appendix D-4

SPEC # RS004 RUN # P023 DATE 2/1/83 Abstinence 4

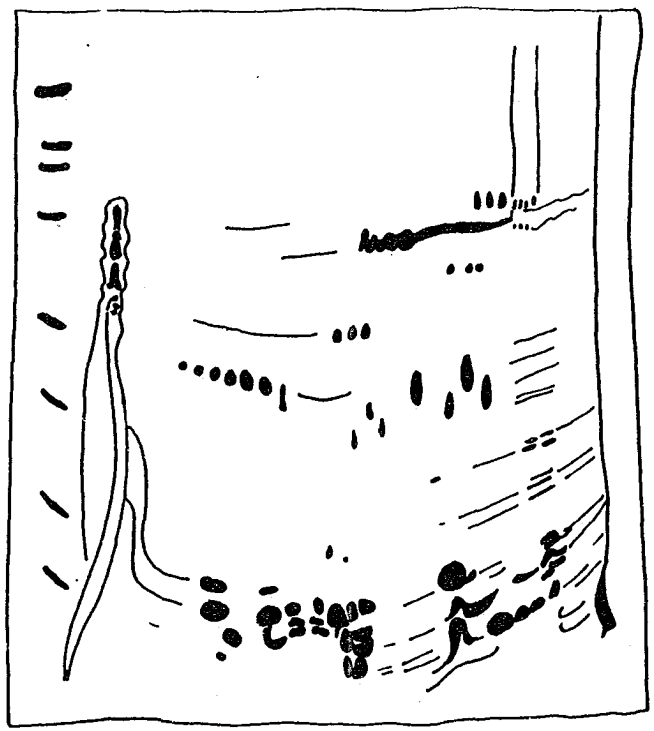
Count 16 M/ml Volume 3.2 ml, pH 7.5

Mobility: 7% QP, 23% S, 70% NM, 68% Dead.

Morphology: Viscous

Diagnosis: No findings

Appendix D-5



SPEC # RS005 RUN # P023 DATE 2/2/83 Abstinence 4

Count 15 M/ml Volume 1.2 ml, pH ?

Mobility. 37% QP, 16% S, 47% NM, 8% Dead.

Morphology:

Diagnosis: (Same patient as RS003)

Appendix D-6

SPEC # RS06 RUN # P027 DATE 2/14/83 Abstinence ?

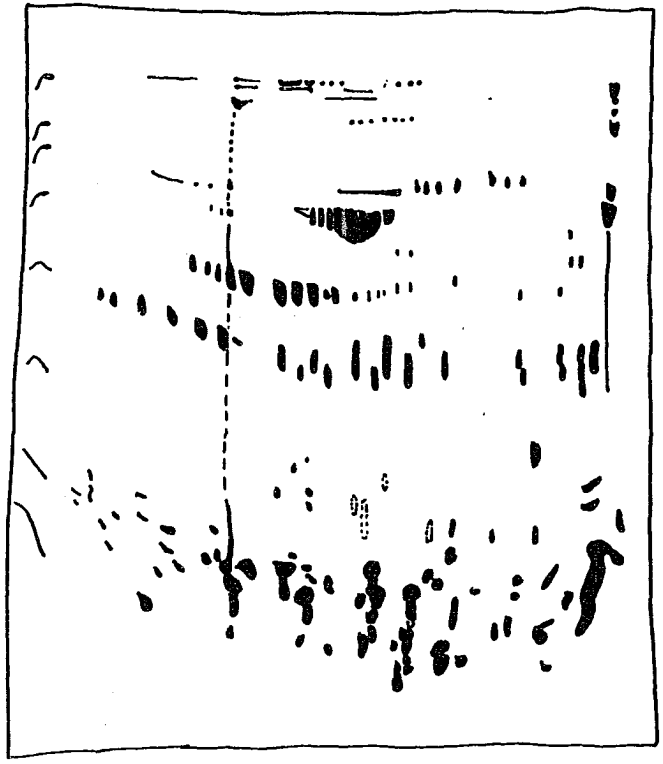
Count 412 M/ml Volume 3.8 ml, pH 8

Mobility: 50% QP, --% S, 50% NM, 60% Dead.

Morphology: Large heads, marked clumping

Diagnosis: Left varicocele, Polyzoospermic

Appendix D-7



SPEC # RS007 RUN # P027 DATE 2/14/83 Abstinence ?

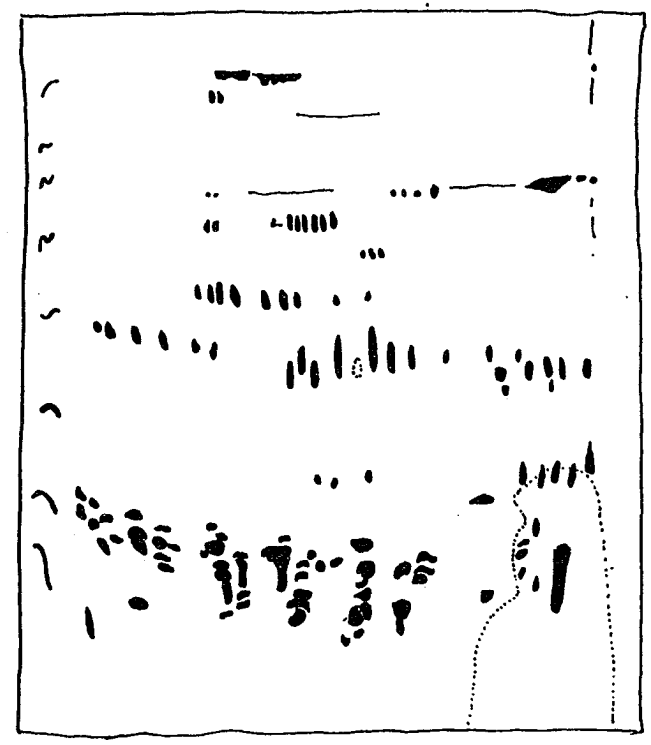
Count 37.5 M/ml Volume 2.7 ml, pH 7.5

Mobility: 11% QP, 39% QP, 50% S, 41% Dead.

Morphology: occ 'tids, few constrix heads, abnl necks

Diagnosis Right epididymitis in 1975

Appendix D-8



SPEC # RS008 RUN # P027 DATE 2/14/83 Abstinence ?

Count 13.8 M/ml Volume 5.2 ml, pH 8

Mobility: 47% QP, 31% S, 22% NM, 64% Dead.

Morphology: moderate spermatids, occasional pinheads
Diagnosis: idiopathic oligospermic

Appendix D-9

SPEC # RS009 RUN # P027 DATE 2/15/83 Abstienence?

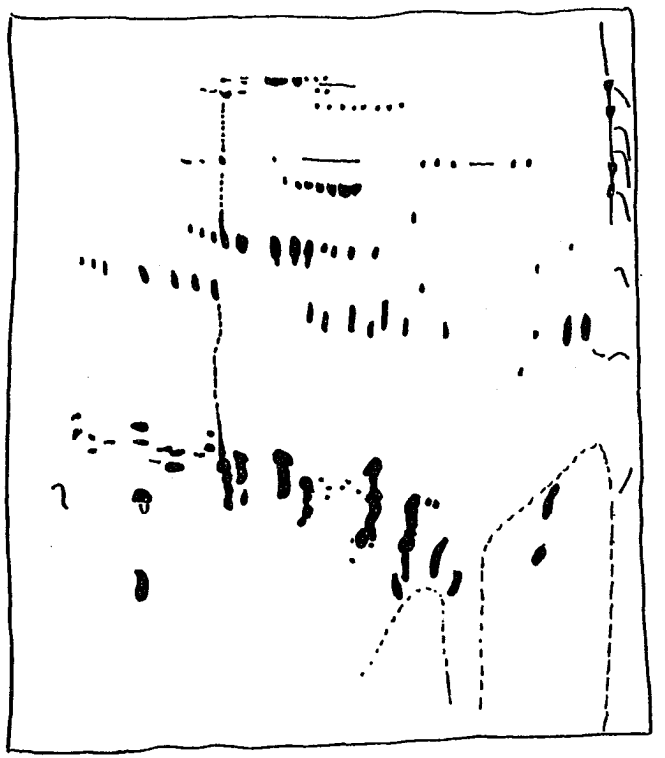
Count 125 M/ml Volume 4.1 ml, pH ?

Mobility: 35% QP, 21% S, 44% NM, 56% Dead.

Morphology: bent necks, few pinheads; viscous, clumping

Diagnosis Normal Exam, analysis

Appendix D-10



SPEC # RS010 RUN # P027 DATE 2/15/83 Abstinence ?

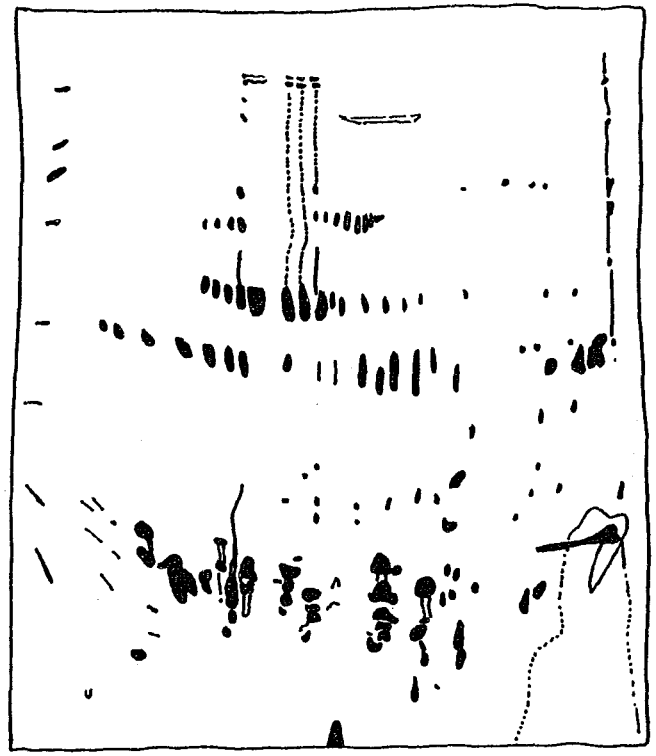
Count 18.8 M/ml Volume 1.9 ml, pH 7.5

Mobility: 60% QP, 3% S, 37% NM, 53% Dead.

Morphology: viscous, clumping

Diagnosis: Vasovasostomy, Jan,'83 (3 weeks post-op).

Appendix D-11



SPEC # RS011 RUN # P027 DATE 2/21/83 Abstinence 5

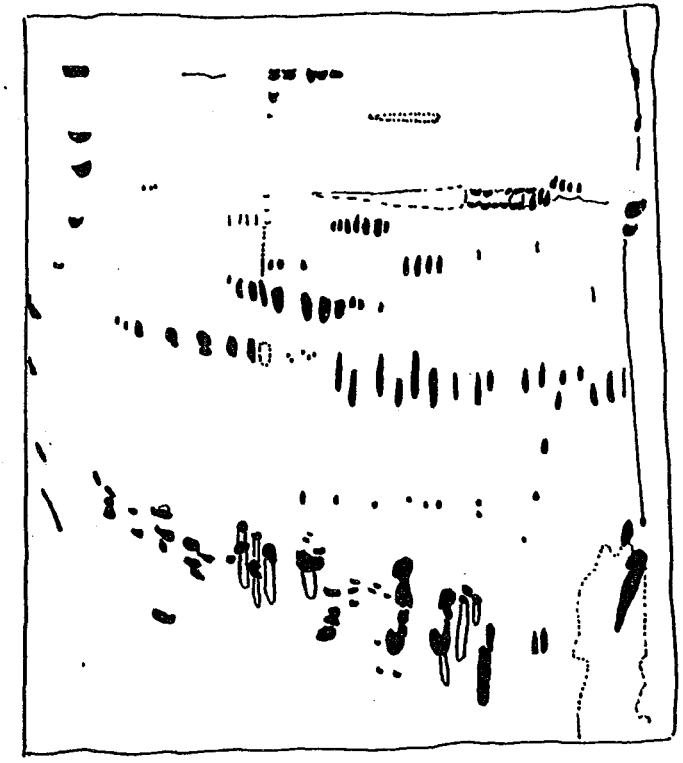
Count 66.3 M/ml Volume 55.2 ml, pH 7.5

Mobility: 55% OP, 19% S, 26% NM, 35% Dead.

Morphology:

Diagnosis: Small left varicocele

Appendix D-12



SPEC # RS012 RUN # P027 DATE 2/21/83 Abstinence ?

Count: AZOOSPERMIC

Diagnosis: Hodgkins Disease: Pan-Testicular Failure as a result of 10 days radiation treatment and 11 months Bleomycin therapy

Appendix D-13

SPEC # RS013 RUN # P027 DATE 2/21/83 Abstinence ?

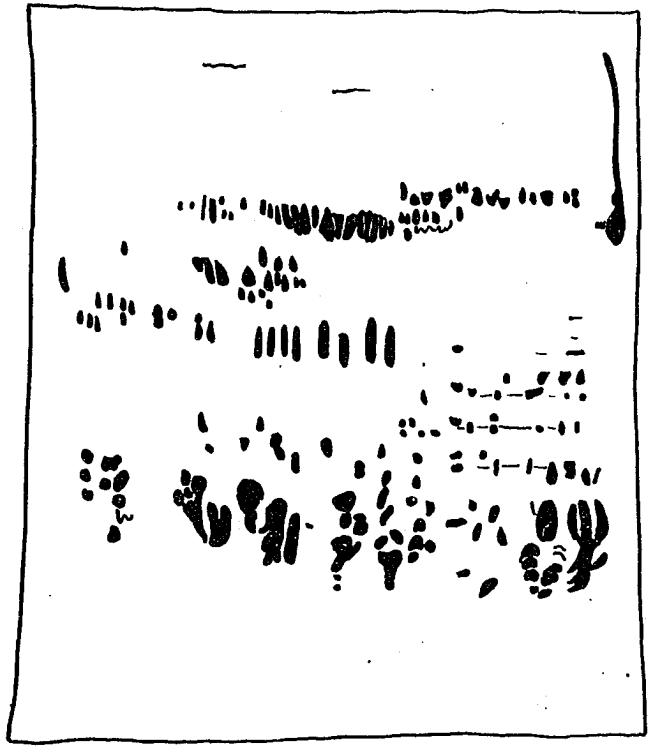
Count 6.3 M/ml Volume 1.8 ml, pH 8

Mobility: 44% QP, 11% S, 45% NM, 55% Dead.

Morphology. very viscous

Diagnosis: Large varicocele with left testicular atrophy

Appendix D-14



SPEC # RS014 RUN # P030 DATE 2/21/83 Abstinence 6

Count 117.5 M/ml Volume 0.7 ml, pH ?

Mobility: 3% QP, 8% S, 89% NM, 33% Dead.

Morphology:

Diagnosis: Diabetic

Appendix D-15

SPEC # RS015 RUN # P030 DATE 3/1/83 Abstinence ?

Count AZOOSPERMIC

Diagnosis: Trans-urethral resection for ejaculatory duct stricture (congenital?).ff

Appendix D-16

SPEC # RS016 RUN # P030 DATE 3/1/83 Abstinence >5

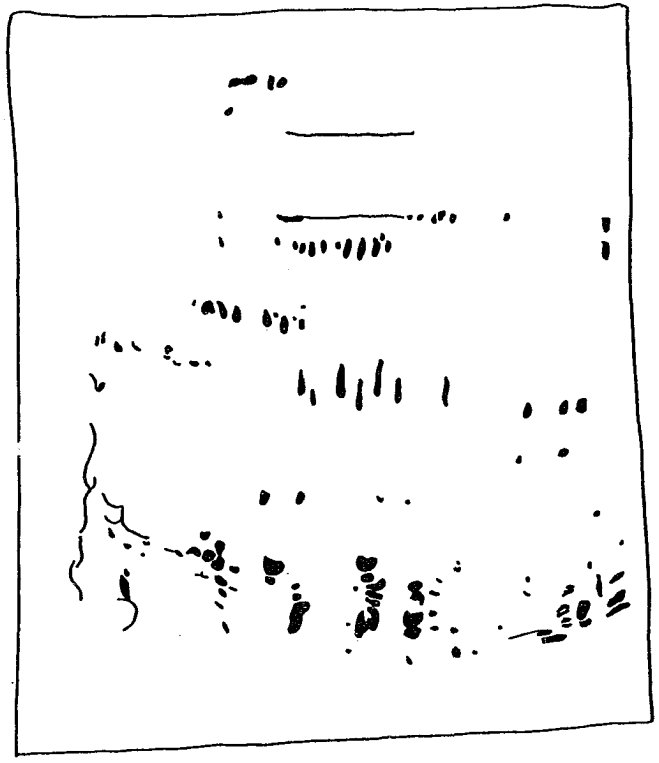
Count 12.5 M/ml Volume 2.0 ml, pH ?

Mobility: 17% QP, 32% S, 5% NM, 48% Dead.

Morphology: occasional ringtails, pinheads, double heads

Diagnosis: post varicocelectomy, August, 1982

Appendix D-17



SPEC # RS017 RUN # P028 DATE 3/7/83 Abstinence ?

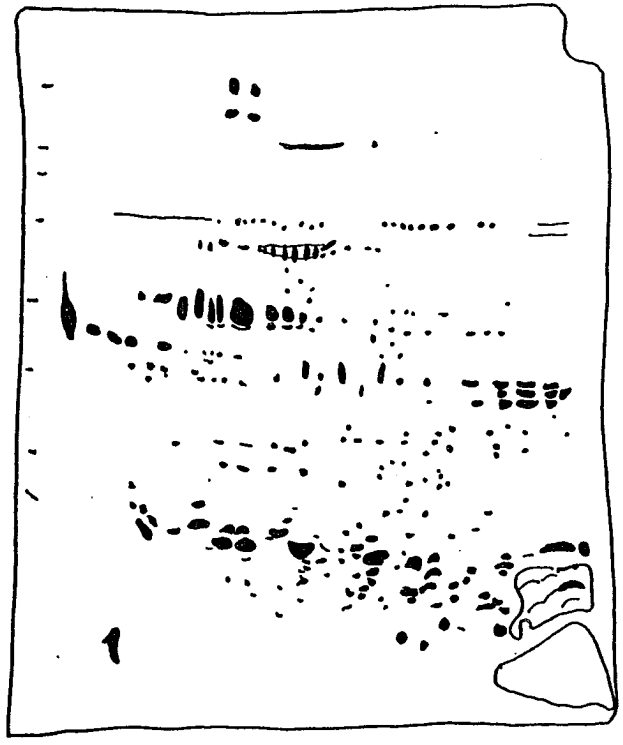
Count 3.8 M/ml Volume 2.3 ml, pH ?

Mobility. 7% QP, 32% S, 61% NM, 34% Dead.

Morphology: marked spermatids without tails

Diagnosis. Small left varicocele

Appendix D-18



SPEC # RS018 RUN # P034 DATE 3/7/83 Abstinence ?

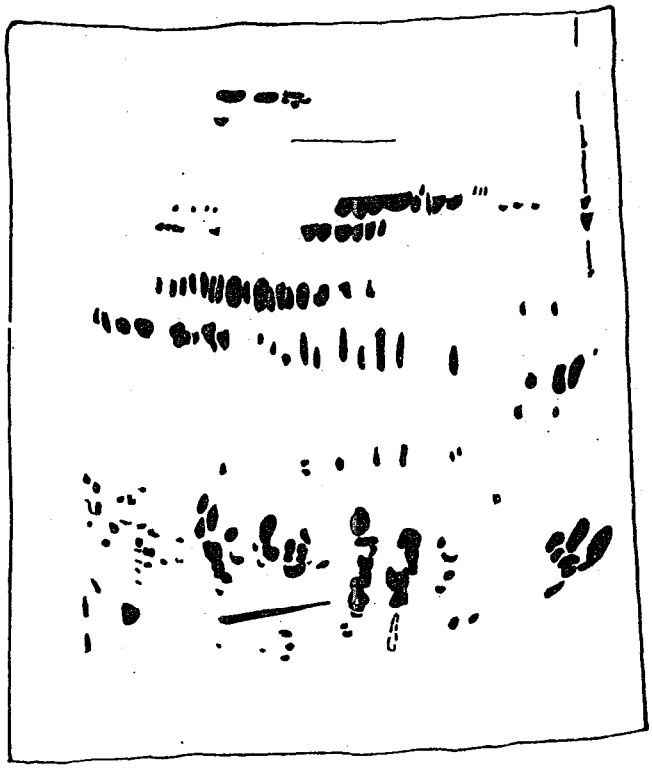
Count 85 M/ml Volume 5.2 ml, pH ?

Mobility: 47% QP, 15% S, 38% NM, 53% Dead.

Morphology: clumping

Diagnosis: Small left varicocele

Appendix D-19



SPEC # RS019 RUN # P030 DATE 3/7/83 Abstinence 5

Count 3.8 M/ml Volume 2.4 ml, pH 8

Mobility: 2% QP, 19% S, 79% NM, 39% Dead.

Morphology: marked abnormal heads

Diagnosis: Varicocelectomy, Aug., '82.

Appendix D-20

SPEC # RS020 RUN # P028 DATE 3/8/83 Abstinence 4

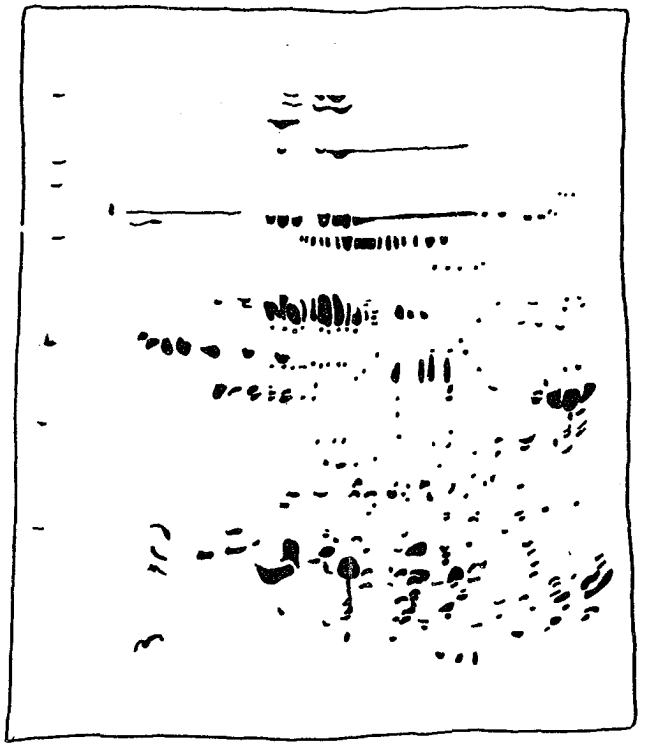
Count 33.8 M/ml Volume 2.8 ml, pH ?

Mobility: 9% QP, 28% S, 63% NM, 40% Dead.

Morphology: few spermatids, globoid bodies

Diagnosis: Bilateral varicocele, corrected in Sept., 82: Wife is pregnant!

Appendix D-21



SPEC # RS021 RUN # P038 DATE 3/14/83 Abstinence ?

Count 33.8 M/ml Volume 2.8 ml, pH ?

Mobility: 12% QP, 27% S, 61% NM, 58% Dead.

Morphology: moderate clumping

Diagnosis:

Appendix D-22

SPEC # RS022 RUN # P029 DATE 3/14/83 Abstinence ?

Count 3.8 M/ml Volume 2.1 ml, pH 7.5

Mobility: 20% QP, 39% S, 41% NM, 38% Dead.

Morphology: marked spermatids, *few pinheads*

Diagnosis: idiopathic oligospermic

Appendix D-23

SPEC # RS023 RUN # P029 DATE 3/14/83 Abstinence 4

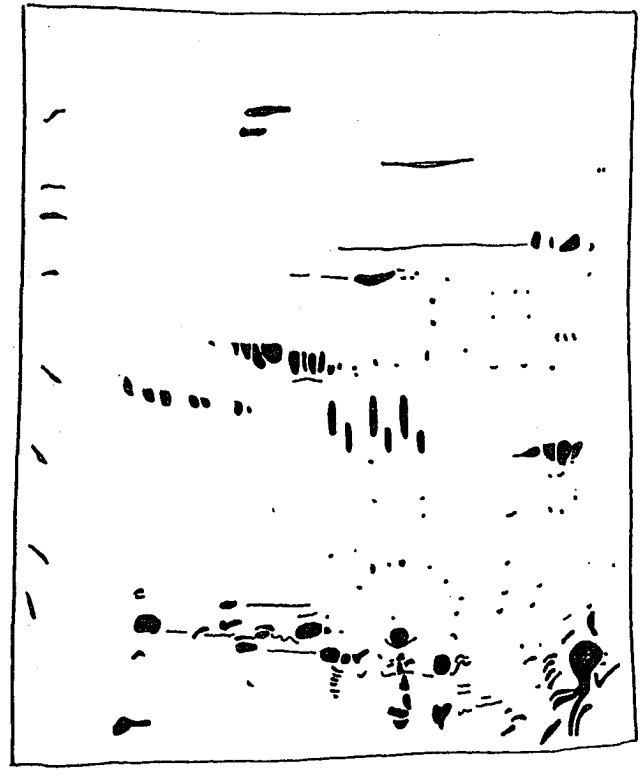
Count 52.5 M/ml Volume 3.1 ml, pH ?

Mobility. 12% OP, 46% S, 42% NM, 25% Dead.

Morphology:

Diagnosis Idiopathic oligozoospermic

Appendix D-24



SPEC # RS024 RUN # P029 DATE 3/14/83 Abstinence 4

Count 41.3 M/ml Volume 7.2 ml, pH 8

Mobility: 9% OP, 13% S, 78% NM, 45% Dead.

Morphology: moderate clumping

Diagnosis: Bilateral varicocele, hyperspermic

Appendix D-26

SPEC # RS026 RUN # P035 DATE 4/5/83 Abstinence ?

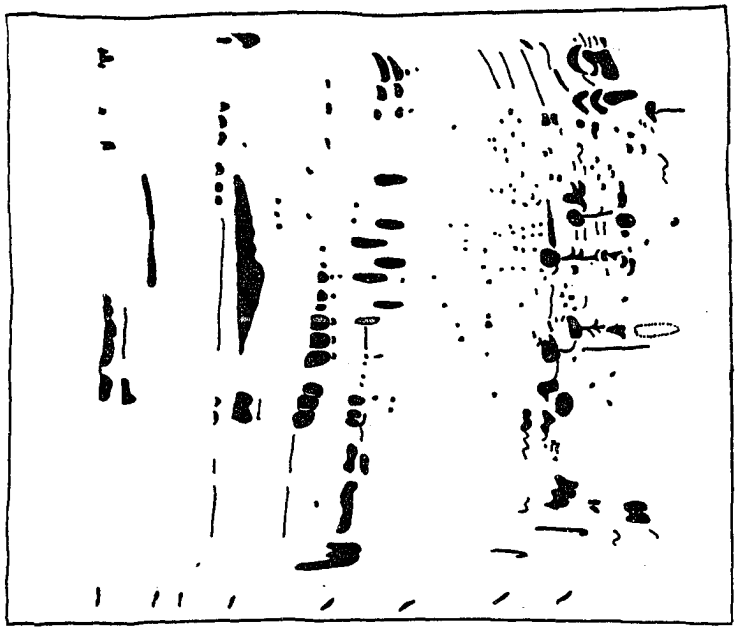
Count 18.8 M/ml Volume 5.6 ml, pH ?

Mobility: 51% OP, 8% S, 41% NM, 50% Dead.

Morphology: moderate spermatids, occasional pinheads

Diagnosis: ?

Appendix D-25



SPEC # RS025 RUN # P029 DATE 3/14/83 Abstinence ?

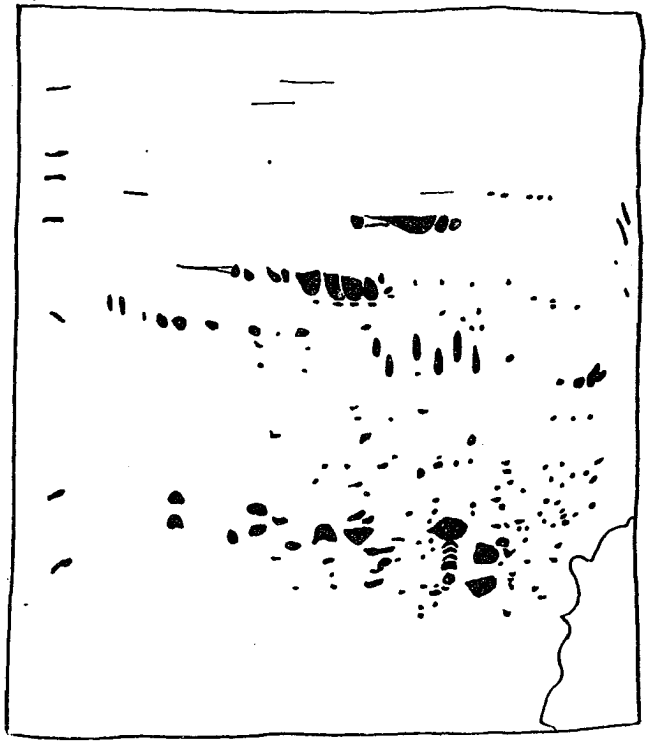
Count 48.8 M/ml Volume 1.9 ml, pH ?

Mobility: 4% OP, 38% S, 58% NM, 43% Dead.

Morphology: occasional spermatids, few constricted heads

Diagnosis: ?

Appendix D-27



SPEC # RS027 RUN # P035 DATE 4/5/83 Abstinence ?

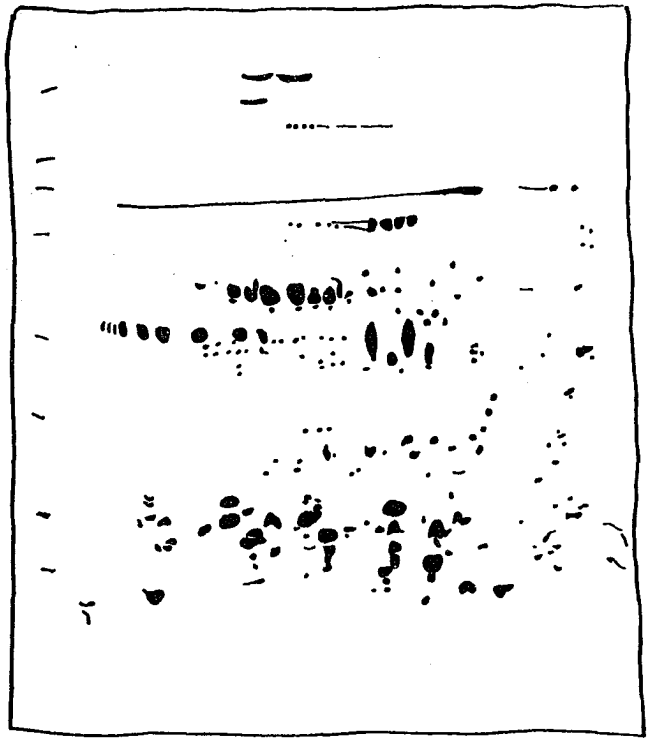
Count 87.5 M/ml Volume 2.5 ml, pH ?

Mobility: 34% QP, 29% S, 37% NM, 71% Dead.

Morphology: viscous, marked clumping

Diagnosis: Left varicocelectomy, June, '82.

Appendix D-28



SPEC # RS028 RUN # P035 DATE 4/5/83 Abstinence ?

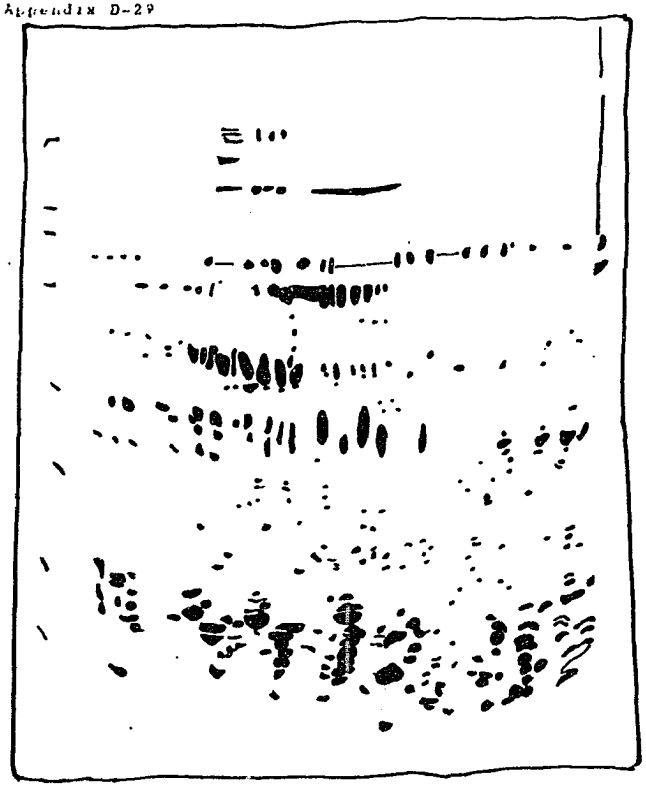
Count 2.5 M/ml Volume 5.2 ml, pH 7.5

Mobility: 4% QP, 15% S, 81% NM, 5% Dead.

Morphology: few pinheads and spermatids

Diagnosis: Small left varicocele

Appendix D-29



SPEC # RS029 RUN # P033 DATE 4/11/83 Abstinence ?

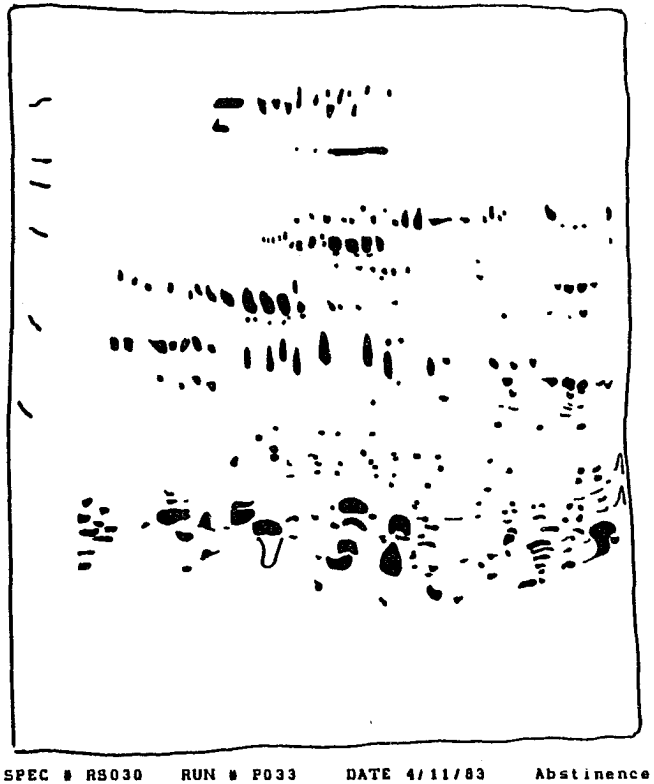
Count 186.3 M/ml Volume 4.4 ml, pH 8

Mobility: 43% QP, 9% S, 48% NM, 68% Dead.

Morphology: occasional spermatids, few large heads.

Diagnosis: taking Corgard (heart blocker), polyzoospermic

Appendix D-30



SPEC # RS030 RUN # P033 DATE 4/11/83 Abstinence 4

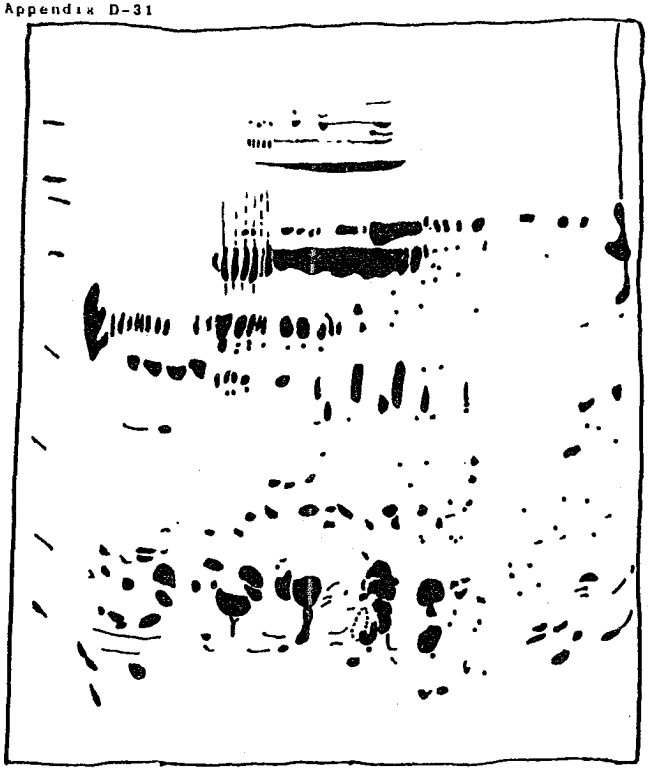
Count 20 M/ml Volume 4.3 ml, pH ?

Mobility: 50% QP, 21% S, 29% NM, 54% Dead.

Morphology:

Diagnosis: Left varicocelectomy, Dec. '82.

Appendix D-31



SPEC # RS031 RUN # P033 DATE 4/11/83 Abstinence 4

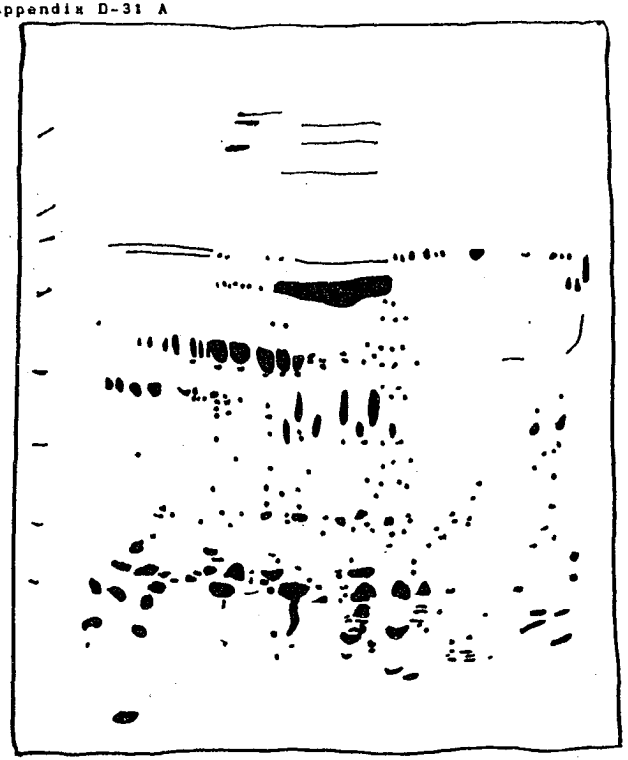
Count 225 M/ml Volume 3.1 ml, pH 8

Mobility. 71% QP, 2% S, 278% NM, 51% Dead.

Morphology. few spermatids, slight clumping

Diagnosis: Polyzoospermic

Appendix D-31 A



SPEC # RS031 A RUN # P034 DATE 4/20/83 Abstinence 4

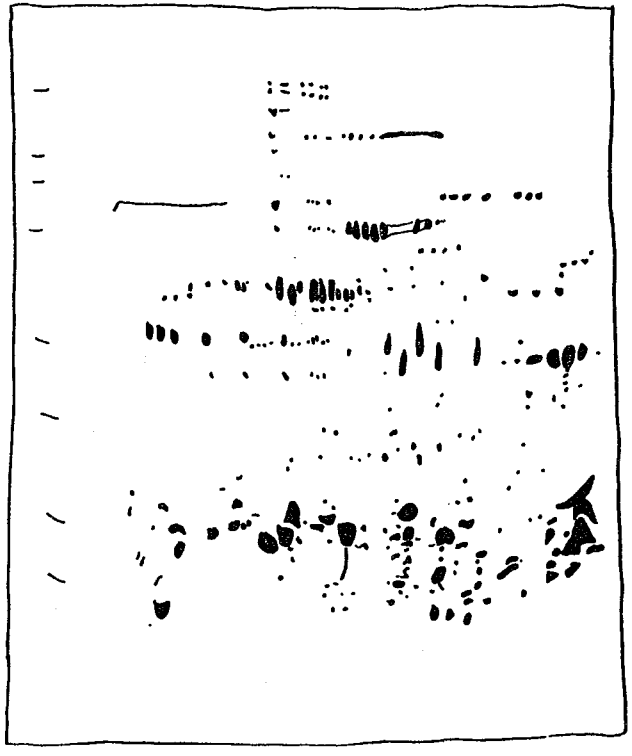
Count 225 M/ml Volume 3.1 ml, pH 8

Mobility: 71% QP, 2% S, 27% NM, 51% Dead.

Morphology: few spermatids, slight clumping

Diagnosis: Polyzoospermic. Same specimen as RS031 - run week later.

Appendix D-32



SPEC # RS032 RUN # P038 DATE 4/11/83 Abstinence ?

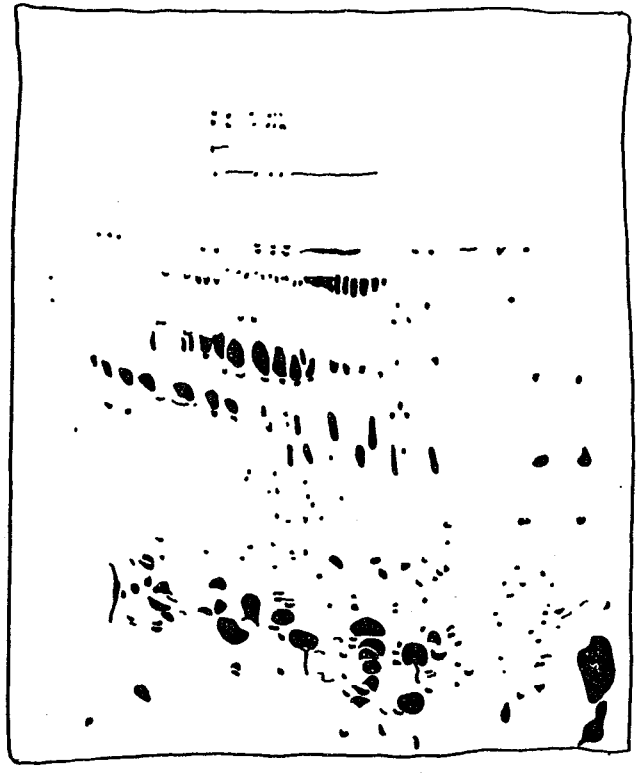
Count 11.3 M/ml Volume 3.5 ml, pH 7.5

Mobility. 46% QP, 14% S, 40% NM, 52% Dead.

Morphology: few spermatids, abnl shaped heads; viscous

Diagnosis: Right orchiopexy for undescended testicle, age 4.

Appendix D-33



SPEC # RS033 RUN # P033 DATE 4/11/83 Abstinence ?

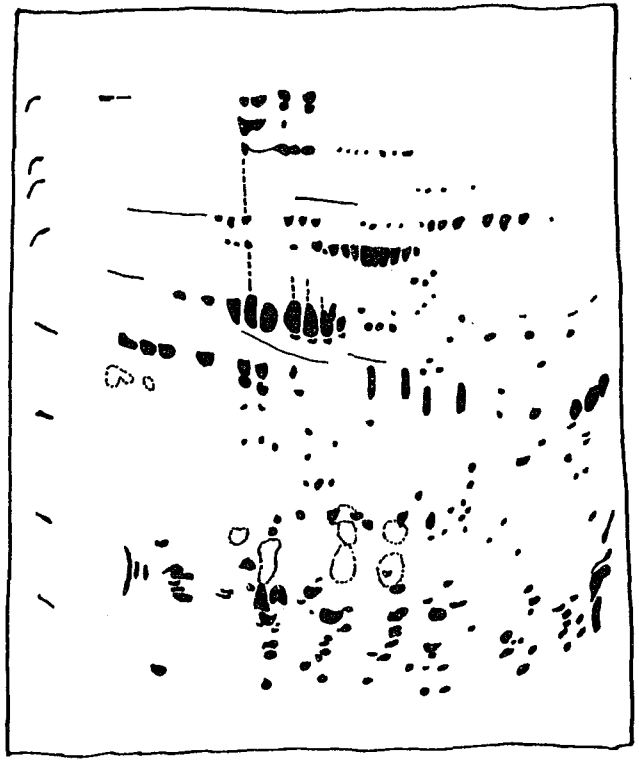
Count 31.3 M/ml Volume 3.4 ml, pH 8

Mobility: 16% QP, 23% S, 61% NM, 12% Dead.

Morphology: moderate spermatids

Diagnosis: No findings, low motility

Appendix D-34



SPEC # RS034 RUN # P033 DATE 4/12/83 Abstinence 5

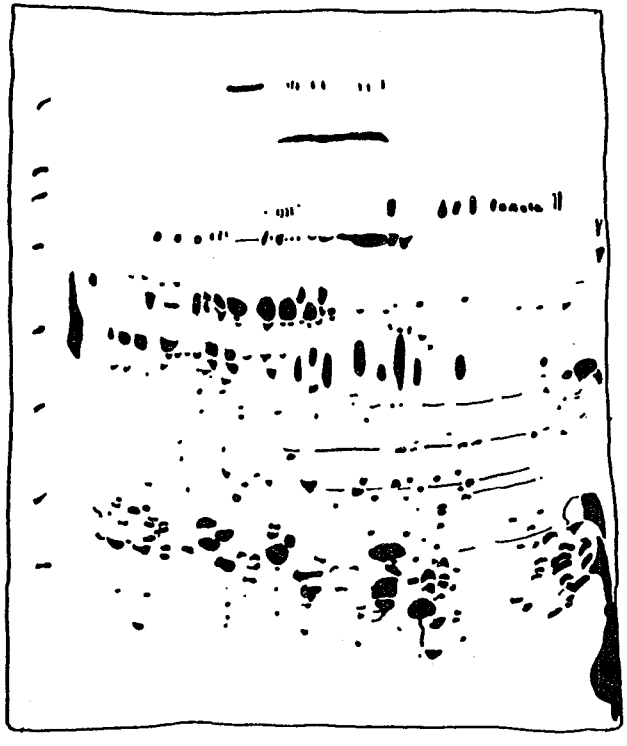
Count 3.8 M/ml Volume 2.8 ml, pH 8

Mobility. 8% QP, 15% S, 74% NM, 68% Dead.

Morphology: fairly viscous

Diagnosis. Idiopathic oligozoospermia

Appendix D-35



SPEC # RS035 RUN # P034 DATE 4/18/83 Abstinence ?

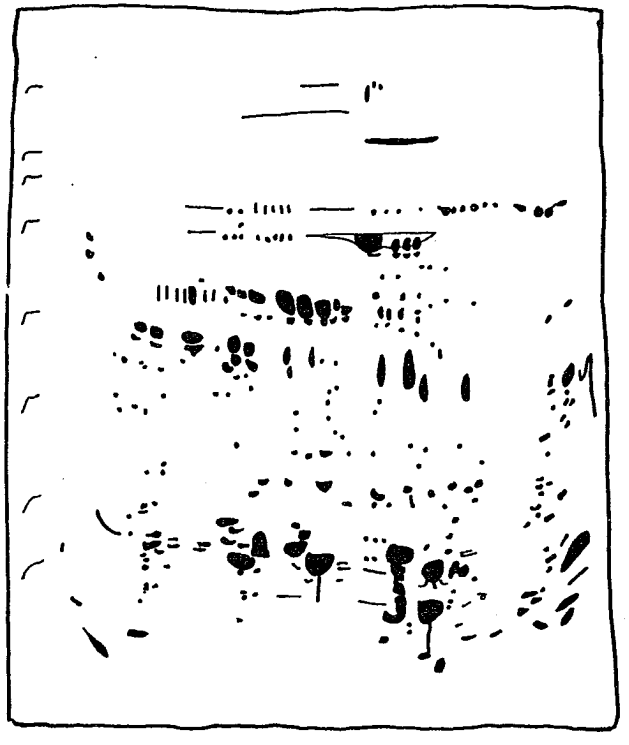
Count 65 M/ml Volume 3.0 ml, pH ?

Mobility: 22% QP, 18% S, 60% NM, 557% Dead.

Morphology: moderate constricted heads

Diagnosis: Small left varicocele.

Appendix D-36



SPEC # RS036 RUN # P034 DATE 4/19/83 Abstinence ?

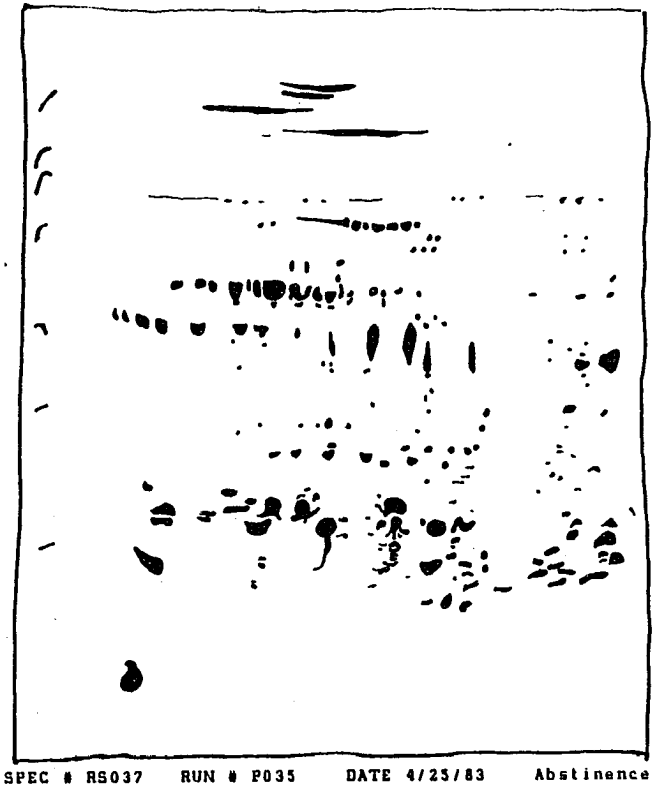
Count 337.5 M/ml Volume 1.4 ml, pH 8

Mobility: 16% QP, 28% S, 56% NM, 43% Dead.

Morphology: head/tail clumping

Diagnosis: Polyzoospermic

Appendix D-37



SPEC # RS037 RUN # P035 DATE 4/25/83 Abstinence ?

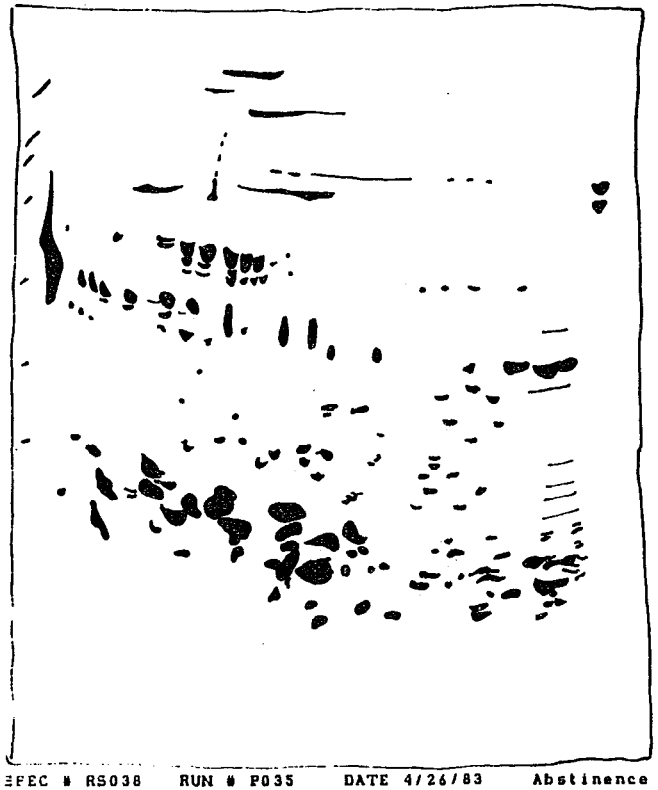
Count 43.8 M/ml Volume 3.1 ml, pH 8

Mobility: 34% QP, 33% S, 33% NM, 53% Dead.

Morphology: few spermatids

Diagnosis: ?

Appendix D-38



SPEC # RS038 RUN # P035 DATE 4/26/83 Abstinence ?

Count 140 M/ml Volume 3.1 ml, pH 8

Mobility: 55% GP, 10% S, 33% NM, 25% Dead.

Morphology: few spermatids

Diagnosis: ?

Appendix D-39

SPEC # RS039 RUN # P035 DATE 4/26/83 Abstinence ?

Count 25 M/ml Volume 4.1 ml, pH 8

Mobility: 47% GP, 29% S, 24% NM, 32% Dead.

Morphology: normal

Diagnosis: ?

Appendix D-40

SPEC # RS040 RUN # P037 DATE 5/2/83 Abstinence ?

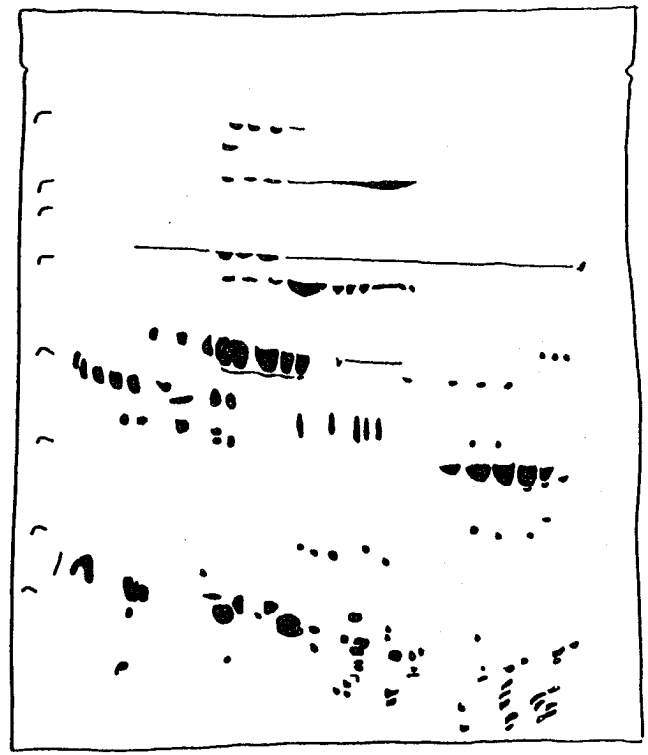
Count 150 M/ml Volume 4.8 ml, pH 8

Mobility: 57% QP, 7% S, 36% NM, 38% Dead.

Morphology: occasional spermatids

Diagnosis: Small lef varicocele, Normal analysis

Appendix D-41



SPEC # RS041 RUN # P037 DATE 5/2/83 Abstinence ?

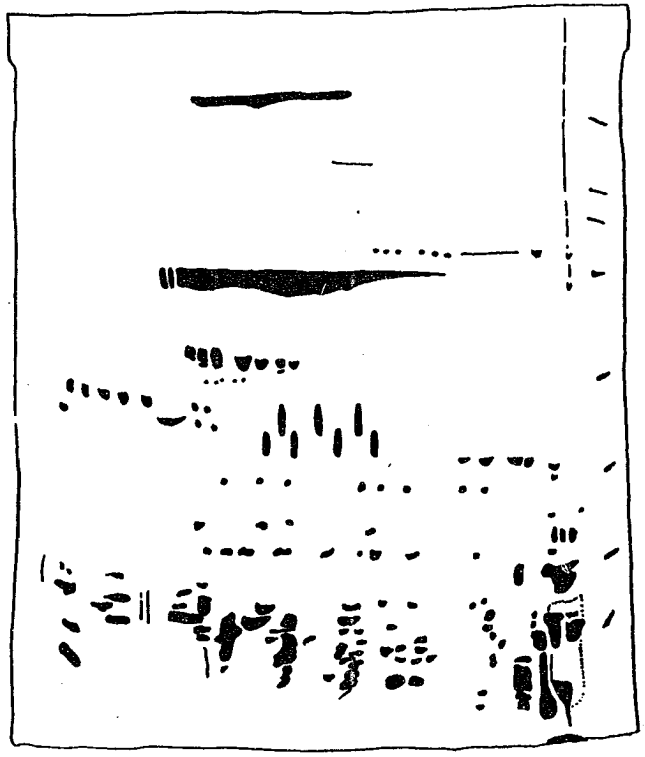
Count 10 M/ml Volume 3.4 ml, pH 8

Mobility: 23% QP, 10% S, 67% NM, 73% Dead.

Morphology: few spermatids, few ringtails

Diagnosis: ?

Appendix D-42



SPEC # RS042 RUN # P037 DATE 5/3/83 Abstinence 5

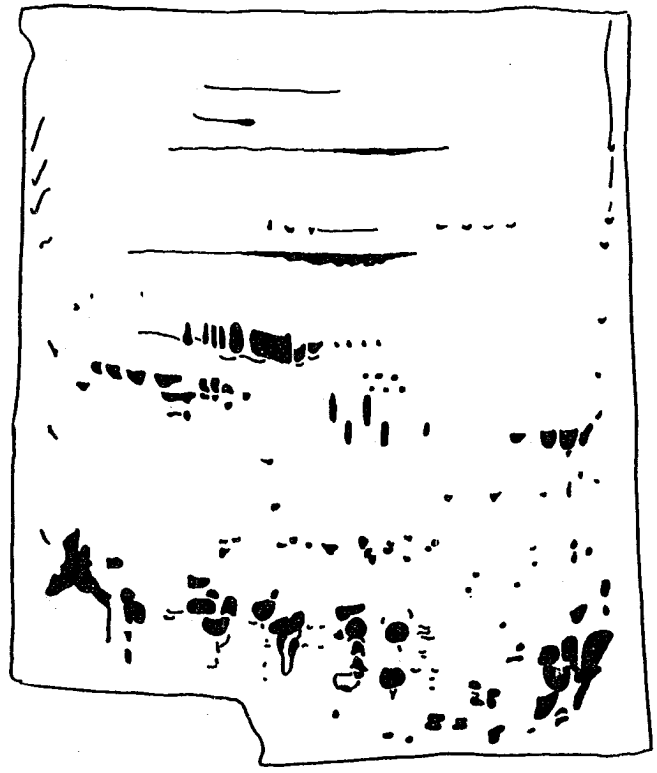
Count 16 3 M/ml Volume 3.6 ml, pH 8

Mobility: 39% GP, 18% S, 43% NM, 33% Dead.

Morphology: marked spermatids, few pinheads and ringtails

Diagnosis: ?

Appendix D-43



SPEC # RS044 RUN # P037 DATE 55/9/83 Abstinence ?

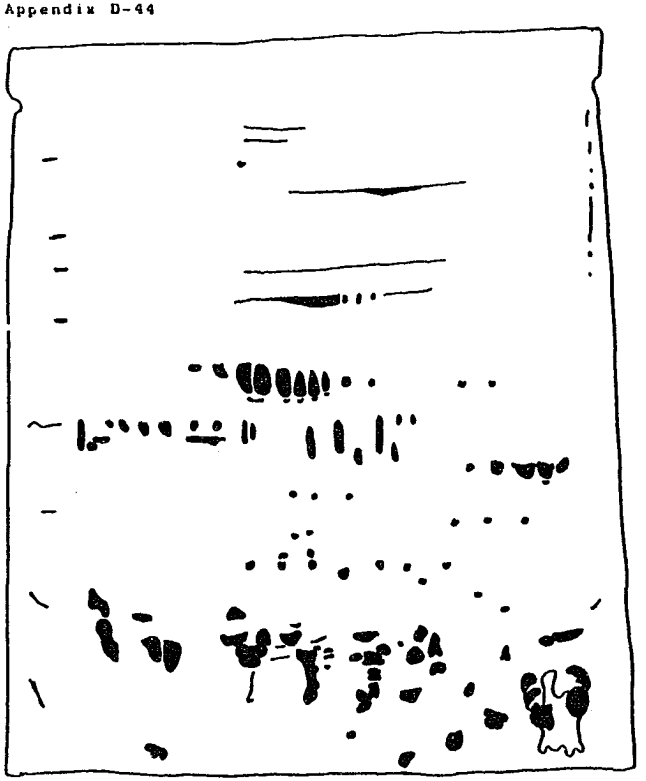
Count 173.8 M/ml Volume 36 ml, pH 7.5

Mobility: 59% GP, 77% S, 34% NM, 37% Dead.

Morphology: moderate spermatids; slightly viscous

Diagnosis: No findings

Appendix D-44



SPEC # RS045 RUN # P037 DATE 5/9/83 Abstinence ?

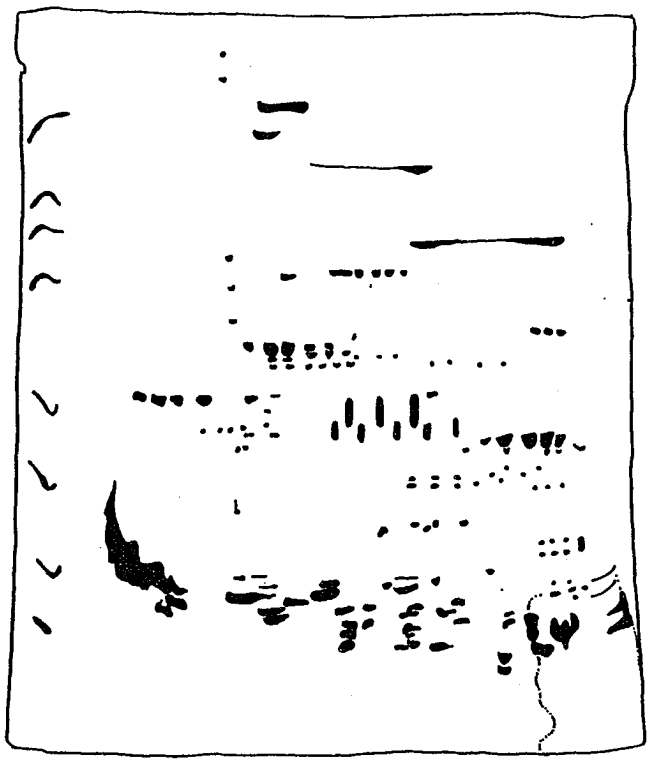
Count 35 M/ml Volume 3.9 ml, pH 8

Mobility: 43% QP, 16% S, 41% NM, 60% Dead.

Morphology: moderate spermatids, Head/tail clumping

Diagnosis: Moderate sized left varicocele

Appendix D-45



SPEC # RS046 RUN # P037 DATE 5/10 Abstinence ?

Count 1.3 M/ml Volume 5.0 ml, pH 8

Mobility: 10% QP, 10% S, 80% NM, 24% Dead.

Morphology: marked spermatids, few pinheads, few WBCs.

Diagnosis: Severe oligozoospermic

Appendix D-46

SPEC # RS047 RUN # P037 DATE 5/10/83 Abstinence ?

Count 38.8 M/ml Volume 6.5 ml, pH 8

Mobility: 35% QP, 26% S, 39% NM, 43% Dead.

Morphology:

Diagnosis:post varicocelectomy (Jan. 83)

Appendix D-47

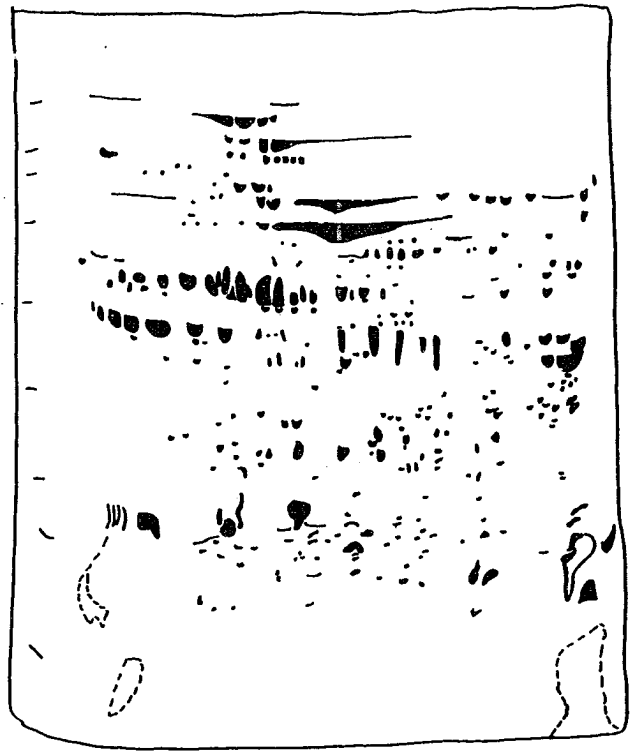
SPEC # RS048 RUN # P037 DATE 5/10/83 Abstinence 4

Count 17.5 M/ml Volume 2.8 ml, pH 8

Mobility: 56% QP, 19% S, 25% NM, 38% Dead.
Morphology:

Diagnosis: Small left varicocele, pyuria

Appendix D-48

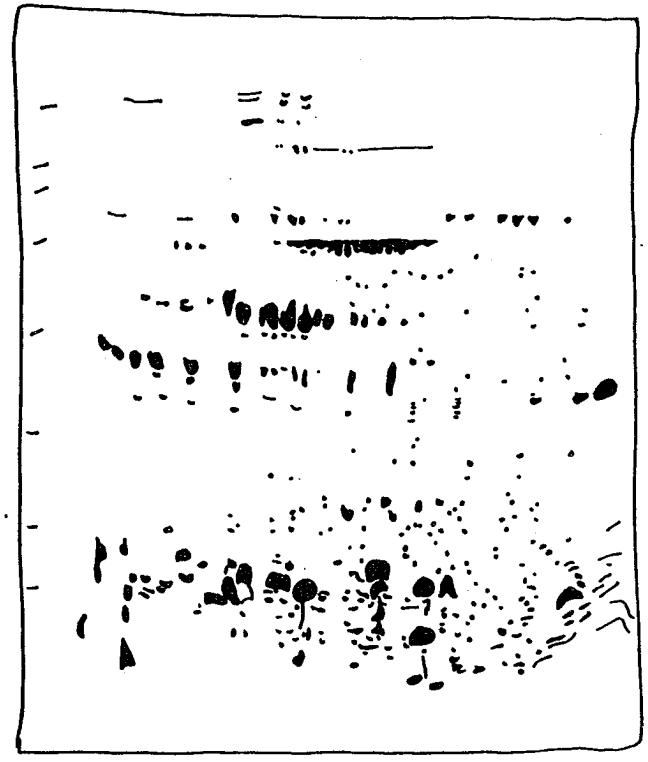


SPEC # RS049 RUN # P038 DATE 5/17/83 Abstinence ?

Count POST VASECTOMY

Diagnosis: Bilateral vasectomy

Appendix D-49



SPEC # RS050 RUN # P038 DATE 5/17/83 Abstinence ?

Count 28.8 M/ml Volume 3.0 ml, pH 8

Mobility: 43% QP, 22% S, 35% NM, 24% Dead.

Morphology: moderate spermatids; few pinheads, ringtails.

Diagnosis: ?.

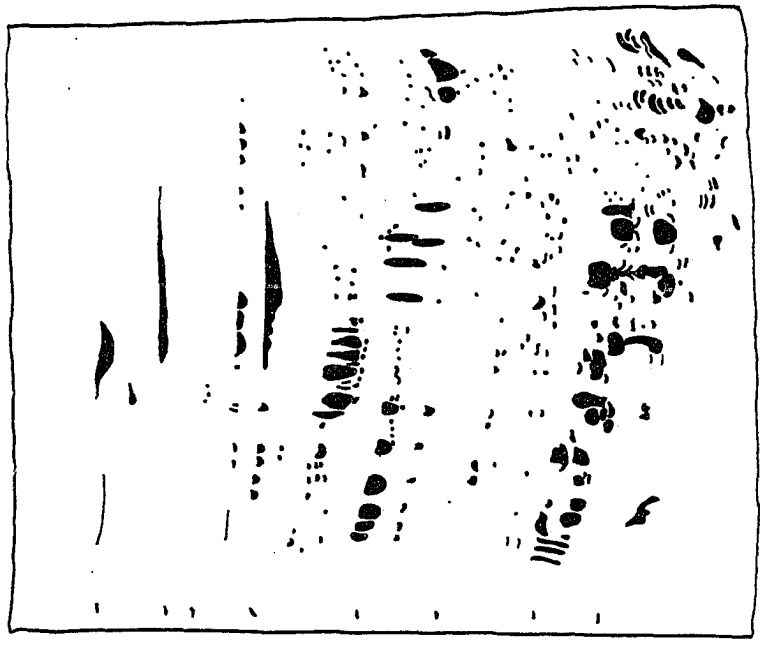
Appendix D-51

SPEC # RS052 RUN # P038 DATE 5/24/83 Abstinence ?

Count AZOOSPERMIC

Diagnosis: No vasa deferentia palpated on physical exam

Appendix D-50



SPEC # RS051 RUN # P038 DATE 5/23/83
Abstinence ?

Count 1.25 M/ml Volume 3.1 ml, pH 7.5

Mobility. 0% GP, 10% S, 90% NM, 65% Dead.

Morphology: marked spermatids

Diagnosis: Severe oligozoospermic

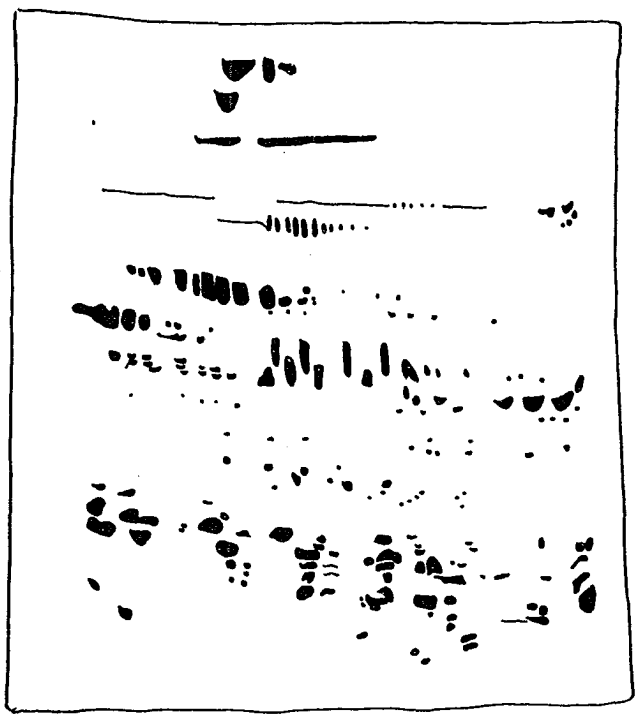
Appendix D-52

SPEC # RS053 RUN # P038 DATE 5/24/83 Abstinence ?

Count NO SPERM SEEN

Diagnosis: Post vasectomy

Appendix D-53



SPEC # RS054 RUN # P039 DATE 6/6/83
Abstinence ?

Count 120 M/ml Volume 1.1 ml, pH 7.5

Mobility: 57% OP, 9% S, 34% NM, 24% Dead.

Morphology: occasional spermatids, macrocephalics

Diagnosis:?

Appendix D-54

SPEC # RS055 RUN # P039 DATE 6/6/83 Abstinence ?

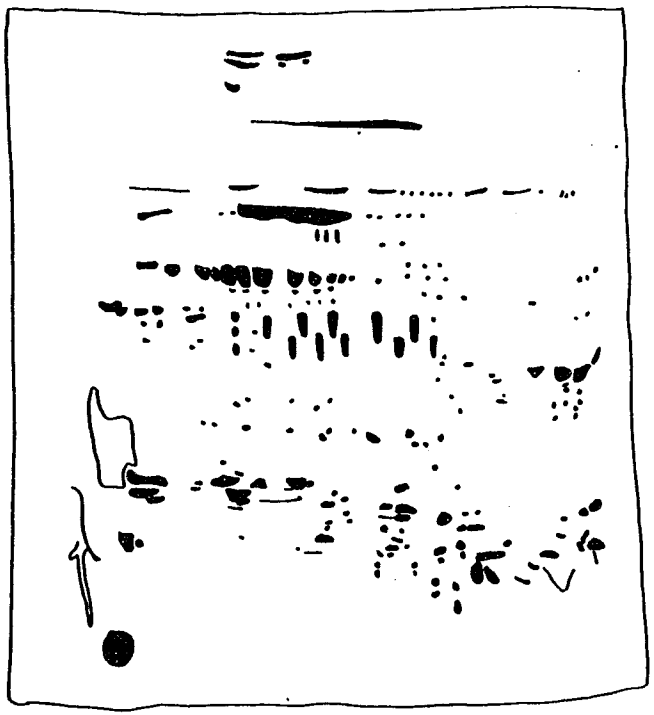
Count 12.5 M/ml Volume 4.2 ml, pH 8

Mobility: 21% QP, 29% S, 550% NM, 55% Dead.

Morphology: marked spermatids

Diagnosis.?

Appendix D-55



SPEC # RS056 RUN # P039 DATE 6/6/83 Abstinence ?

Count 88 M/ml Volume 2.4 ml, pH 8

Mobility: 50% QP, 14% S, 36% NM, 24% Dead.

Morphology: occasional spermatids

Diagnosis: ?

Appendix D-56

SPEC # RS057 RUN # P040 DATE 6/7/83 Abstinence ?

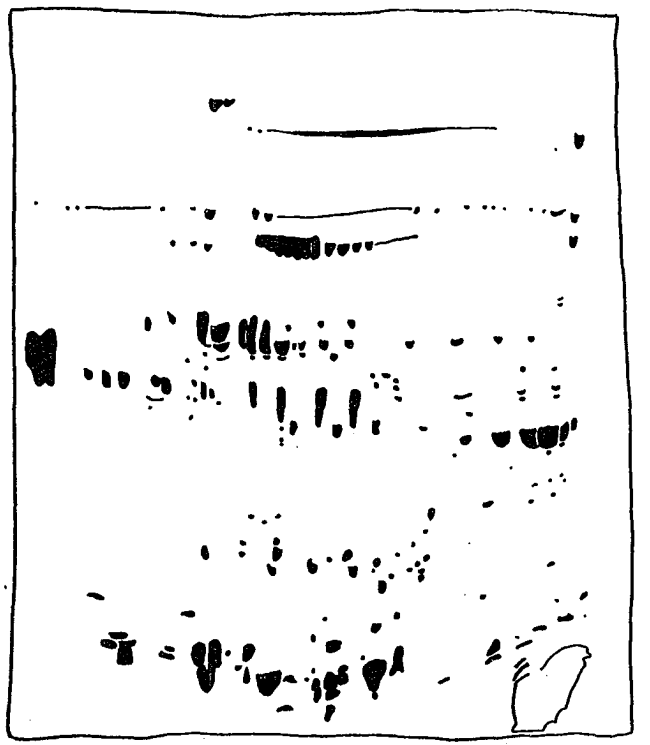
Count 27.5 M/ml Volume 2.4 ml, pH 78

Mobility: 40% GP, 26% S, 34% NM, 50% Dead.

Morphology: moderate spermatids; few constricted heads.

Diagnosis:?

Appendix D-57



SPEC # RS058 RUN # P040 DATE 6/13/83 Abstinence ?

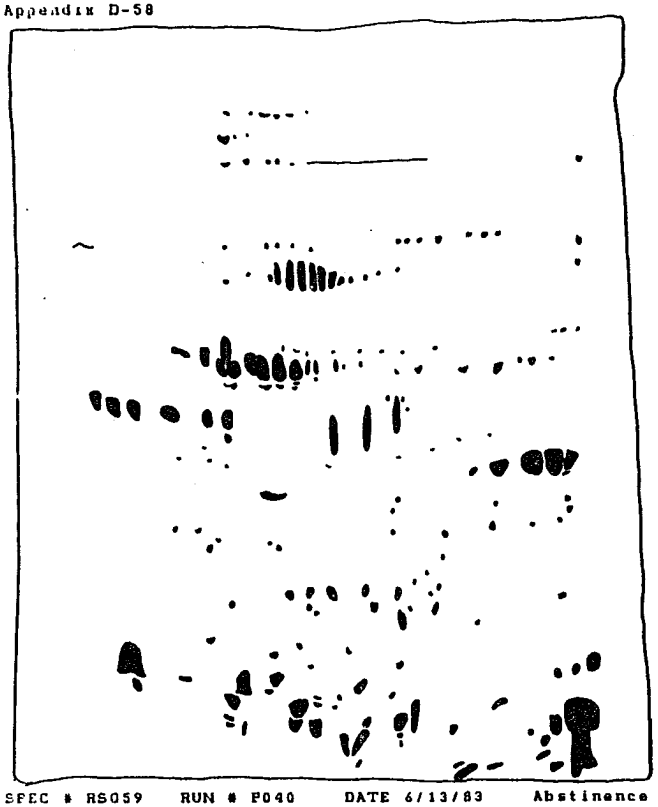
Count 50 M/ml Volume 3.0 ml, pH 7.0

Mobility: 100% non-motile, 85% dead

Morphology: marked spermatids

Diagnosis: Asthenozoospermia/Necrospermia

Appendix D-58



SPEC # RS059 RUN # P040 DATE 6/13/83 Abstinence ?

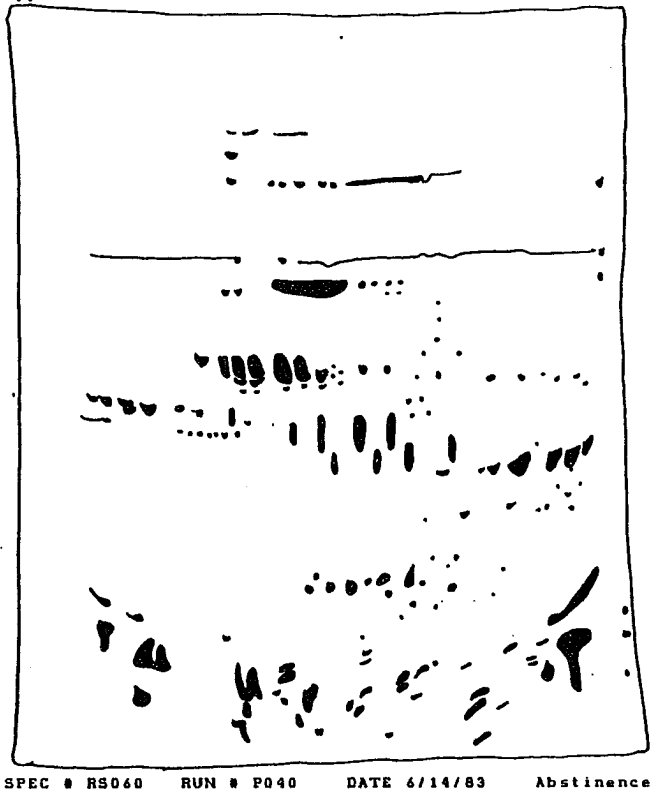
Count 20 M/ml Volume 3.0 ml, pH 7.0

Mobility: 3% QP, 21% S, 76% NM, 46% Dead.

Morphology: marked spermatids

Diagnosis.?

Appendix D-59



SPEC # RS060 RUN # P040 DATE 6/14/83 Abstinence ?

Count 8.3 M/ml Volume 2.2 ml, pH 7.5

Mobility: 22% QP, 17% S, 61% NM, 50% Dead.

Morphology: occasional double heads, few spermatids.

Diagnosis:?

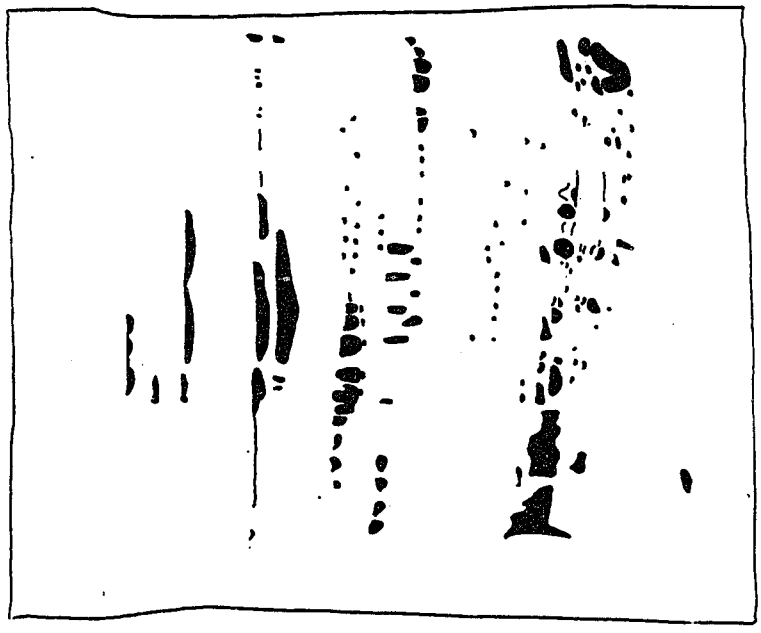
Appendix D-61

SPEC # V012 RUN # F042 DATE 6/21/83 Abstinence ?

Count 64.1 M/ml Volume 4.7 ml, pH ?

Mobility: not done

LAMAZE donor



Appendix D-60

SPEC # V010 RUN # F041 DATE 6/15/83 Abstinence 4

Count 101 M/ml Volume 4.5 ml, pH ?
Mobility: Okay

Morphology: Normal

LAMAZE donor

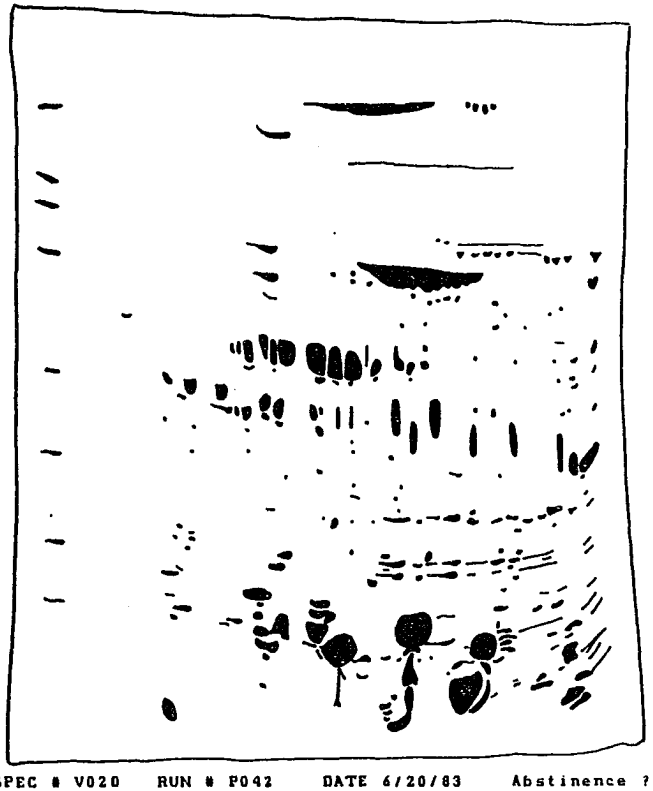
Appendix D-62

SPEC # V017 RUN # P041 DATE 7/7/83 Abstinence 4

Count NO SPERM SEEN

Diagnosis: Post vasectomy donor

Appendix D-63



SPEC # V020 RUN # P042 DATE 6/20/83 Abstinence ?

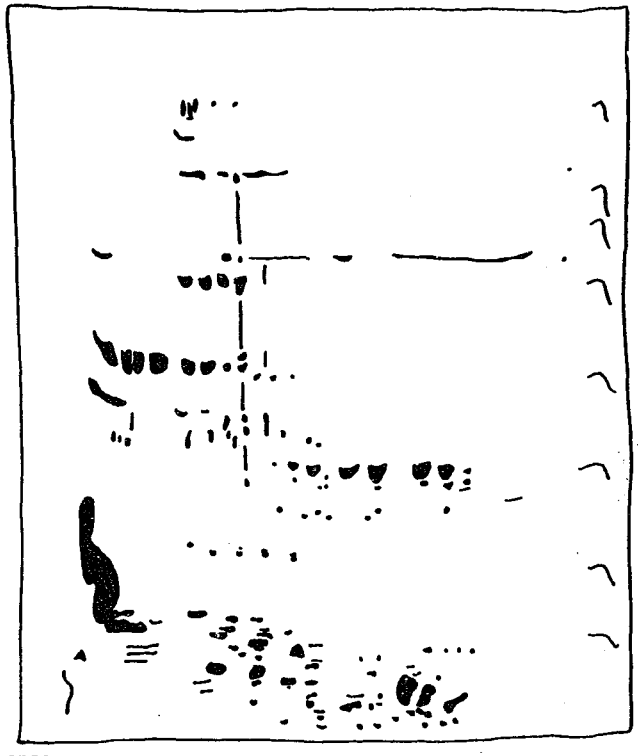
Count 118 M/ml Volume 1.9 ml, pH ?

Mobility: 28% QP, 34% S, 38% NM, 17% Dead.

Morphology: immature forms (TRICHOMONAS?) bent necks

Diagnosis: Previously treated for Trichomoniasis (1981)

Appendix D-64

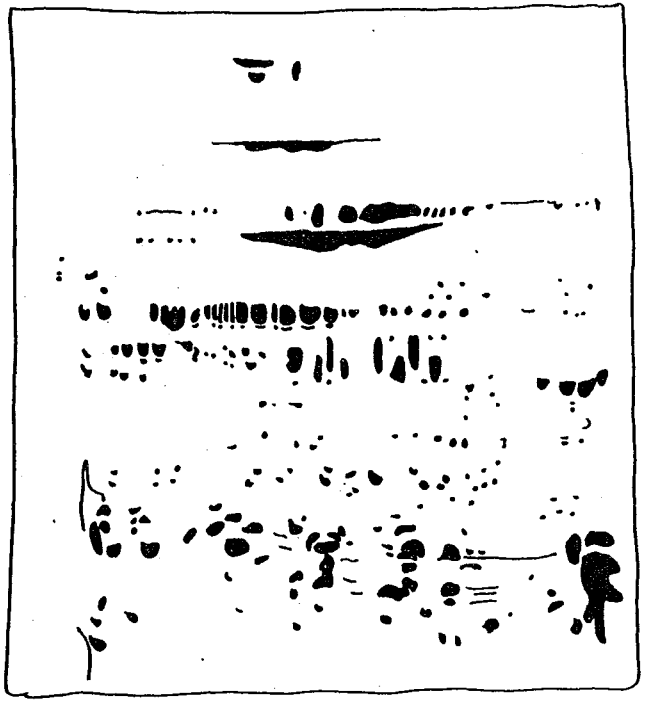


SPEC # V025 RUN # P044 DATE 7/1/83 Abstinence 5

Count NO SPERM SEEN Volume 5.5 ml

POST VASECTOMY DONOR

Appendix D-65



SPEC # V027 RUN # P039 DATE 5/30/83 Abstinence 10

Count 121 M/ml Volume 4.1 ml, pH ?

Mobility: 46% OP, 14% S, 40% NM, 38% Dead.

Morphology: marked ringtails, few bent necks

LAMAZE DONOR

Appendix D-66

SPEC # V028 RUN # P039 DATE 5/30/83 Abstinence 4

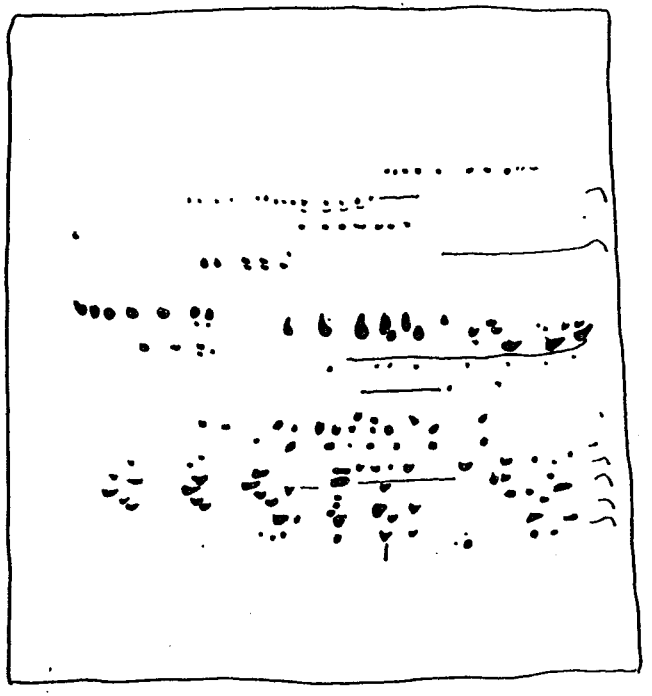
Count 131 M/ml Volume 1.8 ml, pH 7.0

Mobility: 59% QP, 17% S, 24% NM, 40% Dead.

Morphology: few spermatids, pinheads

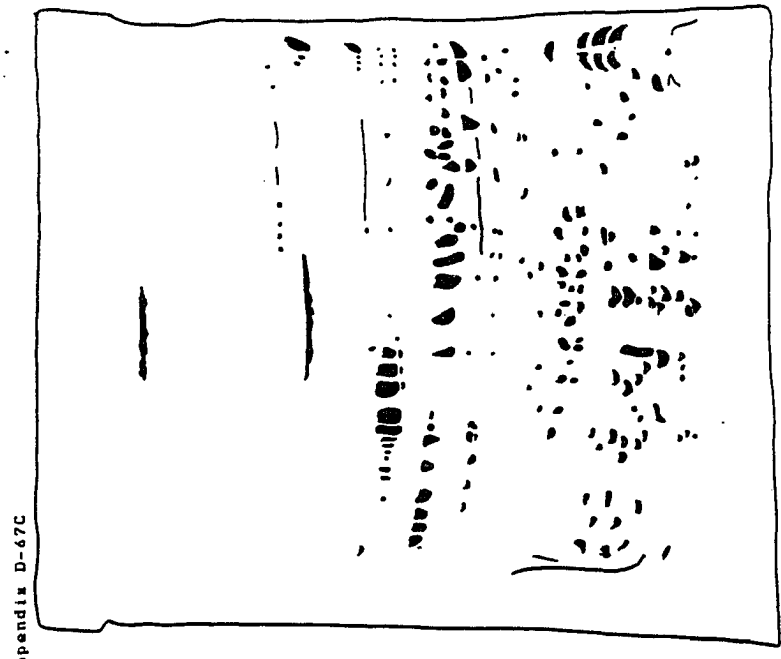
LAMAZE DONOR

Appendix D-67A

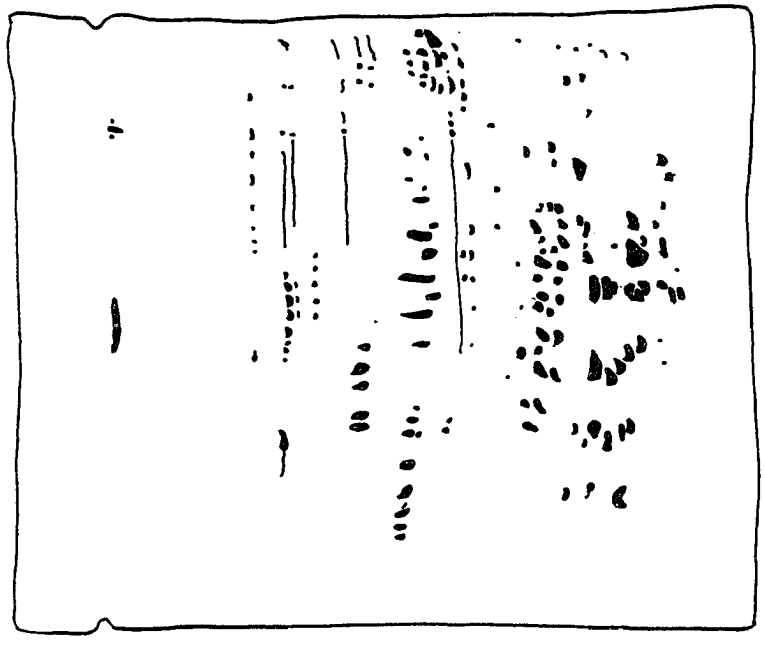


SPEC # SE01 RUN # P036 DATE 5/9/83

SPLIT EJACULATE: Fraction 1



Appendix D-67C

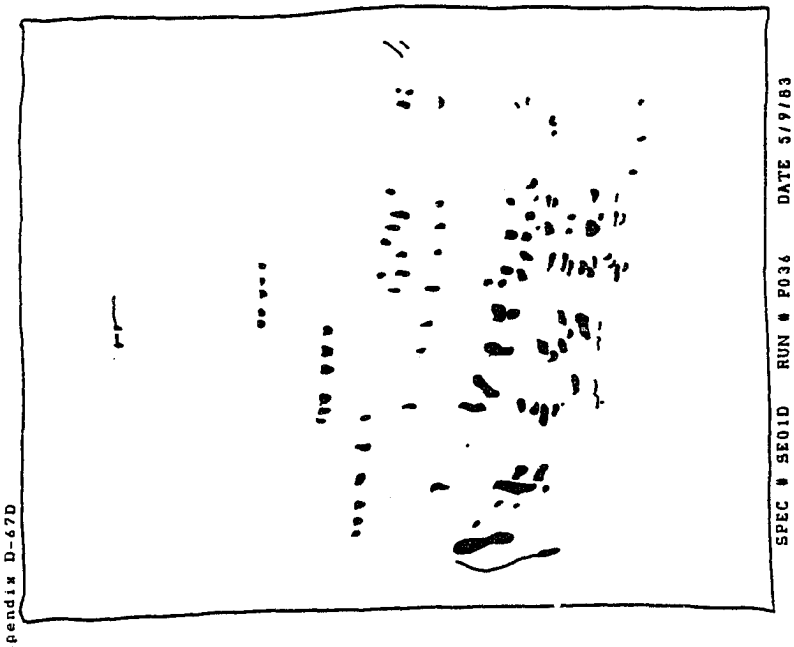


Appendix D-67B

Appendix D-67E

SPEC # SE01E RUN # P036 DATE 5/9/83

SPLIT EJACULATE: Fraction 7



Appendix D-67D

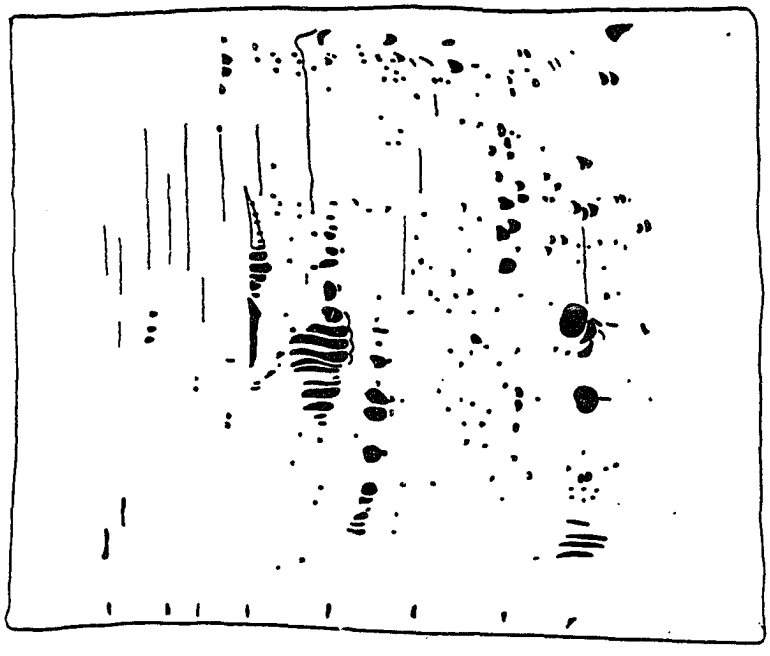
SPEC # SE01D RUN # P036 DATE 5/9/83

SPLIT EJACULATE: Fraction 6

Appendix D-68B

SPEC # SE02B RUN # P043 DATE 7/1/83

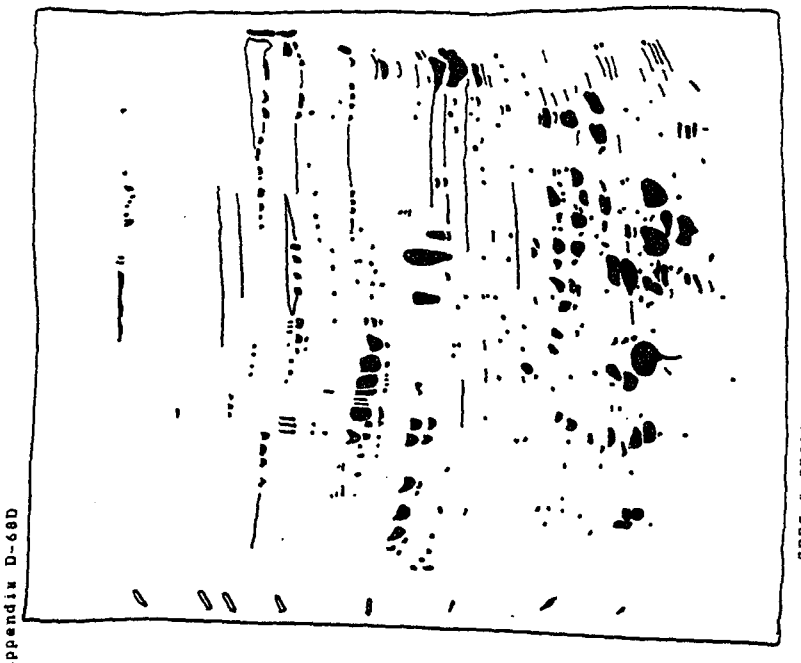
SPLIT EJACULATE: Fraction 2



Appendix D-68A

SPEC # SE02A RUN # P043 DATE 7/1/83

SPLIT EJACULATE: Fraction 1



Appendix D-68D

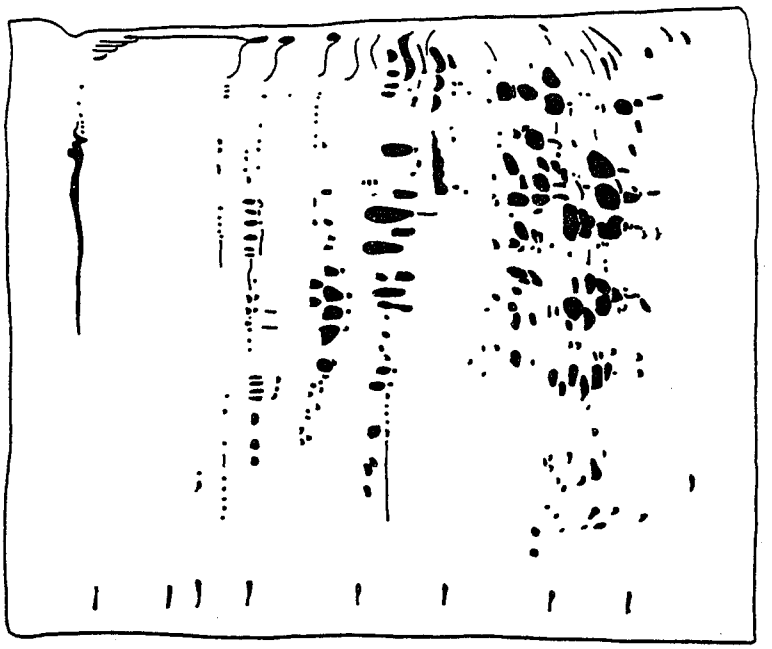
SPEC # SE02D RUN # P043 DATE 7/1/83

SPLIT EJACULATE: Fraction 4

Appendix D-68C

SPEC # SE02C RUN # P043 DATE 7/1/83

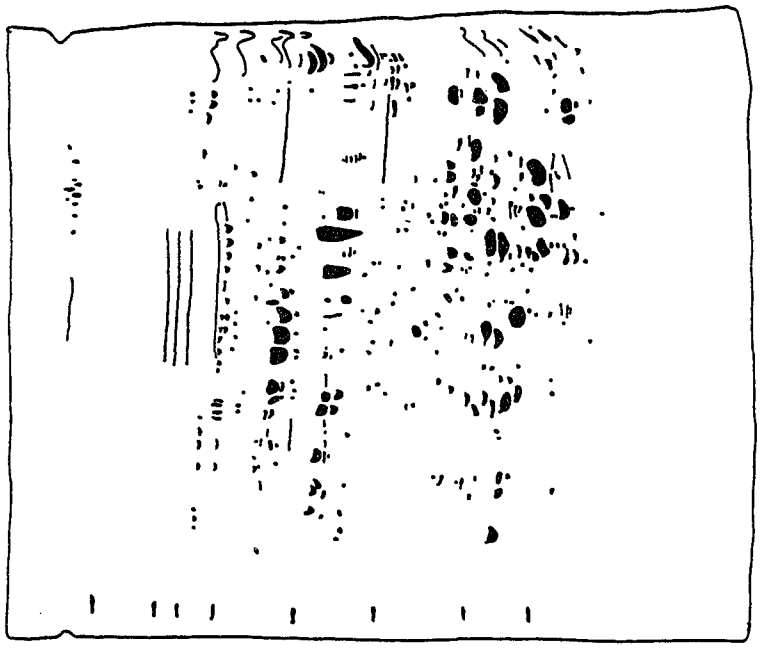
SPLIT EJACULATE: Fraction 3



SPEC # SE02F RUN # F043 DATE 7/1/83

SPLIT EJACULATE: Fraction 6

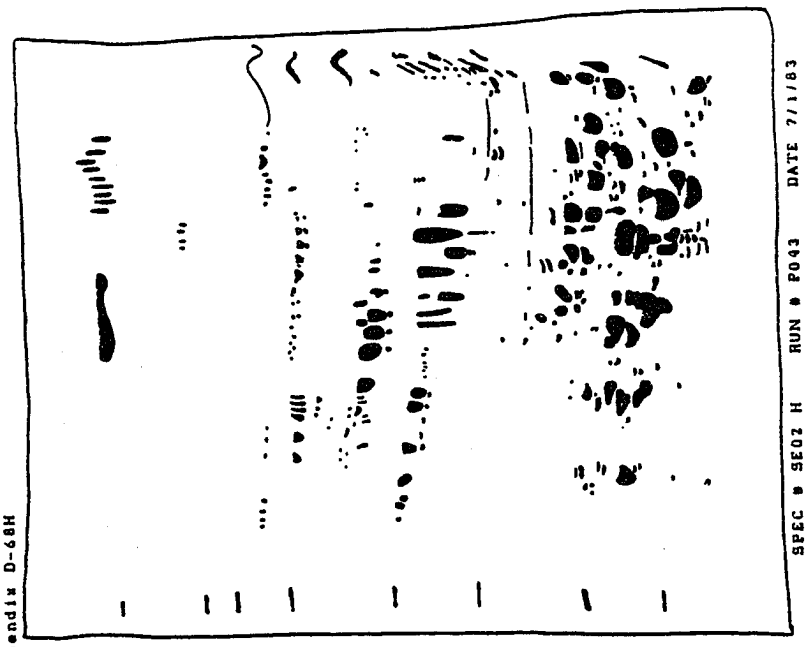
Appendix D-68F

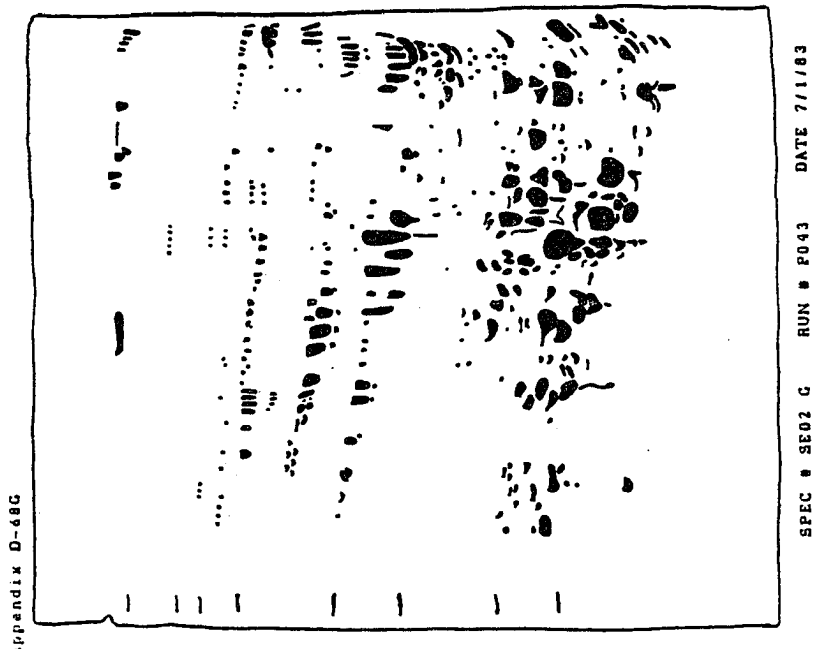


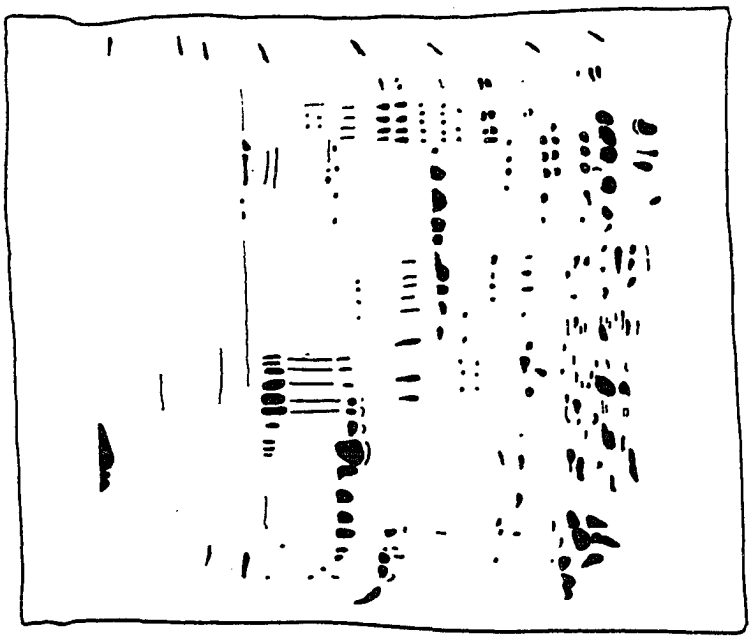
SPEC # SE02E RUN # F043 DATE 7/1/83

SPLIT EJACULATE: Fraction 5

Appendix D-68E



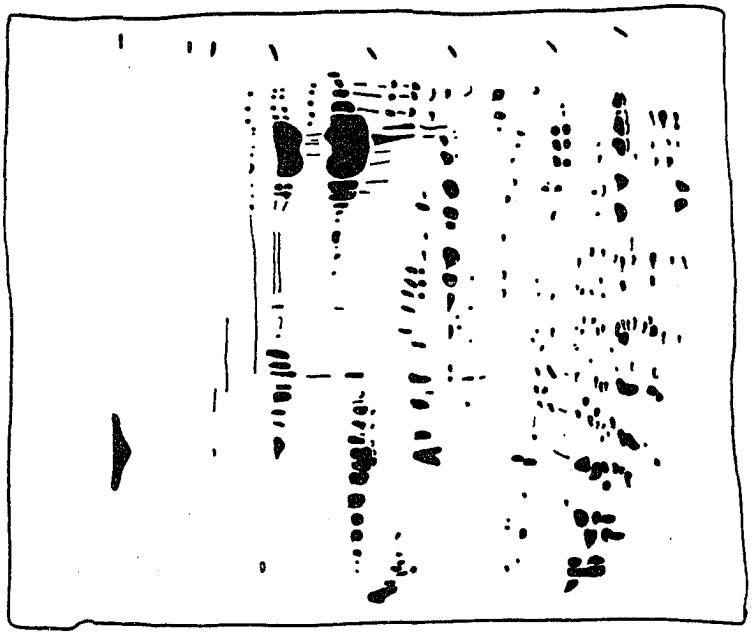




Appendix D-69 B

SPEC # SE03 B RUN # P046 DATE 7/5/83

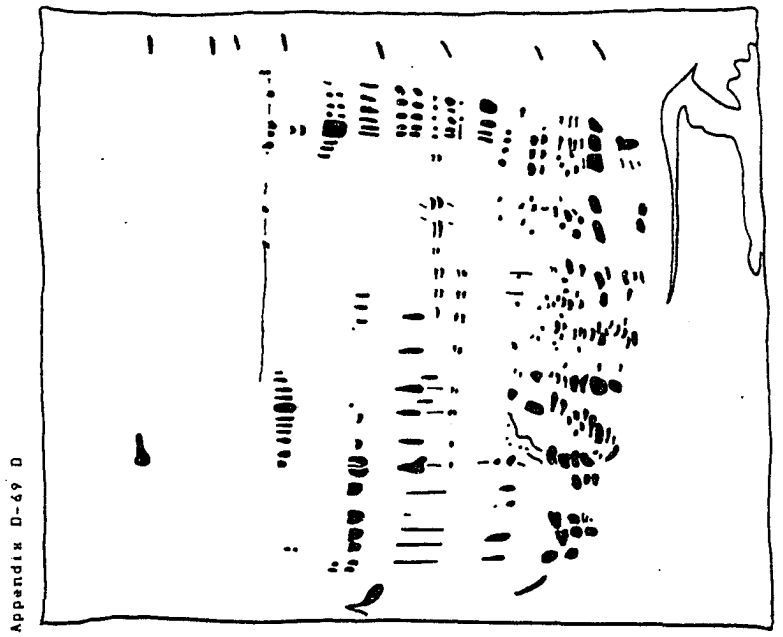
SPLIT EJACULATE: Fraction 2



Appendix D-69 A

SPEC # SE03 A RUN # P046 DATE 7/5/83

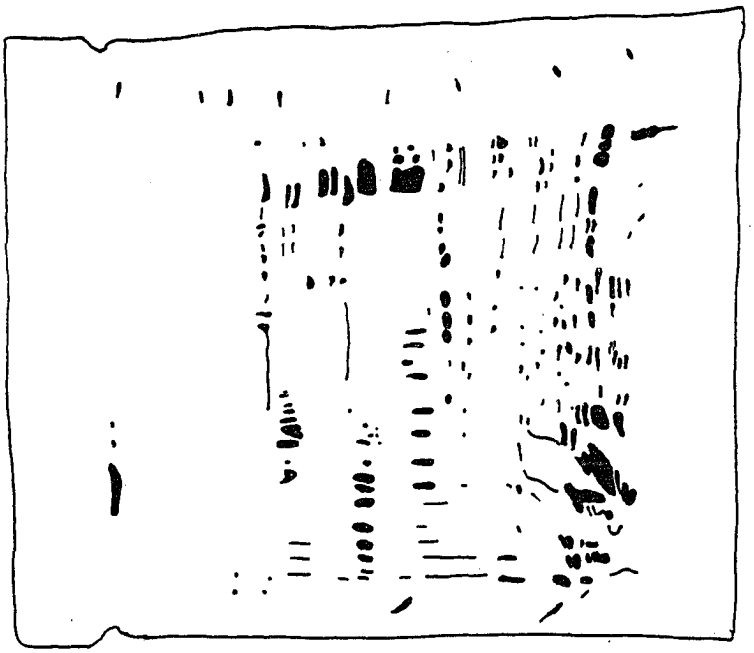
SPLIT EJACULATE: Fraction 1



Appendix D-69 D

SPEC # SE03 D RUN # P046 DATE 7/5/83

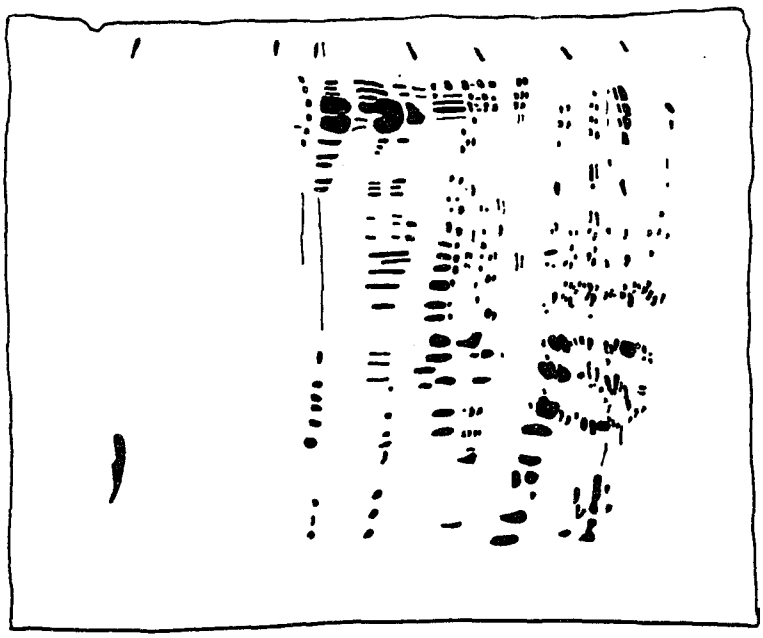
SPLIT EJACULATE: Fraction 4



Appendix D-69 C

SPEC # SE03 C RUN # P046 DATE 7/5/83

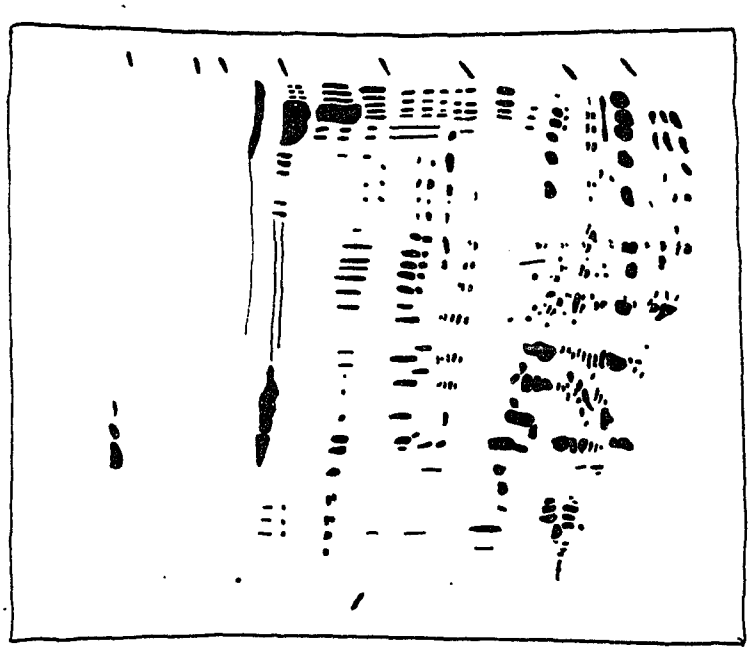
SPLIT EJACULATE: Fraction 3



Appendix D-69 F

SPEC # SE03 F RUN # P046 DATE 7/5/83

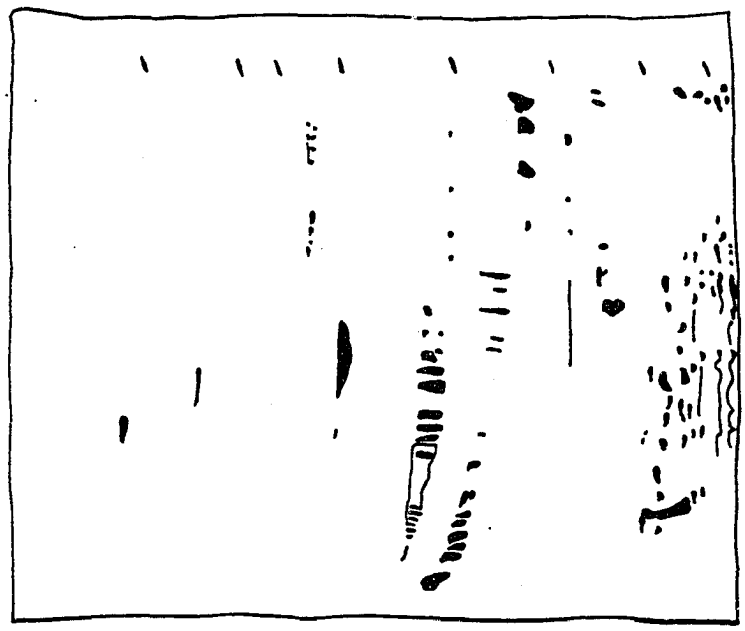
SPLIT EJACULATE Fraction 6



Appendix D-69 E

SPEC # SE03 E RUN # P046 DATE 7/5/83

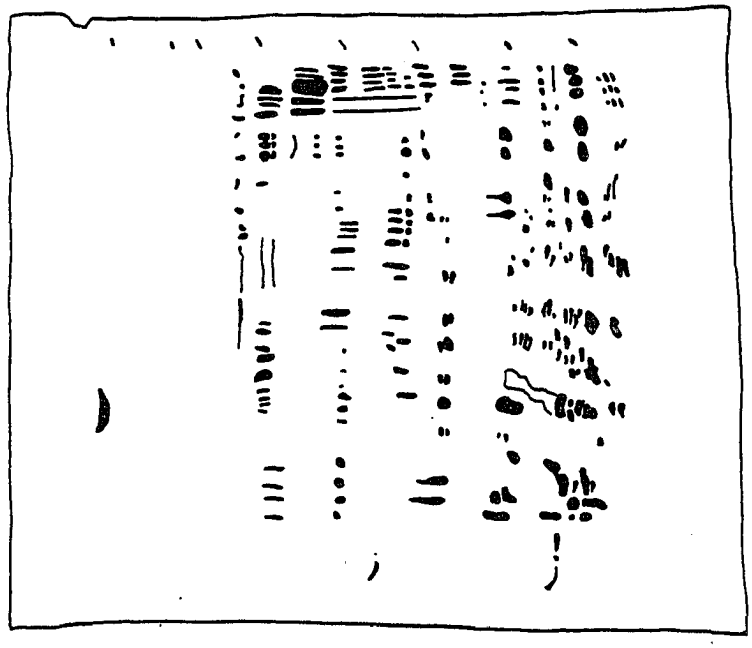
SPLIT EJACULATE Fraction 5



Appendix D-70 A

SPEC # SE04 A RUN # P049 DATE 8/8/83

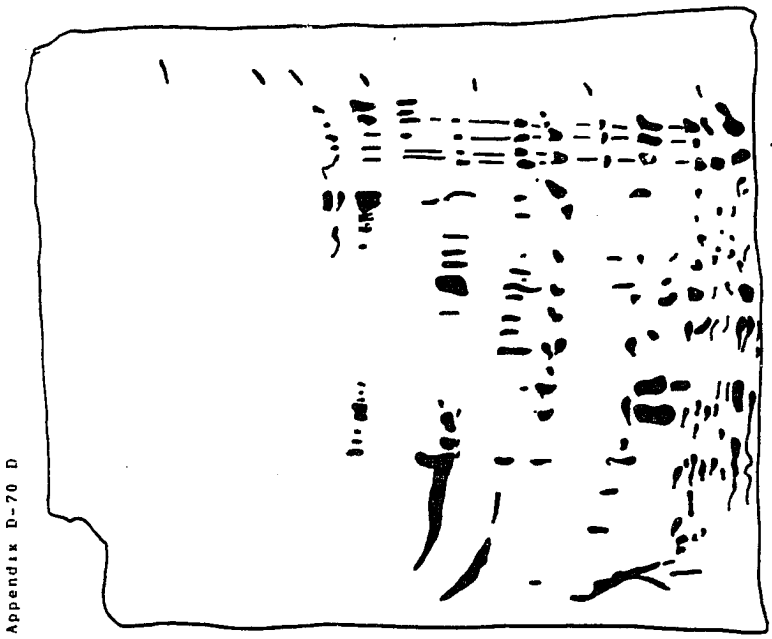
SPLIT EJACULATE. Fraction 1



Appendix D-69 C

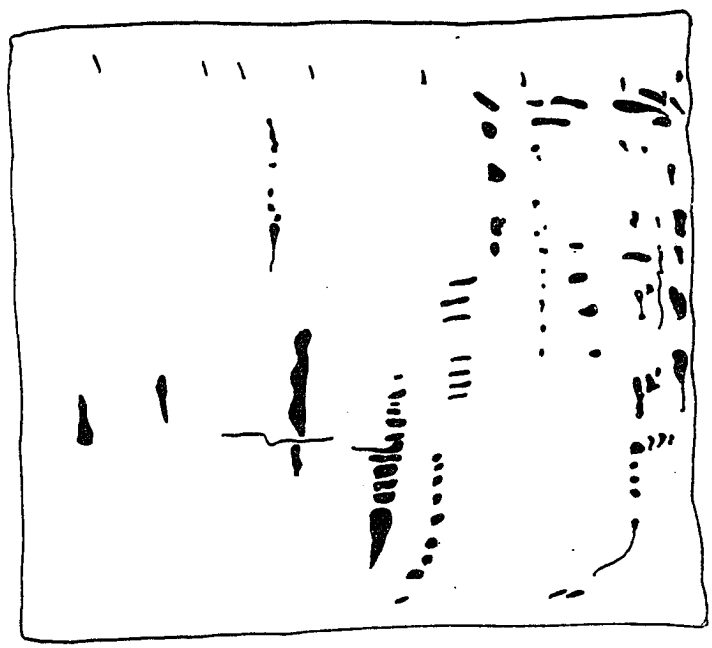
SPEC # SE03 C RUN # P046 DATE 7/5/83

SPLIT EJACULATE. Fraction 7



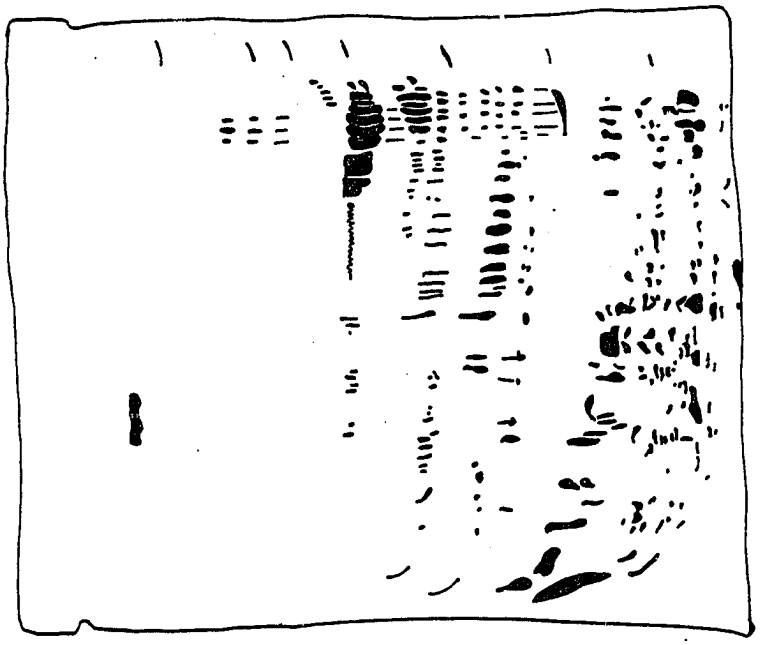
Appendix D-70 D

SPEC # SE04 D RUN # P049 DATE 8/8/83
SPLIT EJACULATE: Fraction 4



Appendix D-70 C

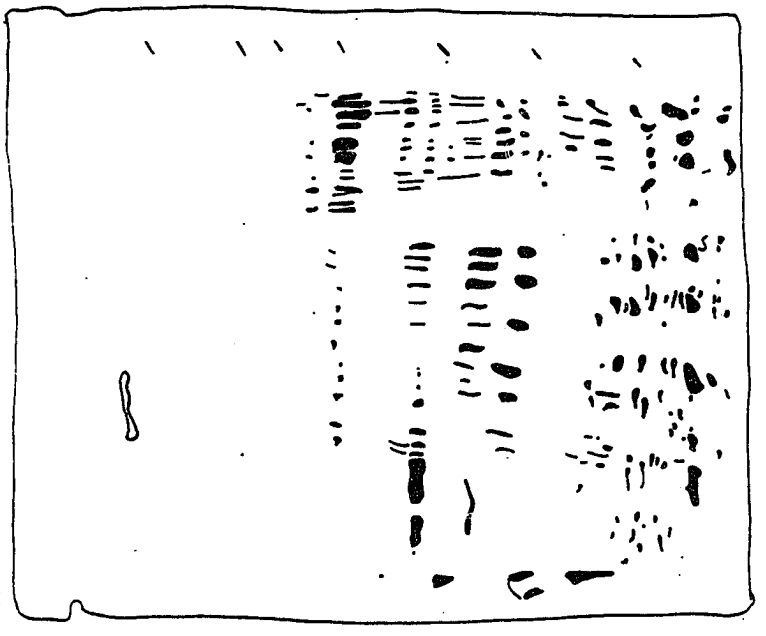
SPEC # SE04 C RUN # P049 DATE 8/8/83
SPLIT EJACULATE Fraction 3



Appendix D-70 F

SPEC # SE04 F RUN # P049 DATE 8/8/83

SPLIT EJACULATE: Fraction 6



Appendix D-70 E

SPEC # SE04 E RUN # P049 DATE 8/8/83

SPLIT EJACULATE: Fraction 5

Appendix D-71 B

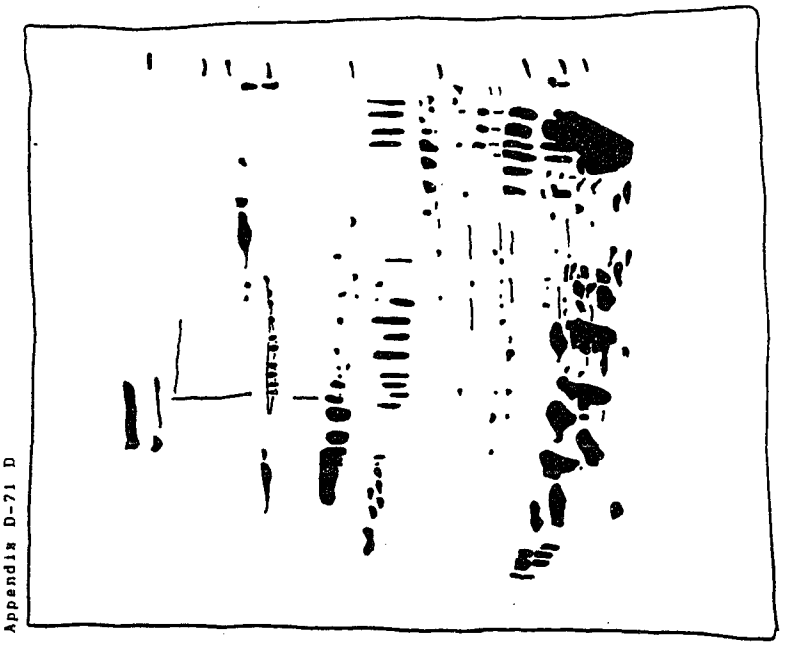
SPEC # SE05 B RUN # P048 DATE 8/8/83

SPLIT EJACULATE: Fraction 2

Appendix D-71 A

SPEC # SE05 A RUN # P048 DATE 8/8/83

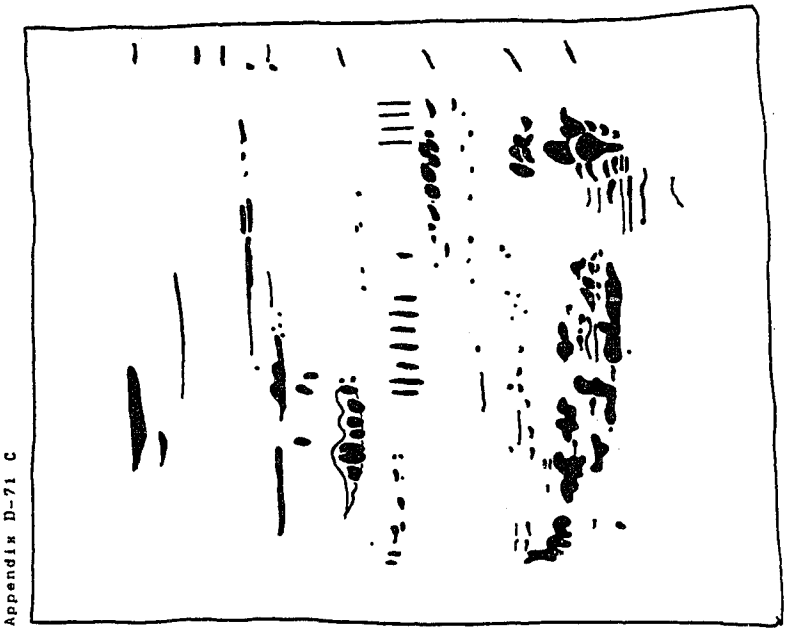
SPLIT EJACULATE: Fraction 1



Appendix D-71 D

SPEC # SE05 D RUN # P048 DATE 8/8/83

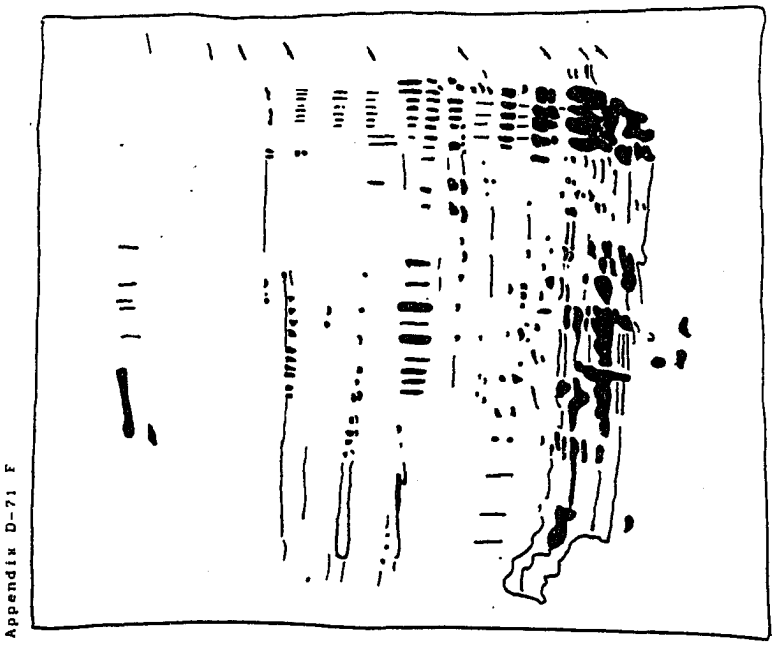
SPLIT EJACULATE Fraction 4



Appendix D-71 C

SPEC # SE05 C RUN # P048 DATE 8/8/83

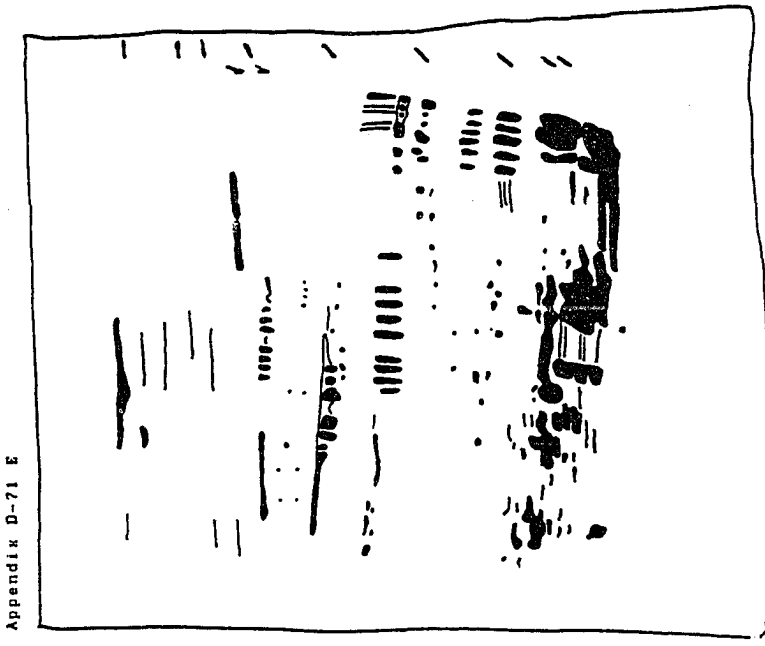
SPLIT EJACULATE: Fraction 3



Appendix D-71 F

SPEC # SE05 F RUN # P048 DATE 8/8/83

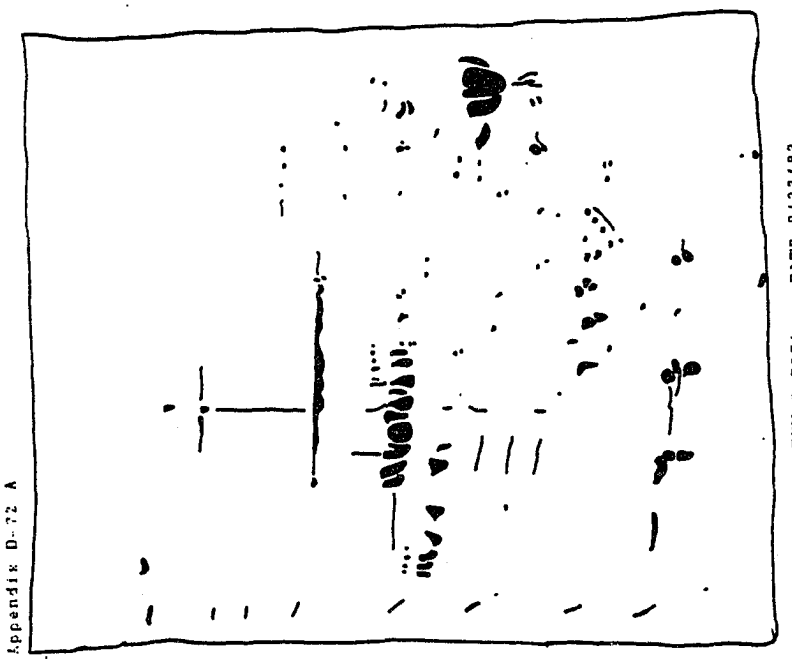
SPLIT EJACULATE: Fraction 6

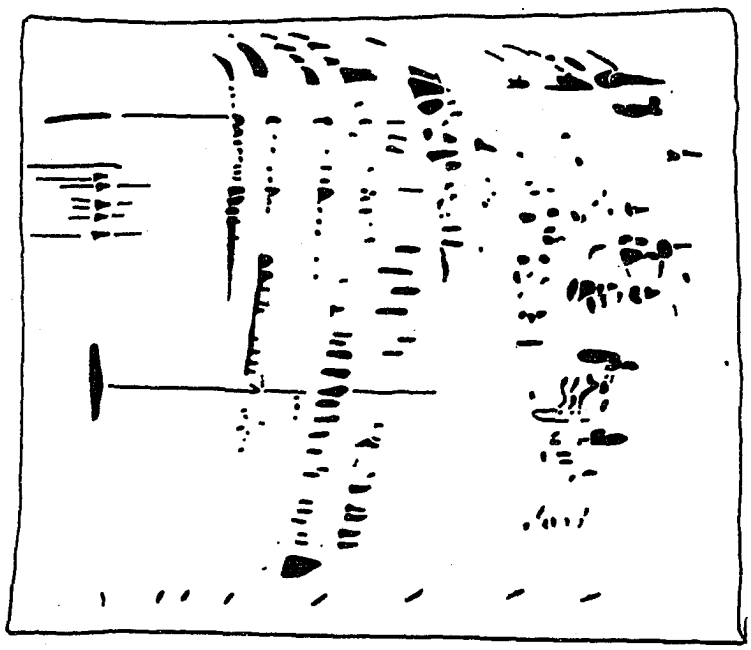


Appendix D-71 E

SPEC # SE05 E RUN # P048 DATE 8/8/83

SPLIT EJACULATE: Fraction 5

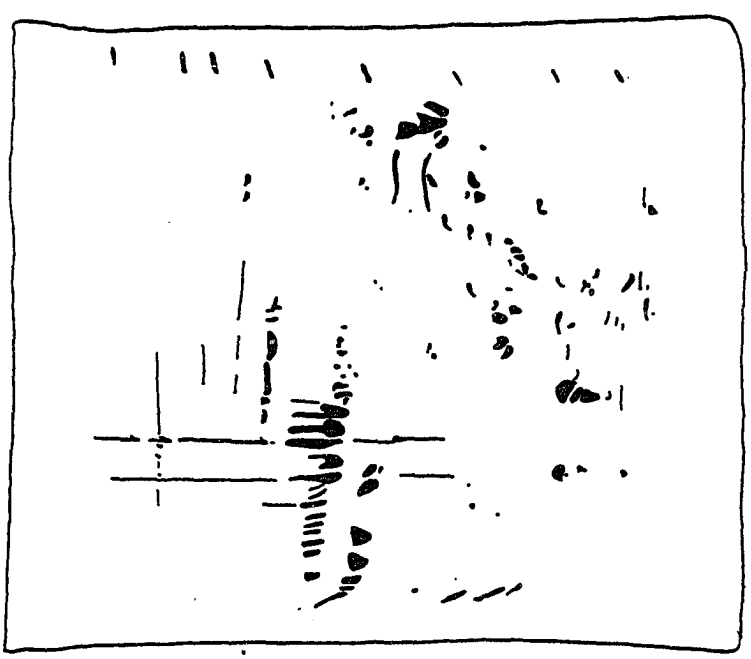




Appendix D-72 D

SPEC # SE07 D RUN # P051 DATE 9/23/83

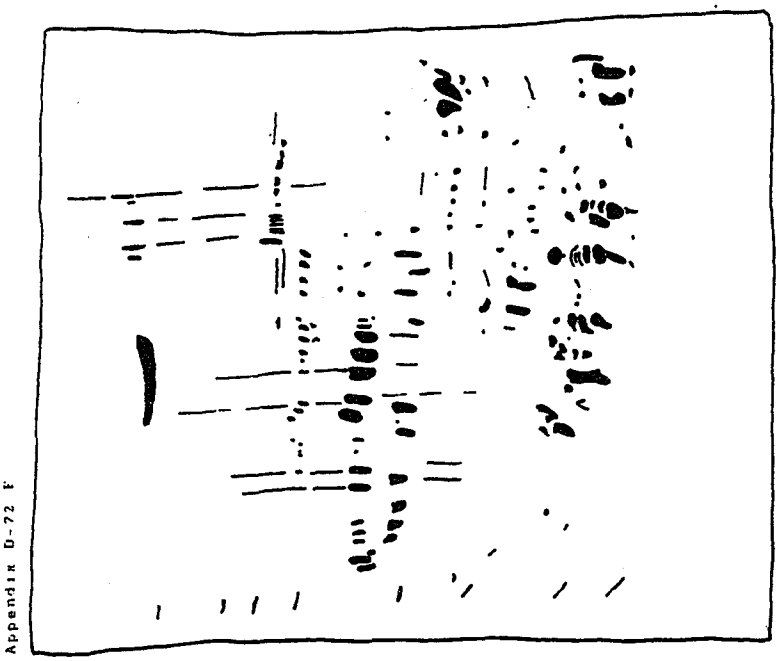
SPLIT EJACULATE: Fraction 4



Appendix D-72 C

SPEC # SE07 C RUN # P051 DATE 9/23/83

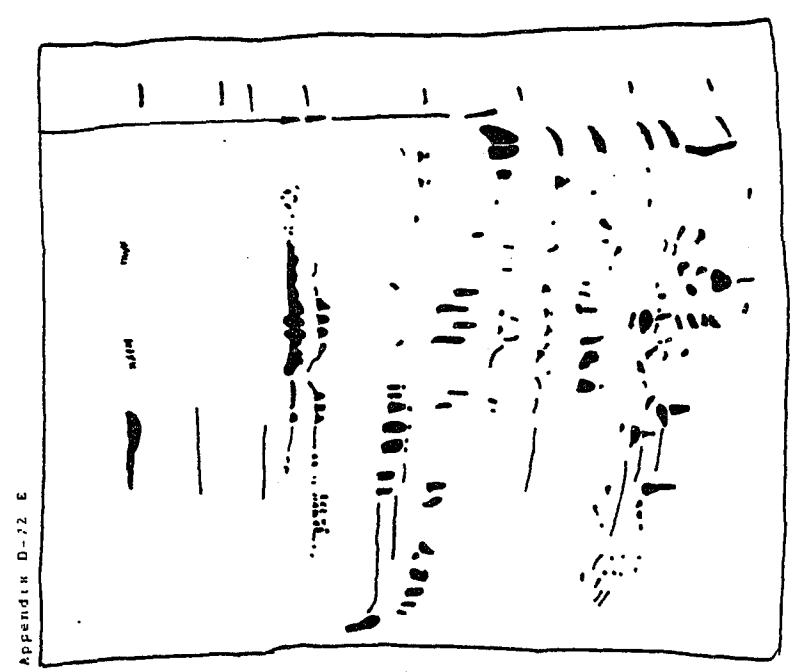
SPLIT EJACULATE: Fraction 3



Appendix D-72 F

SPEC # SE07 F RUN # P051 DATE 9/23/83

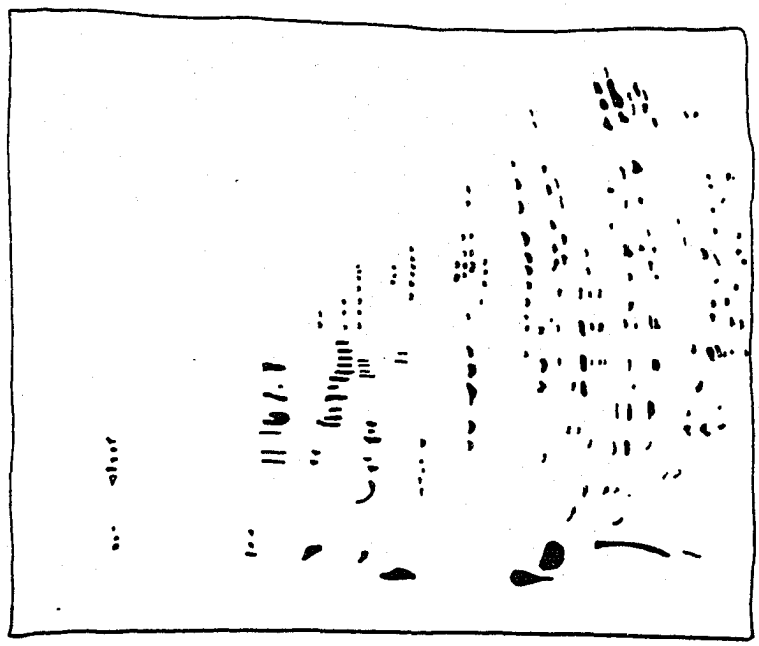
SPLIT EJACULATE: Fraction 6



Appendix D-72 E

SPEC # SE07 E RUN # P051 DATE 9/23/83

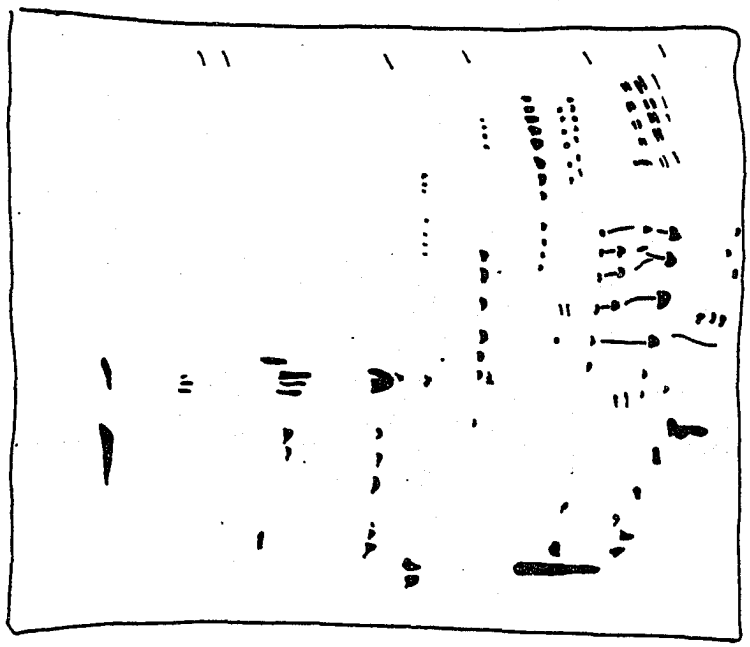
SPLIT EJACULATE: Fraction 5



Appendix D-73 B

SPEC # T01 B RUN # P045 DATE 7/28/83

5 MINUTES



Appendix D-73 A

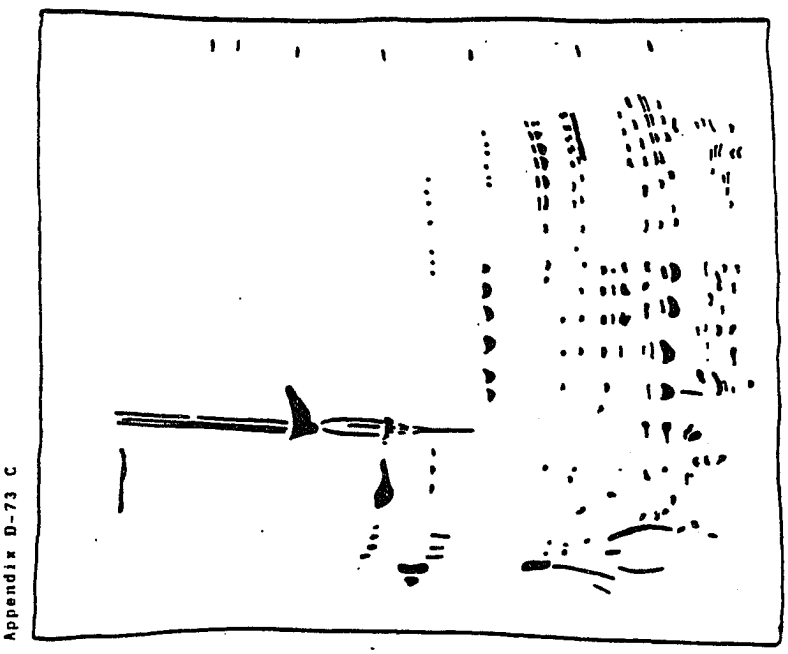
SPEC # T01 A RUN # P045 DATE 7/28/83

3 MINUTES 20 SECONDS

Appendix D-73 D

SPEC # T01 D RUN # P045 DATE 7/28/83

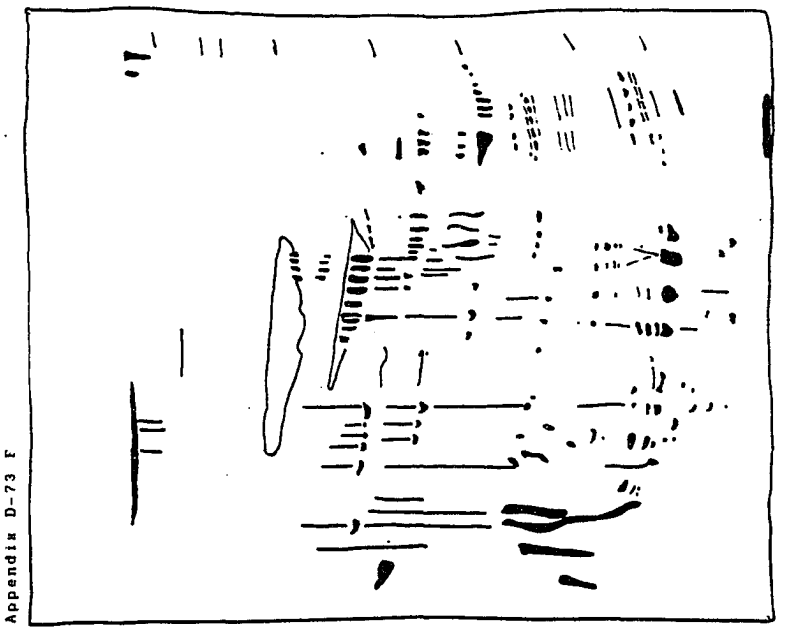
10 MINUTES



Appendix D-73 C

SPEC # T01 C RUN # P045 DATE 7/28/83

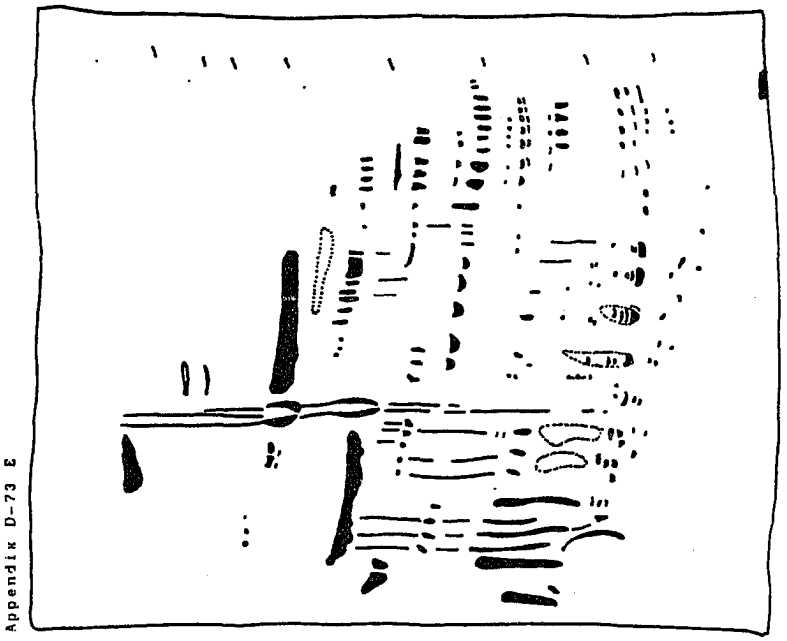
7 MINUTES 30 SECONDS



Appendix D-73 F

SPEC # T01 F RUN # P045 DATE 7/28/83

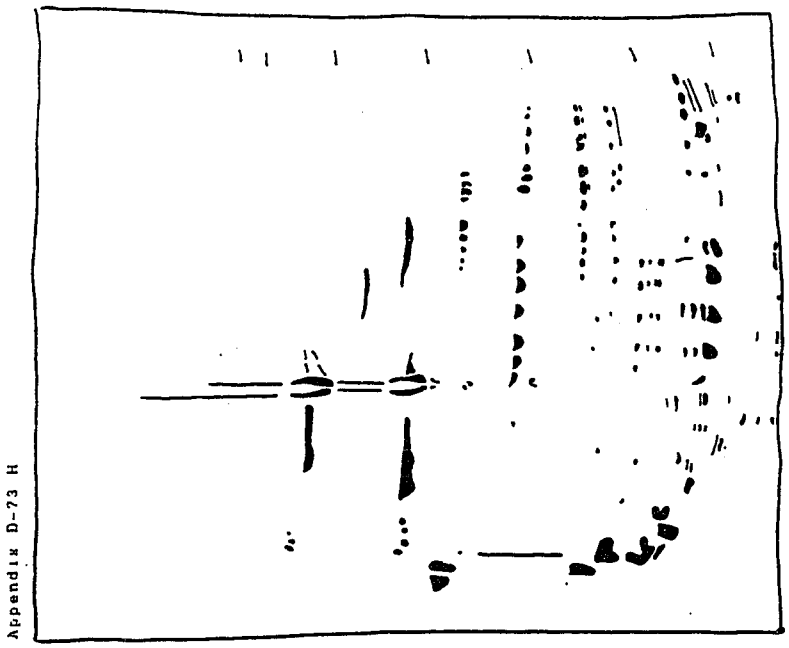
15 MINUTES



Appendix D-73 E

SPEC # T01 E RUN # P045 DATE 7/28/83

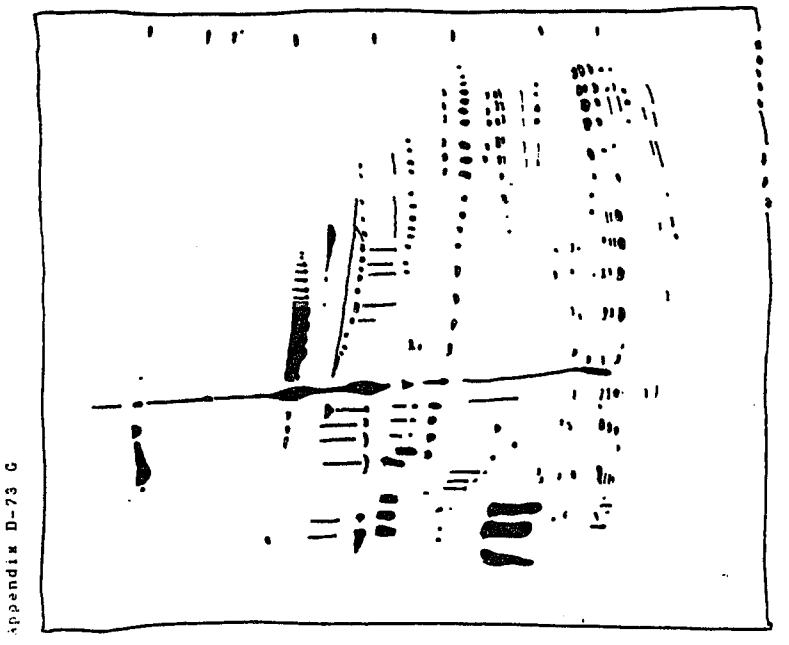
12 MINUTES 30 SECONDS



Appendix D-73 H

SPEC # T01 H RUN # P045 DATE 7/28/83

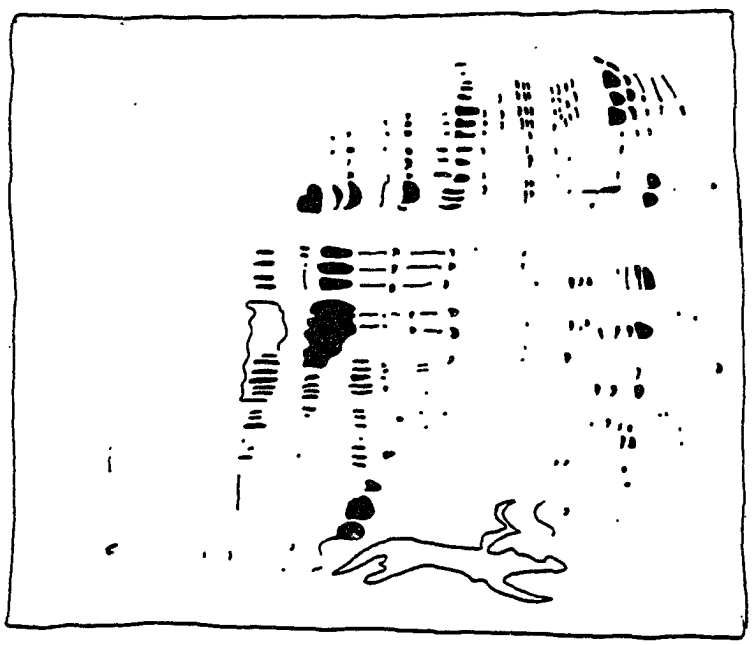
20 MINUTES



Appendix D-73 G

SPEC # T01 G RUN # P045 DATE 7/28/83

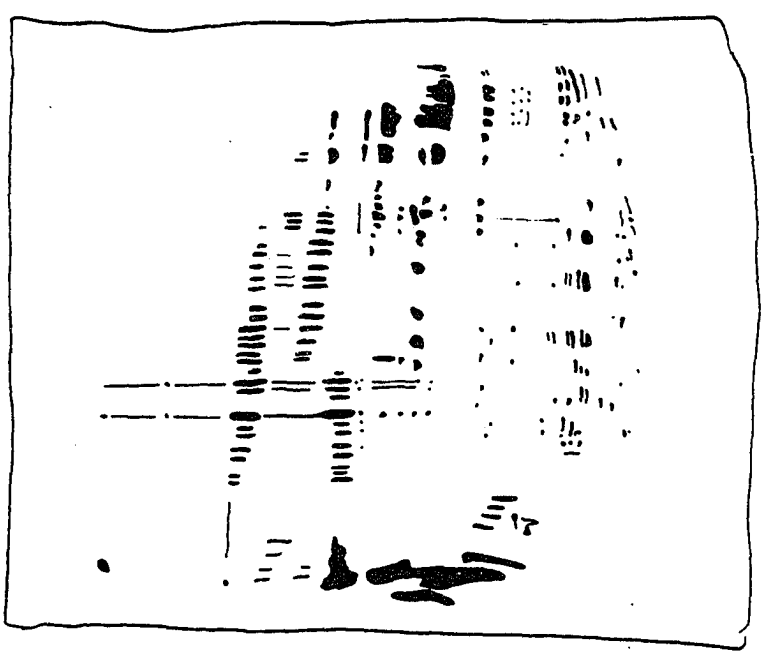
20 MINUTES 30 SECONDS



Appendix D-74 B

SPEC # T02 B RUN # P049 DATE 8/2/83

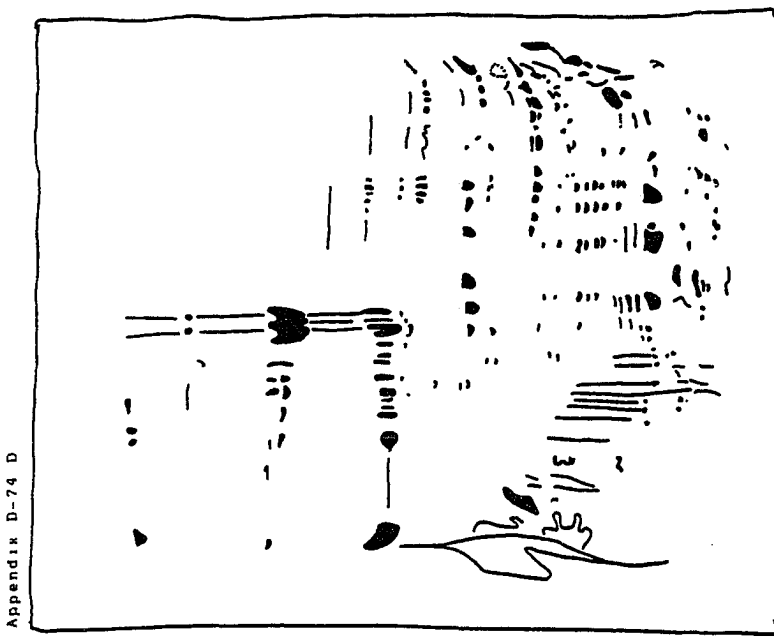
5 MINUTES



Appendix D-74 A

SPEC # T02 A RUN # P049 DATE 8/2/83

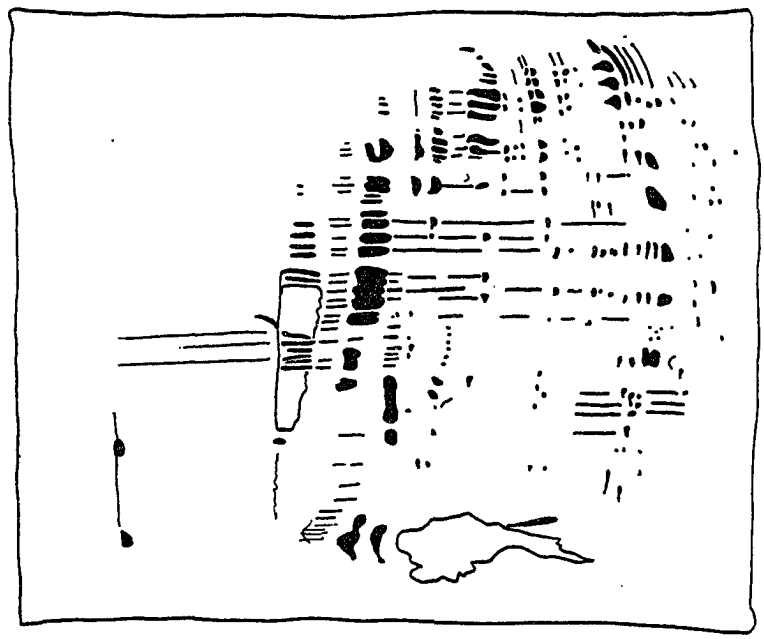
3 MINUTES



Appendix D-74 D

SPEC # T02 D RUN # P049 DATE 8/2/83

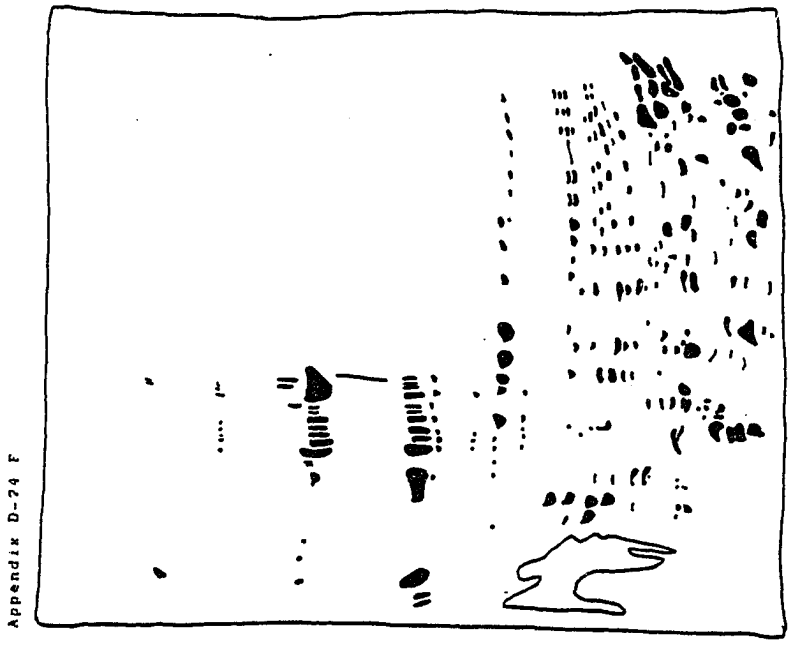
10 MINUTES

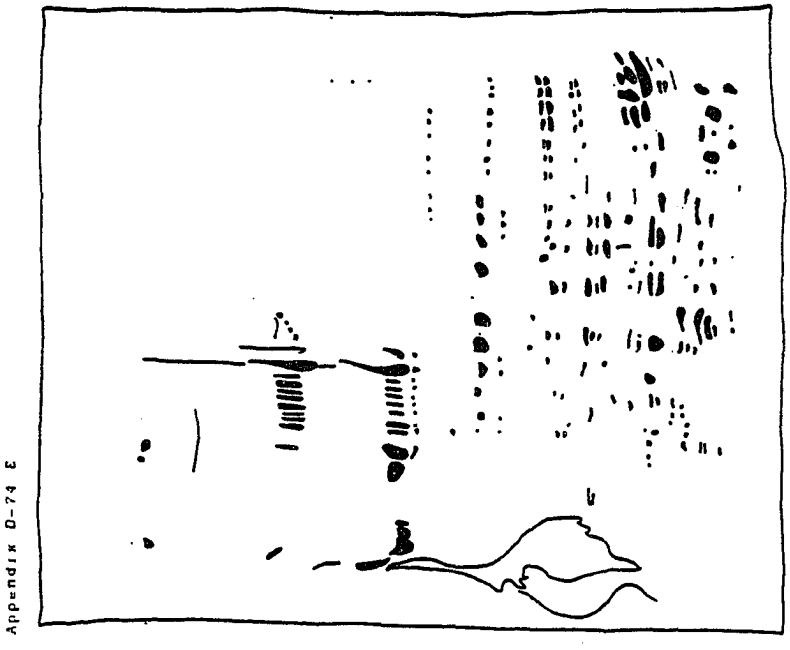


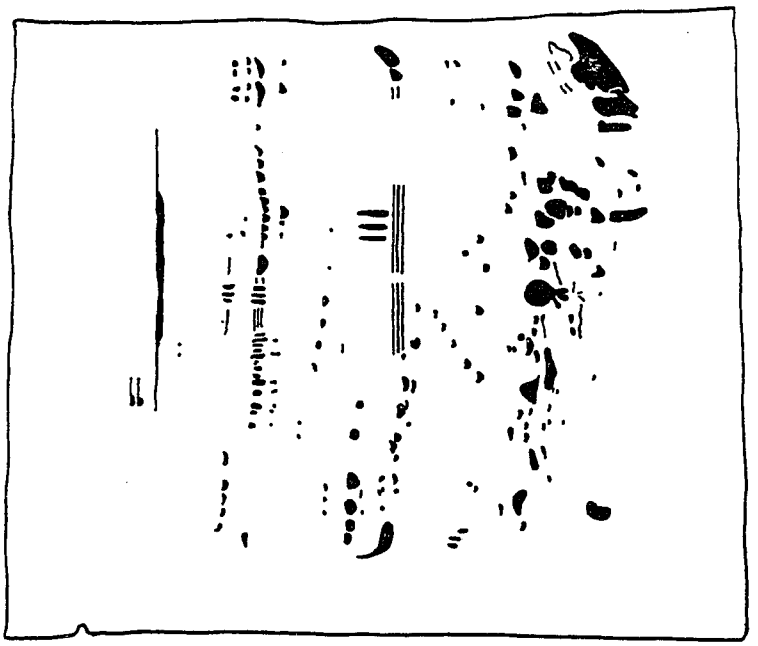
Appendix D-74 C

SPEC # T02 C RUN # P049 DATE 8/2/83

7 MINUTES 30 SECONDS



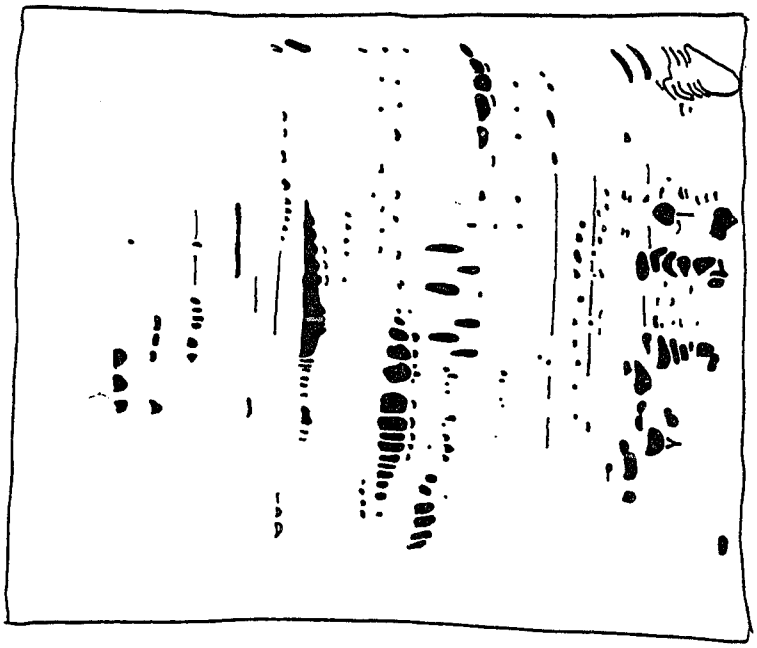




Appendix D-74 H

SPEC # T02 H RUN # P049 DATE 8/2/83

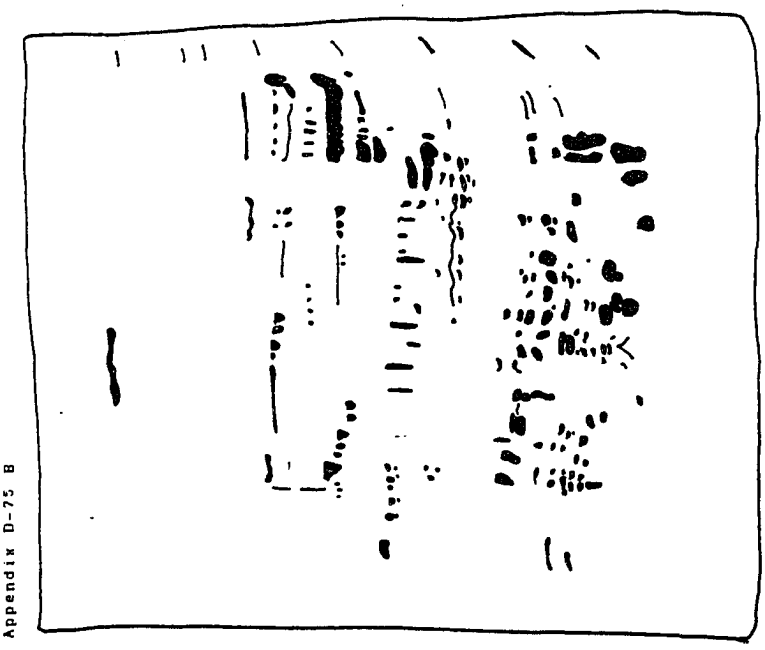
90 MINUTES



Appendix D-74 G

SPEC # T02 G RUN # P049 DATE 8/2/83

30 MINUTES



Appendix D-75 B

SPEC # T03 B RUN # P052 DATE 8/10/83

7 MINUTES 30 SECONDS

Appendix D-75 A

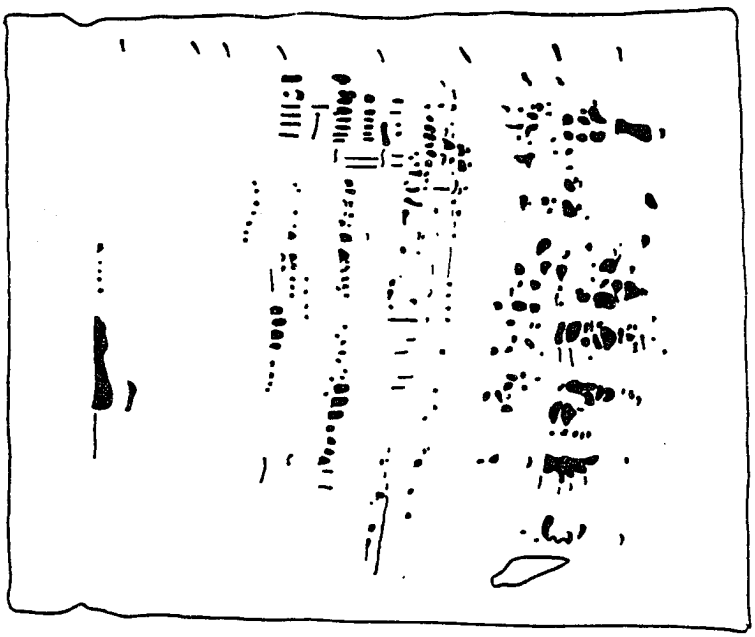
SPEC # T03 A RUN # P052 DATE 8/10/83

4 MINUTES

Appendix D-75 D

SPEC # T03 D RUN # P052 DATE 8/10/83

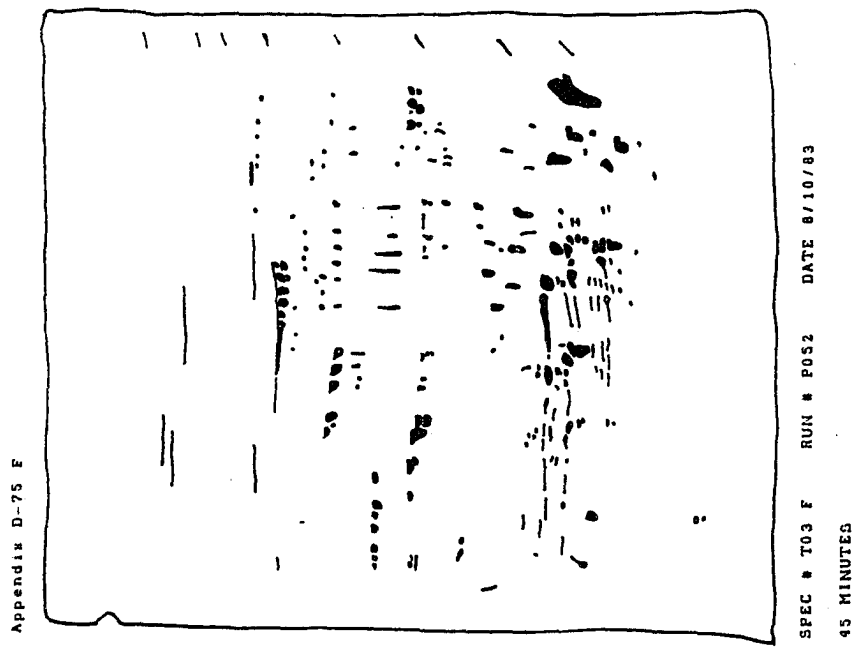
15 MINUTES



Appendix D-75 C

SPEC # T03 C RUN # P052 DATE 8/10/83

10 MINUTES



Appendix D-75 F

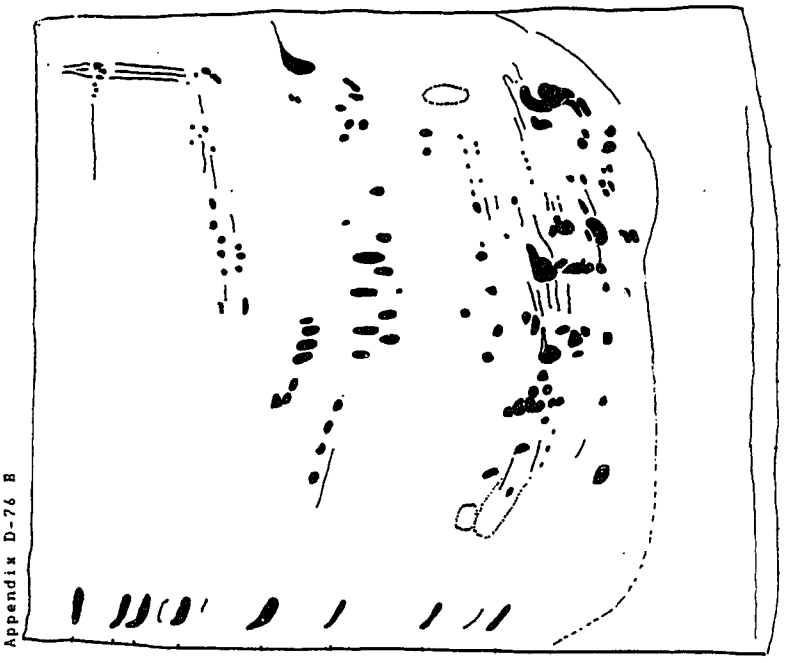
SPEC # T03 F RUN # P052 DATE 8/10/83

45 MINUTES

Appendix D-75 E

SPEC # T03 E RUN # P052 DATE 8/10/83

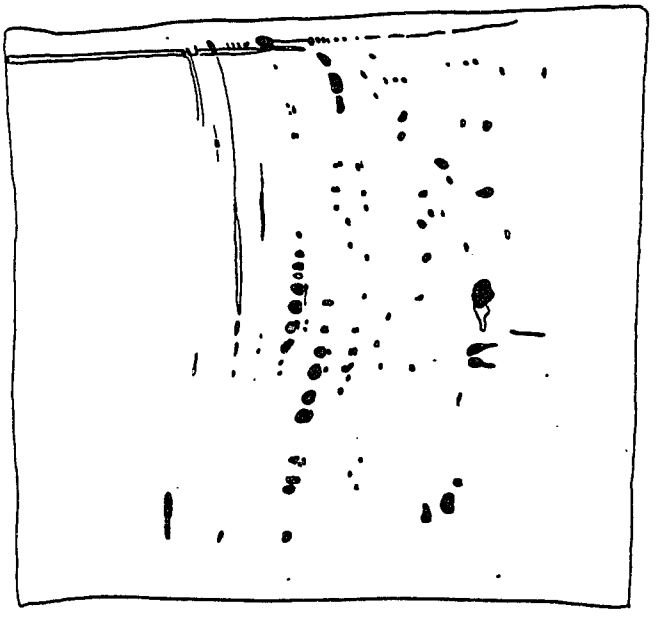
30 MINUTES



Appendix D-76 B

SPEC # HSP 2137 RUN # P021 DATE 1/17/83

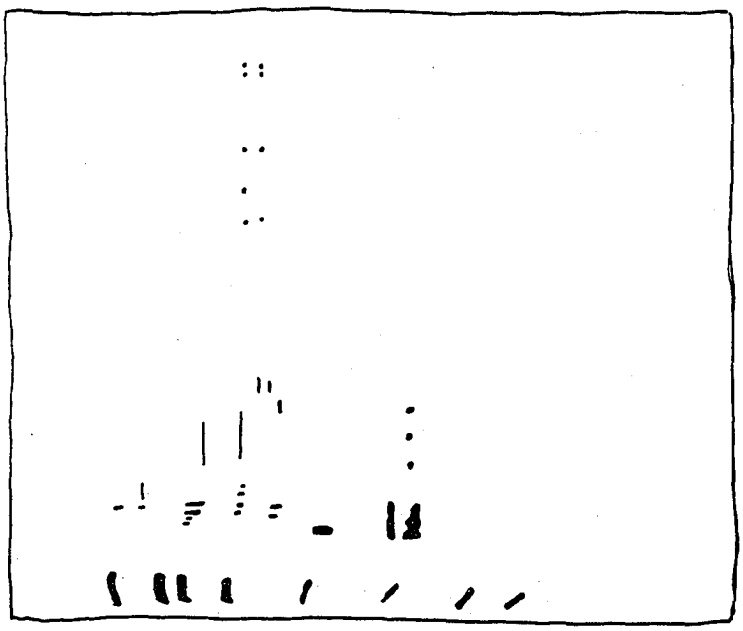
PROSTATIC FLUID



Appendix D-76 A

SPEC # E004 RUN # P010 DATE November, 1980

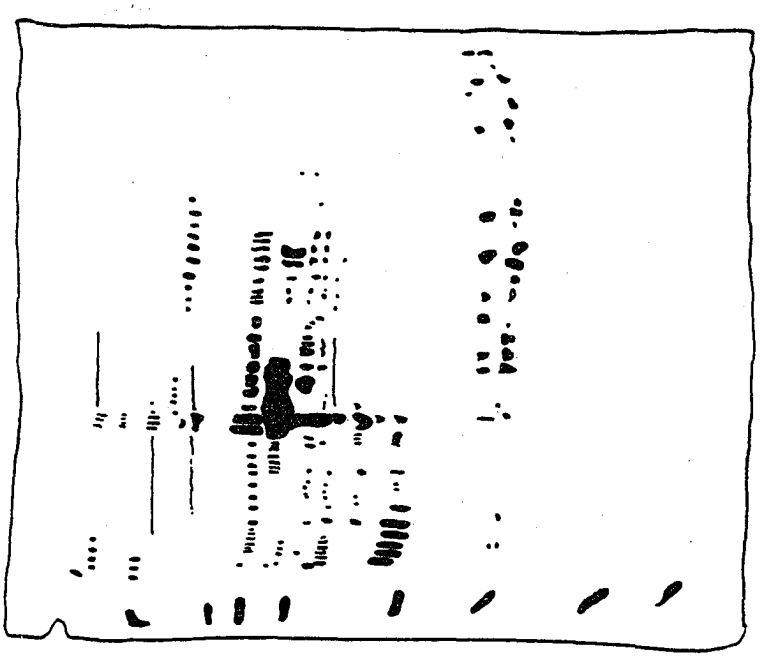
PROSTATIC FLUID



Appendix D-77 B

SPEC H 135 RUN # P048 DATE 8/9/83

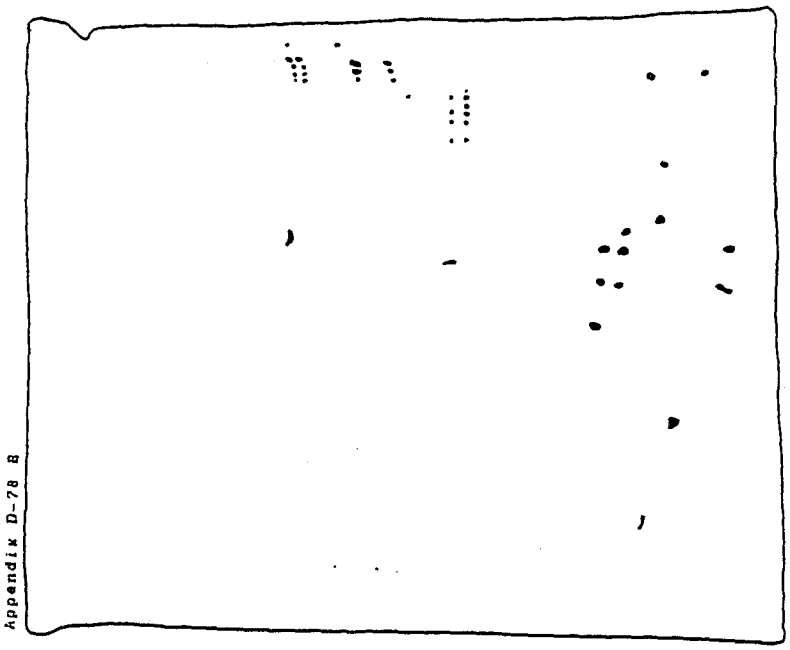
PRE-EJACULATORY FLUID



Appendix D-77 A

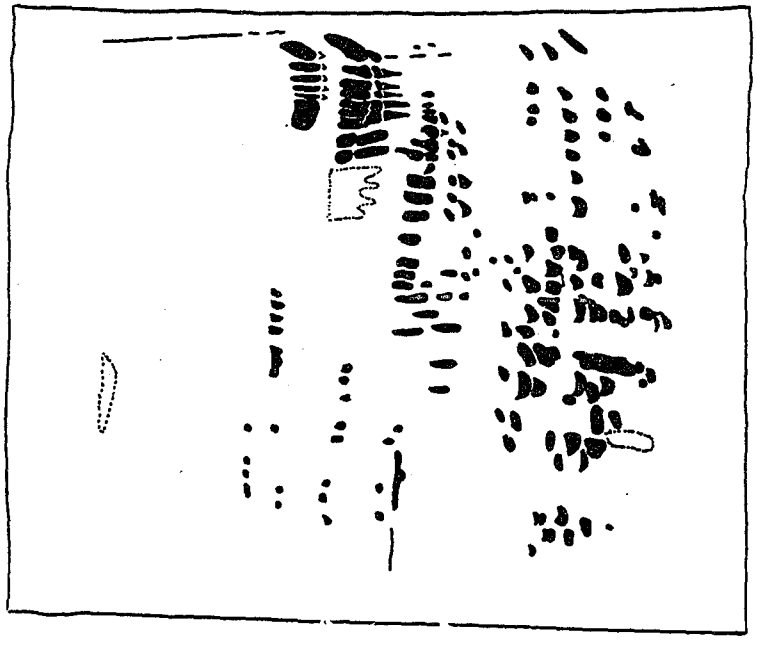
SPEC # H 167-G RUN # P051 DATE 9/21/83

PRE-EJACULATORY FLUID



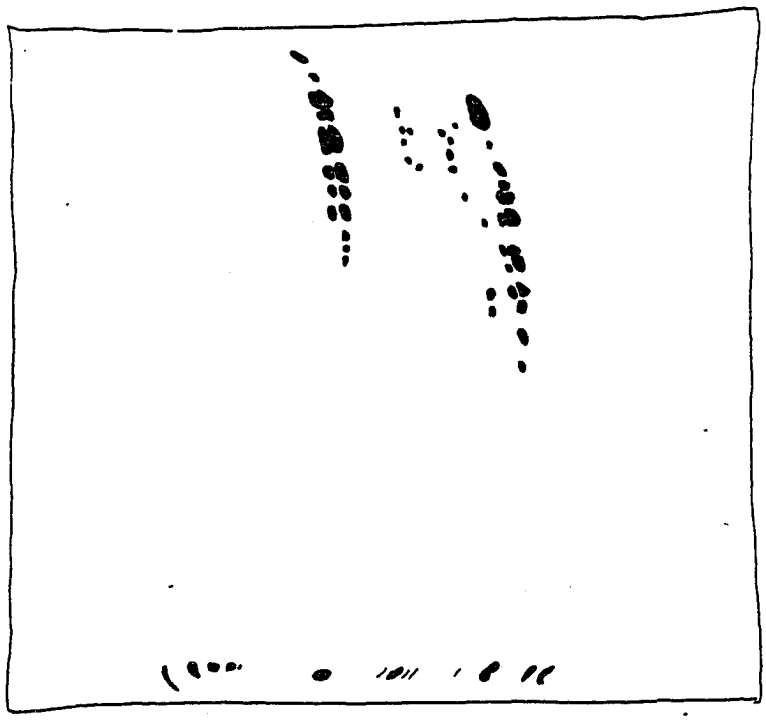
Appendix D-78 B

SPEC HSP 2495 RUN # P040 DATE 6/19/83
WASHED GLOBULES (Incomplete Liquifaction)



Appendix D-78 A

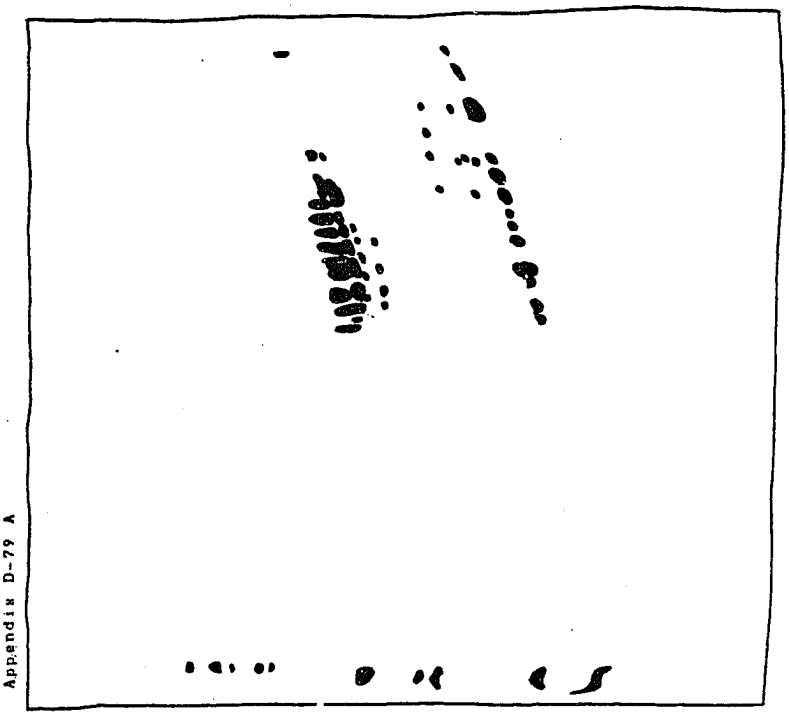
SPEC HSP 2002 RUN # P036 DATE 5/9/83
WASHED GLOBOID BODIES



Appendix D-79 B

RUN P003 DATE 4/2/82

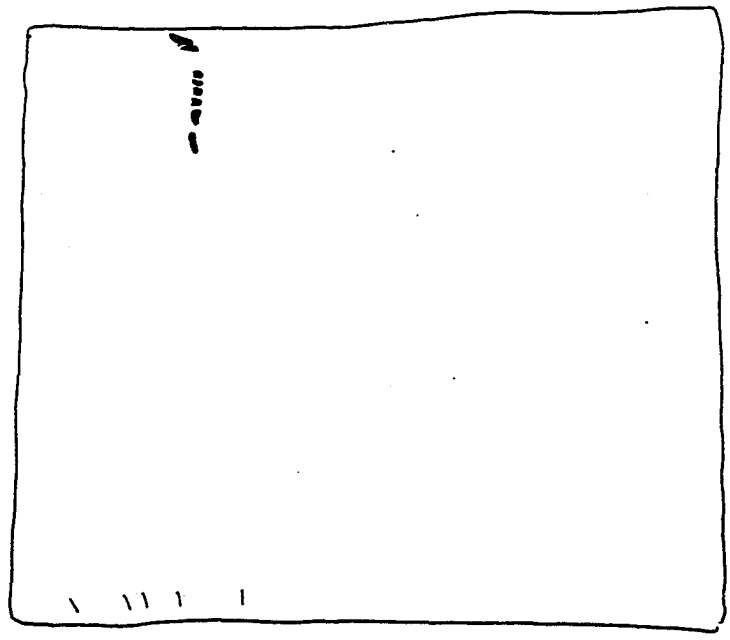
IMMUNOGLOBULIN G (IgG)



Appendix D-79 A

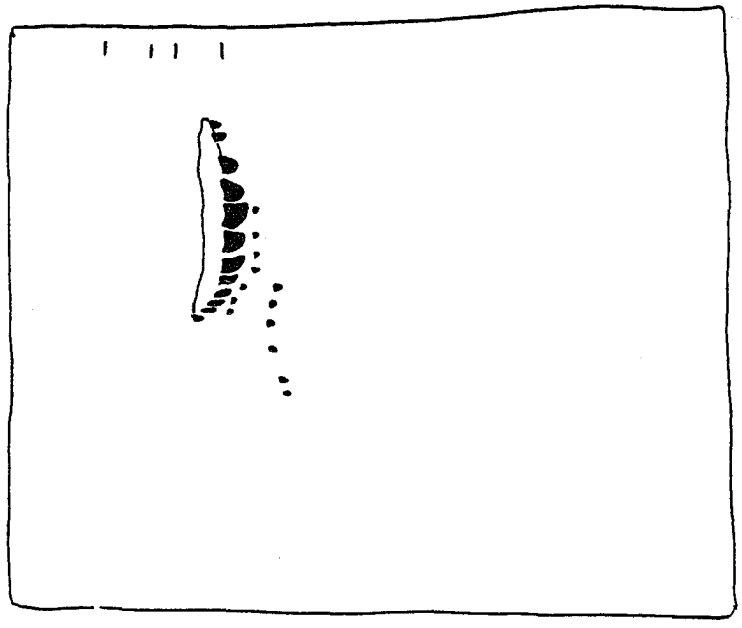
RUN P003 DATE 4/2/82

IMMUNOGLOBULIN A (IgA)



Appendix D-79 D

RUN P031 DATE 3/23/83
Human LACTRFERRIN (Lf)

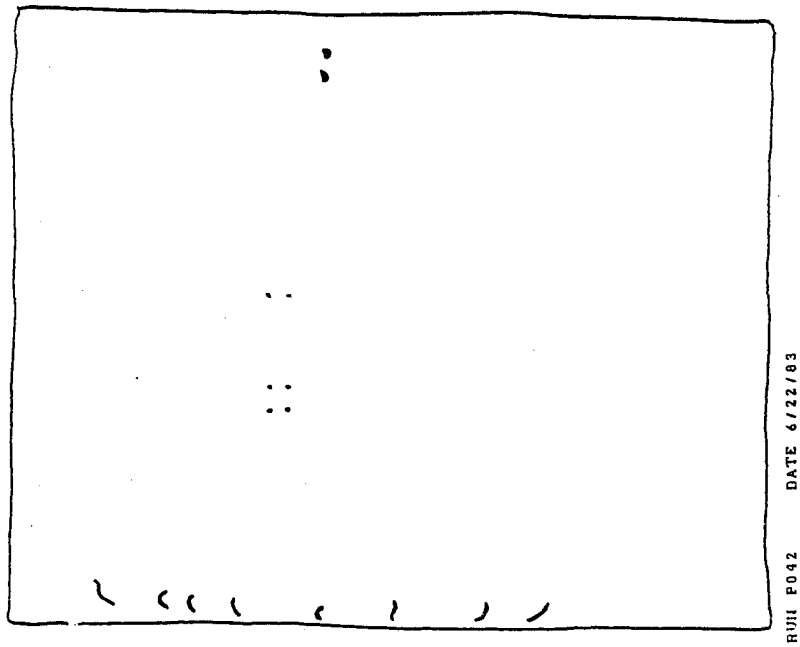


Appendix D-79 C

RUN P031 DATE 3/23/83
Human TRANSFERRIN (hTf)

Appendix D-80

SPEC # HSP 2414 RUN # P041 DATE 6/20/83
Acid Phosphatase missing



Appendix D-79 E

RUN P042 DATE 6/22/83
Mouse LDH-X
(sample provided by Dr. C. Y. Lee)

AUTOBIOGRAPHICAL STATEMENT

Mr. Edward Ellwood Gaunt was born October 28, 1949, in Woodbury, New Jersey to parents C. Richard Moorehouse Gaunt and Janet Horner Gaunt. He married the former Miss Carol Marie Jones of Blackstone, Virginia in August of 1972. He has two children; Kevin Edward, and Ryan Matthew.

Mr. Gaunt graduated from Virginia Polytechnic Institute and State University with a Bachelor of Sciences degree in Biochemistry and Nutrition in June, 1972. He received his Master of Sciences degree in Clinical Chemistry from Old Dominion University in August of 1980.

After graduation in 1972, Mr. Gaunt was commissioned as an Officer in the United States Army and served two tours at the U.S. Army Field Artillery School at Fort Sill, OK, and four years in the Federal Republic of Germany where he was assigned to a LANCE Missile Battalion (1Bn/333FA). While in the Army, he received the National Defense Medal and two Army Commendation Medals. In addition, he was inducted into the Honorary Order of Saint Barbara.

While at Old Dominion University, Mr. Gaunt held several Graduate Teaching Assistantships, an ODU Research Foundation Assistanceship, and the University Doctoral Fellowship.

Mr. Gaunt began study in the joint Old Dominion University and Eastern Virginia Medical School Biomedical

Sciences Program in July, 1980, where he received the Biomedical Sciences Program Fellowship for two terms. Mr. Gaunt will be the first Doctoral Candidate to graduate from the Biomedical Sciences Program.