SECTION 10. Identification of Semen and Vaginal Secretions

10.1 Introduction

Identification of seminal fluid is an old problem in legal medicine, although it was not perceived as such for most of recorded history. Florence (1896) gave an interesting review of the procedures used in examining alleged victims of sexual assault prior to the 19th century (see in Unit IX, Translations). Even though the spermatozoan was discovered in 1677 (see below), acceptance of the facts that the cells were unique to seminal fluid and that the sperm cell was the fertilizing factor in reproduction were not recognized for some time. Consequently, sperm cells were not sought as evidence of the presence of semen in sexual assault cases. It was apparently not until the early years of the 19th Century that any effort was made to try to identify seminal fluid in such cases at all. That no suitable methods were available for the purpose undoubtedly accounts for that situation in part, but perhaps more importantly, examinations of alleged victims were carried out by matrons, and other reputable members of the community, rather than by physicians or scientists.

The first systematic efforts to identify seminal fluid relied on chemical tests, just as was the case with the first efforts to identify blood in medico-legal investigations (see section 3). In 1826, Ollivier d'Angers and Barruel reported on a sexual assault case in which they had been consulted. The suspect claimed that the stains on his clothing were made by fat from uncooked animal meat. The experts examined stained areas of the clothing along with cloth controls, and compared them with respect to wettability with water, the nature and color of the aqueous extract, and the behavior of the extract with absolute alcohol. The extract had a "spermatic" odor, was alkaline, and its residue after drying was sticky. They concluded that the stain could not have been made by animal fat, and that it was a seminal stain.

In 1827, Orfila reported on a series of chemical tests for the identification of seminal fluid. He was one of the most respected medico-legalists of the time (see section 3). He had been consulted in a case in which a 13 year old girl had allegedly been molested. A physician saw the victim nine days later, and issued a report of his findings stating that he thought the victim had been sexually assaulted, this based on the fact that he had recovered semen from the vagina. Orfila objected to the findings on two grounds: first, he said that it was highly improbable that semen would persist in the vagina of the victim for nine days, especially since she was suffering from a mucus discharge; and second, he said that no systematic chemical methods had been used to insure that the identification of seminal fluid was correct. The tests he devised for identifying semen were based on the appearance of the stains, changes in color and consistency upon heating and immersion in water, odor emitted by the moistened stain, and the behavior of the aqueous extract toward a number of reagents and treatments (see in Unit IX, Translations). Seminal stains were compared by these criteria with a number of other types of vaginal discharges, and with nasal mucus and saliva stains. Orfila said in this memoir that, while he had had no difficulty in finding spermatozoa in fresh seminal samples, and even in an 18 year old sample of dried semen, with the microscope, he did not have any confidence in microscopical technique for seminal stains on fabrics. He said that it was very difficult, if not impossible, to find intact cells in stain extracts, and that the chemical procedures should always be employed.

In 1834, Chevallier reported on his examinations in a sexual assault case, the methods employed being essentially those which had been described by Orfila. He did not use a microscope. In 1839, Devergie wrote a paper on the signs of death by hanging (Devergie, 1839a). One of these signs was the finding of spermatozoa in the urethral canal of the victim, accomplished by microscopical examination. He noted that he had found sperm cells in 10 month old seminal stains, and thought that the confirmation of spermatozoa in a stain was a more certain criterion for diagnosing seminal stains than the chemical methods. Orfila (1839) disagreed with Devergie on a number of accounts, and Devergie (1839b) responded to him in print.

In 1837, Rattier had published a paper suggesting that sperm cells could be identified in seminal stain material, and recommending the procedure for medico-legal investigations. Rattier said that he asked the microscopist Charles Chevalier about previous work on the subject, and that he was told that Lebaillif had identified a seminal stain by the microscopical detection of spermatozoa some years before in the Contrafato case, but had not published it. Chevalier himself mentioned this fact indirectly in his book in 1839, and Florence (1896) said that Chevalier had said the same thing to Lassaigne in 1827.

In 1839, Bayard published his extensive paper on the use of the microscope in examining seminal stains for spermatozoa. Careful procedures were set forth for carrying out the technique, and the method began to be widely accepted not long afterwards. The earlier chemical methods were gradually abandoned by many workers. Attempts to find non-microscopical methods for the differentiation of body fluid stains persisted though. Lassaigne, in 1858, indicated a series of reagents which were said to give different sets of reactions with seminal stains and other kinds of stains which might resemble them. Brouardel reviewed the techniques for identifying seminal stains in 1879, emphasizing microscopy

as the principal technique. In the absence of spermatozoa, however, one could not conclude that semen was absent, he said, because it was known that a certain number of men are azoospermic. In 1880, Boutmy and Brouardel were charged by the Society of Legal Medicine with evaluation of a technique which had been proposed by Petel and Labiche for seminal stain identification. Petel and Labiche had noted that many body fluid and other stains took up color from the dye, carmine, but that these decolorized at different rates in a sodium carbonate solution. Seminal stains required 12 hours to decolorize, while other stains they had looked at needed much shorter times. Boutmy and Brouardel could not accept the test as sufficient unto itself for identification, but noted that it might be useful in providing additional evidence in some cases.

None of the non-morphological tests used during most of the 19th Century has survived. Most authorities began to rely upon sperm cell detection for seminal stain identification around 1840. The Florence test for seminal stain identification was introduced in 1896 (see section 10.4.1).

A number of the early papers cited in this introductory section may be read in their entirety in the translations set (Unit IX).

10.2 Detection and Identification of Spermatozoa

The famous Dutch scientist van Leeuwenhoek first described the morphology of the spermatozoan three centuries ago. In a letter written in November of 1677, and published in the Philosophical Transactions of the Royal Society of London, he credited a medical student from Leiden, named Ham, with having made the actual discovery (Kemper, 1976; Schierbeek, 1959).

> I have divers times examined the same matter [human semen] from a healthy man, not a sick man, not spoiled by keeping for a long time and not liquefied after the lapse of some time; but immediately after ejaculation before six beats of the pulse had intervened; and I have seen so great a number of living animals in it that sometimes more than a thousand were moving about in an amount of material of the size of a grain of sand. . . . These animalcules were smaller than the corpuscles which impart a red colour to blood; so that I judge a million of them would not equal in size a large grain of sand. Their bodies which were round, were blunt in front and ran to a point behind. They were furnished with a thin tail, about five or six times as long as the body, and with the thickness of about one twenty-fifth of the body. They moved forward, owing to the motion of their tails like that of a snake or an eel swimming in water; but in the somewhat thicker substance they would have to lash their tails eight or ten times before they could advance a hair's breadth.
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> (from van Leeuwenhoek's 1677 letter; after Schierbeek, 1959.)

Identification of sperm cells in a stain is not the oldest method for the medico-legal identification of seminal stains, but may still be the most reliable one. The techniques are relatively simple, and the finding of spermatozoa constitutes incontrovertible proof that the stain was of seminal origin. For a long time, until around 1900, there were really no other reliable methods for identifying semen. Reese (1891) made this point in his text. Menger (1887) gave an account of a case in San Antonio, Texas, in which an older man was accused of raping a child. In some 30 slides prepared from the stains on the victim's underclothing, however, he could find no sperm cells, and said that he could not testify to the presence of semen.

10.2.1 Isolation and identification of spermatozoa from seminal stains

Dozens of different procedures have been recommended for examining seminal stains for spermatozoa. All may be classified into one of the following categories (Pollack, 1943): (1) separation of cells from the supporting material or substratum and microscopical identification; (2) partial or complete destruction of the supporting material; and (3) identification of sperm cells *in situ*, almost always with various biological stains (dyes).

Separation of the cells from the supporting material, and examination of the extract, is the oldest method (Bayard, 1839). Often, the stain has been soaked in one of a variety of solutions, including acetic, hydrochloric, and nitric acids, KOH, ammonia, saline, sodium carbonate or mercuric chloride, glycerin, alcohol or Paccini's solution (see section 5.3). Some authorities have called for soaking, followed by filtration (Bayard, 1839), or soaking, followed by centrifugation (Corin, 1907), or soaking, followed by squeezing (Schmidt, 1848; Koblanck, 1853). Hamlin (1883) said that the squeezing technique, originated by Schmidt, and advocated by Koblanck, was extremely destructive of the cells, and was to be avoided. Scraping of the stain has also been suggested. Hamlin (1883) scraped the dry stain, and then examined the moistened material. Others (e.g. Williams, 1937b) have moistened the stain first, and then scraped material from the surface of the stain for examination as was first done by Robin and Tardieu (1860). Ellis (1960) recommended collection of the cells from stain extracts on Millipore filters. One of the oldest soaking agents is ammonia (Bayard, 1839; Mezger, 1857), and Eungprabhanth (1973) said that cells could be eluted from cotton and filter paper better in the presence of ammonia than with saline alone. Kivela, in 1964, first suggested the use of sonic oscillation as a means of recovering sperm cells from seminal stains on fabrics. The material was first soaked in distilled water for at least 10 minutes, and then shaken briefly by hand. This procedure did not remove many cells, but did extract some extraneous material from the stain. The piece of fabric was then removed to another tube containing water, and exposed to the sonication bath for 1 minute. The cloth was removed, and the tube centrifuged. Most of the supernatant fluid was removed, and the pelleted material dried onto a slide for examination. Kivela said that the method was a considerable time saver,

although there was some breakage of the cells by the exposure to sonic oscillation. Marcinkowski and Przybylski (1966) suggested exposure of seminal stains to sonic oscillations as a means of detaching sperm cells from fabric substrata, and they credited Lukači as being the originator of the method. After sonication, the material was centrifuged and the pellet examined. Gluckman (1968) found the method to be quite successful, using 30 min. sonication times in saline. After centrifugation, the pelleted material was fixed and stained with hematoxylin-eosin. Hueske (1977) compared sonication with mechanical vibration in terms of sperm cell yields from seminal stains. Sonication yielded more cells than mechanical vibration, but more of them were decapitated. He tried a combination of the methods as well, exposing the stain first to mechanical vibration in saline, and then to sonication. Yields were good, but some cells were broken by the sonication step. It is to be noted that the exposure to sonic oscillations was not carried out directly in these studies, i.e., a sonic probe was not placed into the saline covering the stained material. Exposure was by means of a sonic cleaning bath, or similar device, into which tubes containing bits of stained material and saline had been placed.

Destruction of the fabric support has been carried out both mechanically and chemically. Teasing apart the threads in the fabric is the simplest technique, and is nearly always done after soaking. It can be done after staining of the material too, which may make the spermatozoa easier to visualize. Mezger (1857) used this technique after soaking the stains in ammonia water. Hamlin (1883) thought that this was the best method with thinner, more transparent fabric materials which could then be examined directly under the microscope. Hamlin used no biological dyes. Mueller (1926) examined material which had been soaked in pepsin and HCl, and the fabric threads then teased apart. Bohné and Dieckmann (1956) recommended a somewhat similar procedure using an extracting solution which was 0.01% in pancreatin followed by centrifugation. Optimal extraction time was 24 hours at 37° and the cells were not damaged by the procedure. Mezger (1857) recommended examining the center of the stain, in which it was expected to find the highest density of cells. Others have agreed with this view (Longuet, 1876; Mueller, 1926; Liethoff and Leithoff, 1965b). The supporting fabric may be completely destroyed using chemicals. Sulfuric acid has been commonly employed to destroy all the organic material except the sperm cells, which are relatively resistant. The sulfuric acid technique was first introduced by Vogel in 1882, and recommended by Grigorjew (1902) as well. Greene and Burd (1946) suggested a cuprammonium reagent for the destruction of cellulosic substrata, such as cotton. The reagent was prepared by precipitating CuOH from $CuSO_4$ with NH_4OH, filtering, washing the filtrate with water, and redissolving the material in NH_4OH. It was suggested that the reagent be freshly prepared. Kirk (1953) agreed that this was a good method in some cases.

Some workers have examined stains for spermatozoa *in situ* without destruction of the fabric or supporting material. Bayard (1839) used this technique with thin materials, but in most cases preferred filtration. The nature of the fabric, its thickness, texture, transparency, etc., must all be taken into account in deciding upon the applicability of such methods. Usually, biological stains have been employed in conjunction with these methods, as indeed they have been with the other methods. Hamlin (1883) was an exception, carrying out microscopical examinations without staining. Gabbi (1914) described a technique in which a thin layer of gum acacia may be applied to the stain on cloth or any other substratum, and then transferred to a slide. Cells are transferred with the gum acacia, and the slide may then be stained with Baecchi's acid fuchsin and methylene blue (see below). De Bernardi (1959) describes a technique for transferring spermatozoa from substrate to microscope slides utilizing "Scotch" cellophane tape.

A large variety of biological stains have been recommended for sperm cells, sometimes with prior fixation, and sometimes not. Roussin (1867) used iodine-KI staining to render sperm cells from seminal stains more visible under the microscope. Florence (1896) said that he thought Roussin was probably the first to use a stain for the visualization of spermatozoa. The stains employed appear to be largely a matter of personal preference. Some dyes and stains and their properties are given in Table 5.3. Longuet (1876) used an ammoniacal carmine solution for sperm cell staining. Carmine is a very old biological stain, its use dating to 1770 (Lillie, 1969). It is only slightly soluble in water and is used as an acid (e.g. aceto-carmine), or alkaline aqueous solution, or as an alcoholic solution. The active principle is carminic acid, an anthroquinone derivative. Weigert (1887) used carmine as a Gentian violet counter-stain for bacteria. Best (1906) noted that carmine in potassium carbonate solution stained glycogen, a fact which could be useful in examining vaginal swab smears for sperm cells. Baecchi (1909) originated a sperm cell staining mixture of methylene blue and acid fuchsin which was recommended by Strassman (1921). Tsunenari *et al.* (1971) said that methyl blue could be substituted for methylene blue in Baecchi's stain. The heads of the sperm stain red, while the tails and midpieces are blue. Methyl blue is a triaminotriphenylmethane derivative, structurally related to the fuchsin dyes, but not very similar structurally to methylene blue. Corin and Stockis (1908) used erythrosin in ammonia solution for staining cells after fixation. Fixation and staining could be carried out in one step using an erythrosin solution made up in ammoniacal potassium dichromate and sodium sulfate. Tsunenari *et al.* (1971) got good results with Corin and Stockis' method, as did Ponsold (1957). De Rechter (1914) noted that ammoniacal erythrosin was not very stable; he prepared the erythrosin solution in methanol, diluting it 1:1 with ammonia just prior to use. Erythrosin is a fluorescein dye, and is very closely related to eosin structurally, the only difference being that the four Br atoms of the latter are replaced by I atoms in the former (see Fig. 5.4). Florence (1896) noted that eosin was introduced by Prof. Renaut in Lyon in the 1870's, although its introduction was apparently attributed to Schmitter in 1883 by some writers. De Domincis (1909)

recommended the use of 0.01 g eosin in 6 mℓ ammonia for staining spermatozoa in smears. Nickolls (1956) said that hematoxylin-eosin stain worked well. Casarett (1953) recommended a one-solution stain for smears, consisting of 2 volumes 5% aniline blue, 1 volume 5% eosin B and 1 volume 1% phenol. Eosin B is a close relative of eosin, except that two of the Br atoms are replaced by $-NO_2$ groups. "Aniline blue" is apparently a mixture of methyl blue and water blue I (Lillie, 1969).

Mueller (1926) used May-Grünwald or iron-hematoxylin staining, following the pepsin-HCl soaking treatment of the fabric. May-Grünwald stain is equivalent to Jenner's stain. Alum hematoxylin, with eosin as counterstain, was suggested by Rentoul and Smith (1973). Raitzin (1928) used Giemsa stain after alcohol fixation of the smear. Holbert (1936) used gentian violet with a rose bengal counterstain. Paulsen and Varnek (1953) used a "carbol-gentian violet", or gentian violet in a phenol-glycerin solution. "Gentian violet" is a mixture like "methyl violet", and it is best to substitute crystal violet (see Fig. 6.5), a pure compound, in techniques calling for these stains (Lillie, 1969). Macaggi (1925) used an ammoniacal extraction solution, followed by a crystal violet-tannic acid staining mixture. The tannic acid is said to have a protective effect on the cells. Hankin (1904) placed seminal stain in a boiling solution containing 0.5% tannic acid and 1:1000 dil H_2SO_4. He then treated the stain with dilute ammonia and 2% KCN prior to staining with gentian violet. Williams (1937b) employed a seldom-used biological stain called wool black, with a methylene blue counterstain for spermatozoa. Heads stained a golden yellow, with tails and background remaining a gray color. It is not clear which of the several "wool black" dyes (Gurr, 1960) was actually used. Baima-Bollogne (1968) said that he had been able to identify cells in seminal material that had seeped into wood by means of the Feulgen reaction using basic fuchsin. The Feulgen technique stains nuclei, and could be used for stains on fabrics as well. Döllner (1913) used so-called Ruthenium Red stain for seminal spots. This staining solution is prepared from $RuCl_3$ dissolved in ammonia. Greene and Burd (1946) used a safranin stain, which could have been Safranin O, a mixture of dimethyl- and trimethylphenosafranins, or Methylene Violet. Rentoul and Smith (1973) noted that Papanicoleau stain could be used for examining smears. The staining procedure is somewhat involved, but the solutions required (Clark, 1973) are commercially available. Oppitz (1969) described a staining procedure using Nuclear Fast Red (calcium red), or "kernechtrot", with indigo carmine in saturated picric acid as counterstain. Material is eluted in saline and collected by centrifugation, or scraped off and saline-moistened. Heads are stained red, midpieces, pink to green, and tails green with this procedure. Stone (1972) described the procedure in detail, and provided a translation of some of Oppitz's original observations. A number of the biological stains mentioned in this section were discussed in section 5.3.

The material in section 10.2.1 was comprehensively and excellently reviewed by Pollack in 1943. The older literature was reviewed by Florence (1895 and 1896) and by Lecha-Marzo in 1907 and again in 1918. A complete review of the pre-1943 literature did not, therefore, seem warranted, and the discussion has been an attempt to cover major points. Pollack (1943) carried out a large series of experiments on the methods as well. He recommended moistening stains with water or alcohol, and teasing the fabric into fine fibers. Staining was carried out with erythrosin and iron hematoxylin, or with Giemsa stain. Alternatively, the material could be placed into concentrated H_2SO_4 at 50° and checked every hour for chemical destruction of the substratum. Still a third technique involved boiling a portion of the stain in water, and passing the material through a fixation process before embedding in paraffin, and sections being cut, stained and examined. Hankin (1904) had used boiling water as a fixation device for seminal stains. Gültingen (1961) compared the recovery of spermatozoa from seminal stains using the methods of Corin and Stockis (1908), Bohné and Dieckmann (1956) and the sulfuric acid technique of Pollack (1943). Best results were obtained with Pollack's method, relatively good results with Corin and Stockis' method, cells being recovered from an 11 year old seminal stain. Bohné and Dieckmann's method was not very satisfactory, failing to yield cells in a number of stains from 1 month up to 11 years old. Pollack noted especially the tendency of cells to adhere to the fine fibers of fabric. Failure to find large numbers of cells in a normal stain was explained by the frequent failure of the method used for separating them from the substratum to actually do so. Kirk (1953) made the same point. Walther (1967) studied the effect of washing of seminal stains on the subsequent detection by sperm cell identification and acid phosphatase methods. Even after an hour of washing in 20° detergent solution, sperm cells, or at least heads, could still sometimes be found after a careful search. Janssen and Kiesling (1967) noted that motile sperm survive only a short time in seminal stains. Only about 10% were motile after 5 mins. and all were non-motile in 50 min. There was some individual variation as well.

It may be mentioned that more sophisticated types of microscopy have been recommended in looking for spermatozoa. Mueller *et al.* (1966) got good results with phase contrast microscopy, as did Dérobert *et al.* (1966) with fluorescence microscopy using specific fluorescent staining agents. Cortner and Boudreau (1978) recently discussed the value of phase contrast and differential interference contrast microscopy in searching for sperm cells, and the situations in which one or the other of these might prove advantageous.

10.2.2 Survival of spermatozoa in the vagina

The question of the length of time that sperm cells survive in the vagina following ejaculation can sometimes be of importance. Demonstration of the presence of sperm cells in the vagina may not be adequate evidence in itself in a sexual assault case, if sufficient time has elapsed between sexual contact and examination to leave open the possibility that the sperm cells found are unrelated to the alleged assault.

The information available has come primarily from the fertility-sterility literature, and from data collected by medical examiners in cases or in controlled experiments. A number of factors appear to be involved in determining survival time. The number of sperm present in the ejaculate may have an effect. Shorter survival times have been reported if the initial count is low. There is some dependence on the cycle stage at which coitus occurs, and there is quite a bit of individual variation as well.

Menger (1887) mentioned a case in which sperm cells were recovered from a child victim 14 days after sexual contact had occurred. In living, sexually mature women, time estimates are generally considerably shorter. The manner of collecting the sample swab may make some difference to the conclusions, for it is known that sperm survive longer in the cervix than in the vagina *per se*. Sharpe (1963) said that motile sperm persist for 30 min and up to 6 hrs in the vagina, but may persist in the cervix from 7 hrs to over 5 days. Non-motile sperm may be found in the vagina from 7–12 hrs after coitus, much less often up to 24 hrs, and exceptionally as long as 3 to 4 days later. In the cervix, however nonmotile sperm may be found after 17 days. Sperm cells could be recovered in anal swabs up to 24 hrs following an episode of sodomy. Enos and Beyer (1977) said that rectal swabs could be sperm positive up to 20 hrs after attack. They also noted that in cases where oral sex has been reported by the victim, oral swabs must be examined. Sperm could be identified by Papanicolaou staining in oral swabs up to 6 hrs after the incident, and could survive the use of a toothbrush, mouthwash or the ingestion of various liquids. In 1978, Enos and Beyer said that the presence of spermatozoan heads in the rectum or anal area should be interpreted with great caution. They found heads on rectal or anal swabs in some cases where no history of anal sex was reported, and they thought that the findings were due to contamination from the vagina. Sperm identification in vaginal swabs was normally not a problem for up to 3 days following coitus. Nicholson (1965) studied 85 patients, and said that sperm survived in the cervix up to 8 days, and that motile sperm could be found sometimes even after six days. Rupp (1969) noted that studies on a series of 84 rape case samples indicated that there was an approximately equal chance of finding motile and nonmotile sperm within 8 hrs of coitus, and that nonmotile sperm were found in vaginal aspirates for up to 14 hrs after intercourse. Morrison (1972) thought that sperm cells survived longer following coitus in the first 14 days after menstruation. In 104 subjects studied, the ability to find sperm dropped markedly after 48 hours postcoitus. Sperm could be found in the vagina up to 9 days after intercourse which had occurred on the 5th postmenstrual day. In one case, sperm was found on a cervical smear 12 days after coitus which had taken place on the 8th day after menstruation. This case was exceptional, no other cervical smears being positive after 10 days following intercourse. In women who are pregnant, sperm could survive up to 7 days in the vagina. Morrison noted that, in rare instances, sperm could be found on cervical smears taken after the end of a menstrual period where coitus had occurred during menstruation. Georgiades and Schneider (1972) reported that motile sperm could be recovered from the cervical mucus up to about 8 days postcoitus, and that nonmotile sperm persisted for up to about 10½ days. Davies and Wilson (1974) studied the persistence of spermatozoa and of other seminal fluid constituents in the vagina following single coitus. Cells could ordinarily be identified on swabs for up to 3 days, less often up to 6 days. However, swabs with no cells could be found in some cases at 28 hours after coitus. Wallace-Haagens *et al.* (1975) studied the numbers and motility of sperm in 22 subjects throughout a complete menstrual cycle. Motility declined rapidly at about 12 hours postcoitus, and only 6% of the samples showed spermatozoa 48 hrs after intercourse. In most samples, the number of sperm was very small in the washings as compared with the number in a single ejaculate. Brown (1977) reported that studies on 22 subjects indicated that motile sperm may survive up to 9 hrs, while sperm could be found up to 72 hrs after intercourse. Breen *et al.* (1972) said that motile sperm survive up to 28 hrs in the vagina, while nonmotile sperm may be found up to 48 hours postcoitus. Evrard (1971) said that motile sperm could survive up to 96 hrs in the vagina. Duenhoelter *et al.* (1978) reported their findings in 288 cases of women examined because of alleged sexual assault. Almost all of them had been examined within 24 hours of the attack. Motile sperm were found in about a third of the cases seen within 6 hours; nonmotile sperm were identified in a larger percentage. The value for motile sperm decreased significantly for cases examined from 7 to 24 hours after the incident. Dahlke *et al.* (1977) reported results on 500 patients who had been examined in connection with alleged sexual assault. Semen was identified by the finding of sperm in about 60% of the cases. The longest known interval between the attack and the examination in a case where sperm was found was 48 hours.

Sperm apparently survive longer in the vaginas of women who are dead. Wilson (1974) reported a case in which sperm were recovered from a rape-murder victim 16 days after the incident. Although the environmental temperature was low for most of this time, it was not always below freezing (the body was outside, in a mountainous area). The conditions to which the body is subjected undoubtedly play a major role in determining sperm survival. Willott (1975) mentioned that sperm had been found in one of the Christie victims after she had been dead between 3 and 4 months.

These considerations have implications for the doctors who carry out the actual examinations of the victims. In most cases, the person carrying out the physical examination and the person examining the evidence collected are different, the latter having not much control over, and sometimes not much knowledge of, the actions of the former. While this situation is not the most desirable from a medico-legal standpoint, it persists in many places as a matter of practicality and/or lack of communication.

Much of what can be concluded from the detailed examination of the evidence is determined not only by what is

found, but by the manner in which it was collected. A number of authorities have addressed this question, recommending procedures that should be followed (Enos *et al.*, 1972; Vitullo, 1974; Paul, 1975; Enos and Beyer, 1975). Paul (1977) described special procedures that should be employed in the case of children who are victims of sexual assault. Paul (1975) made the point that contamination of the samples during the physical examination is entirely possible, and renders the evidence useless. An example would be accidental contamination of a vaginal swab with sperm traces from the perineum. Since there are differences in the time of survival of spermatozoa in the vagina as against the cervix, the manner in which the swab is taken, or washings collected, may make a difference to the interpretation of the findings. Pollack (1943) said that only findings from the vagina should be used to try to assess elapsed time since last coitus. He said that, although there may be occasional exceptions, one may expect to find sperm from about 30 min to 24 hours after intercourse in most situations. Most authorities have agreed that it would be desirable to have physicians familiar with medico-legal practice carry out the physical examinations of victims, but it is recognized that this is seldom practical.

Some authorities have noted that the finding of isolated parts of the sperm cell may suffice for seminal stain diagnosis, especially the finding of heads (Willott, 1975), but Hektoen and McNally (1923) and Pollack (1943) said that no conclusions should be drawn unless intact cells are found. Fibers, certain bacteria and molds could suggest tails, while yeast, certain Monilia or various other cells could suggest heads in a stain extract preparation.

10.2.3 Spermatozoan morphology—medico-legal implications

For a number of years there has been an interest in the relationship between variable spermatozoan morphology and male infertility. The subject was opened by Moench in 1927. Prior to his work, it was fairly generally accepted that male fertility had mainly to do with sperm count, usually expressed as the number of cells per mℓ semen. It was usually said that a sperm count of about $60 \times 10^6/m\ell$ was the minimum for fertility. Moench, however, began to examine the cells themselves in detail, noting that there were quite a number of morphologically variant sperm cell types which could occur (1927a) and that studies of the numbers and kinds of these cells in particular individuals might provide an alternative explanation for some cases of infertility (1927b). In addition to establishing profiles for variant morphological types, measurements of sperm cell heads were made on a large number of cells, and this plotted as a function of the numbers of each size observed. The curves thus obtained were called biometric curves, and were fairly characteristic of individuals over the course of time. By 1934 it was clear that the morphological and biometric profiles were fairly characteristic of individuals. At least 300 cells had always to be examined, and up to 500 were often included. Moench (1934a) noted that the characteristic individual patterns were obtainable not only in smears prepared from fresh semen, but also in "reconstituted" semen, i.e. from dried material. He thought that this finding might have particular applicability in rape cases, and published a paper in the medico-legal literature (1934b) suggesting the possibilities. It may also be noted that his initial inclination for taking up the studies, the relationship of aberrant sperm morphology to male infertility, proved to be well founded (Moench, 1944 and 1955; Joel, 1953).

Williams (1937a) published a classification scheme for morphological variants of spermatozoa, and suggested (1937b) that individualization of the sample might be possible in some medico-legal cases. MacLeod took up seminal cytological studies in 1951, in connection with impaired male fertility (MacLeod, 1951; MacLeod and Gold, 1951). He confirmed that the seminal cytological profile exhibited individuality, and that there was a definite relationship between sperm quality and fertility (MacLeod and Gold, 1953). There was also a definite correlation between increased numbers of aberrant morphological types of sperm, and various kinds of stress (MacLeod, 1967). Infections, allergic reactions and varicocele all affect the morphological type of the sperm produced. Psychological and behavioral stress are apparently factors as well, there being fewer morphological variants in seminal samples from prison populations, in which "stress" is minimized by the managed environment, than in comparable samples from the general population. Drugs of the bis-dichloroacetyldiamine family cause increases in the numbers of morphologically aberrant sperm cells, as does an elevation of 17-ketosteroids (MacLeod, 1962). Hartmann *et al.* (1964) said that they believed that the degree of individualization which could be achieved by studies of sperm cell morphology was of the same order as that of fingerprints.

It should be noted that there is, as yet, no general agreement on exactly what constitutes a morphologically aberrant sperm cell, nor on a classification scheme for structural variants. In some cases, of course, the variance and interpretation are obvious, such as in the case of two-headed cells, but in other cases, the differences are considerably more subtle. The role of the observer is very critical in that different observers may well obtain different "profiles" after examination of the same specimen. MacLeod and Gold recognized this problem in 1951, and noted the importance of having the same observer carry out all examinations in which results were to be internally compared. MacLeod reiterated this view in 1967. Freund (1967) underscored the point by conducting a "blind study" wherein photomicrographs of 500 cells were sent to many laboratories engaged in this sort of work for determination of normal vs aberrant cell numbers, and for classification of the variants. There were enormous discrepancies in the results from various observers. Pollack (1943), who presented a detailed classification scheme for the cell types within an ejaculate, noted that material recovered from the female reproductive tract was not suitable for profile determination or biometry. Contamination of the sample with vaginal and/or cervical

material and cells and alterations of the sperm cells by the vaginal environment might give a completely different picture than that which would be obtained from a smear made from a semen sample. There is also the possibility that more than one individual's semen is present. Fredericsson *et al.* (1977) mentioned that in freshly obtained semen samples, a better profile can be obtained through the use of supravital staining with eosin Y in phosphate buffer at pH 7.4. Living cells may be distinguished in this way from nonliving ones, and information about each population of cells separately determined. Fraas and Soldo (1977) were involved in a medico-legal case involving aberrant sperm morphology. Dr. MacLeod examined a sample from an alleged rapist, and compared it with the pattern on the vaginal smear recovered from the victim at the time of the incident. Because there were only about 10 intact cells on the smear slide, however, the court found that no reasonable conclusions could be drawn as to exclusion of the defendant.

Fredericsson and Björk (1977) examined the morphology of spermatozoa in postcoital samples (10 hrs) from the vagina and from the cervix. Vaginal samples exhibited a morphological profile comparable to that of the deposited semen, but the cervical samples showed a much lower fraction of aberrant types of cells. They suggested the existence of a kind of "cervical barrier" to the morphologically aberrant types, especially those with abnormal heads.

10.3 Seminal (Prostatic) Acid Phosphatase and Vaginal Acid Phosphatase

10.3.1 Introduction

It has been known for a very long time that semen may lack spermatozoa. There are many different reasons for this circumstance, including congenital defects, pathologies, and vasectomy. Examination of physical evidence in sexual assault cases is more difficult if the identification of semen lacking sperm cells is required. For many years, forensic scientists have been concerned about this problem, and a number of methods have been offered as solutions to it. Most of the remainder of Section 10 is taken up with discussions of these techniques.

Pollack (1948) drew a distinction between "aspermia" and "azoospermia". The former condition is characterized by a total lack of testicular elements in the ejaculate, only accessory gland secretions being present. In the latter, spermatozoa are absent, but early cells in the spermatogenesis series are usually present. The ejaculate from a person suffering from aspermia, Pollack said, should not be called "semen". Eliasson (1975) gave virtually equivalent definitions for the terms. These distinctions are important in evaluating fertility, but probably matter little in medico-legal examinations in cases of sexual assault. That said, the term "azoospermic semen" will be used in all subsequent discussions to refer to any seminal sample in which mature spermatozoa are not present.

10.3.2 Seminal acid phosphatase detection for medico-legal identification of semen

The "acid phosphatase" test, as it is usually called, is one of the best known and most widely employed techniques for semen identification, apart from sperm cell identification itself. It is based, in its many variations, on the presence in human semen of high levels of a non-specific phosphohydrolase with acid pH optimum. This acid phosphatase (EC 3.1.3.2) is of prostatic origin, and is sometimes abbreviated in what follows as "AP".

In 1935, Kutscher and Wohlbergs reported that male ejaculate contained an enzyme which hydrolyzed various phosphate esters at an acid pH optimum. Kutscher's earlier (1935) studies on phosphatases in urine had prompted the investigation. The seminal enzyme hydrolyzed phenylphosphate better than α-glycerophosphate, and this better than β-glycerophosphate. It hydrolyzed hexose diphosphates slowly, and pyrophosphate almost not at all. The pH optimum for the hydrolysis of phenylphosphate was 4.65, and the enzyme was inactivated by exposure to 60° for 5 minutes. Its origin was established as being in the prostate gland, and the enzyme was called "prostate phosphatase". Kutscher and Wörner (1936) showed that the enzyme had a fairly broad pH optimum with β-glycerophosphate as substrate, and that it was inhibited by fluoride but not by cysteine. It had no demonstrable Mg^{++} requirement. The prostatic origin of the enzyme was confirmed by Gomori in 1941 using histochemical staining techniques.

In 1936, Gutman *et al.* noticed that acid phosphatase activity was greatly elevated in osteoplastic skeletal metastatic tissue in patients suffering from prostatic cancer. They suggested that the metastasizing prostatic tumor cells retained their ability to synthesize the enzyme described by Kutscher and Wohlbergs (1935). In 1938, Gutman and Gutman went on to show that there was a significant increase in acid phosphatase activity in the serum of 11 of 15 patients with metastasizing prostate carcinoma. Sera of 88 other patients having other diseases did not show the high AP values, except for that of one woman suffering from widespread Paget's disease. They assayed the enzyme in pH 4.9 buffers using phenylphosphate as substrate; a unit of activity was defined as that amount of enzyme which liberated 1 mg phenol from phenylphosphate per hour at 37° at pH 4.9. Levels in excess of 4U/100 mℓ serum were considered to be high. The assay was a slight modification of the classical assay devised by King and Armstrong (1934), except that a King-Armstrong unit was originally defined (for alkaline phosphatase) as that amount of enzyme which liberated 1 mg phenol from phenylphosphate at 37.5° in 30 min at pH 9.1. The Gutmans suggested that the assay of serum AP might be valuable in helping to diagnose prostatic carcinoma, an idea which rapidly gained favor in clinical circles (Cf. Benotti *et al.*, 1946; Walker *et al.*, 1954; Woodard, 1959; Südhof *et al.*, 1964).

In 1945, Lundquist, in Copenhagen, suggested that the extraordinarily large amounts of prostatic acid phosphatase

present in human semen be used as the basis for the identification of semen in medico-legal situations. Studies on this possibility were carried out in Denmark by Hansen (1946), Riisfeldt (1946) and Rasmussen (1945).

Rasmussen (1945) did not cite Lundquist's paper, and the publication of his paper seems in fact to have preceded Lundquist's by a matter of months. It would be correct, therefore, to credit the origin of the medico-legal utilization of seminal AP as an identification technique to both of these investigators. Rasmussen carried out quantitative determinations of AP in semen, saliva, urine and vaginal secretions in stains, using units of activity per mg dry weight as a basis of comparison. A unit of activity was that amount of enzyme which liberated 1 mg phenol from phenylphosphate per hour at 37° and pH 5.9. The lowest values observed in seminal material exceeded by nearly 200 times the highest values seen in other materials. Decline of AP activity in stains at ordinary temperatures was fairly rapid at first, i.e., in the first few days, but levelled off thereafter. Different individuals had different levels of seminal AP and these remained relatively constant in the absence of disease, infection, etc. The enzyme activity in a stain was reduced by about 50% by exposure to 74° for an hour, indicating a considerably higher heat stability than had been noted earlier by Kutscher and Wohlbergs (1935) (see above). Rasmussen recommended the test as a useful means of identifying seminal stains in legal medicine, especially azoospermic samples.

Hansen (1946) looked at the AP activity in a substantial number of seminal samples, and of samples of other bodily secretions. Measurements were done in liquids, where units of activity could readily be related to volume, as well as in stains. In the stains, the units of activity were related to the area occupied by the stain in order to be able to compare different materials. Knowledge of the volume of liquid material that might be maximally absorbed on any given substratum area allowed him to relate the units of enzyme activity per volume to units of activity per area, referred to as "voluminal potency" and "areal potency", respectively. Different fractions of urine, including urine collected after ejaculation, gonorrheal discharge from men, vaginal and cervical secretions, gonorrheal discharge and urine from women, feces, blood, saliva, bile and pancreatic juice were all tested for activity. Plant materials were treated as well. Seminal fluid invariably contained higher concentrations of the enzyme than the other materials. Hansen employed the same units of activity as had Rasmussen (1945) (see above), and said that AP activities in excess of 2 units/cm^2 stain were definitely diagnostic for seminal stains. A kind of "qualitative" test was arranged, in which the sensitivity of the assay was simply adjusted such that only seminal materials (i.e. materials with activities of 2–4 units/cm^2 stain) would give positive reactions. It was noted that only human and monkey prostatic secretions contained large AP concentrations, and that this test could therefore be of value in discriminating animal semen should the need arise. The obvious value of the test lay in the identification of azoospermic semen. Hansen noted that the first part of the ejaculate was particularly rich in prostatic AP. This fraction contained minimal spermatozoa. Later fractions are richer in cells and have less AP. In medico-legal exhibits, no cases were found in which sperm was present, but AP was negative. Hansen felt that the test was specific for semen, but nevertheless recommended that it be used in conjunction with, and not as a replacement for, the search for an identification of sperm cells.

The third extensive study was carried out by Dr. Ove Riisfeldt in 1946. High values of acid phosphatase activity were found in all ejaculates except those from prostatectomized subjects. Most samples contained 1500 to 3500 units of activity/mℓ semen, the lowest value noted being 400 units/mℓ. A unit of activity in these studies was defined as that amount of enzyme which liberated 1 mg phenol from phenylphosphate per hour at 37° and pH 4.9, and the enzyme concentration was expressed as "per mℓ semen". Vaginal secretions and cervical mucus from normal women and patients with gonorrhea and salpingitis, urine from men, women and children, serum, feces, male gonorrheal discharge, milk, saliva, gastric juice, tears, sweat, pus and a number of beverages, including coffee, tea, cocoa, beer, wine and distilled spirits were all tested for AP activity. None contained the enzyme at levels higher than 10 units/mℓ, well below the minimum value of 400 observed with seminal fluid specimens. Heating the sample to 60° for 5 min abolished enzyme activity, as did exposure to 37° for 14 days. A sample kept at room temperature for a month showed a 30% reduction in activity. A few chemicals that could conceivably be found as components of vaginal deodorant preparations or spermicidal contraceptives did inhibit the enzyme.

For stains, the assay system was adjusted such that material had to contain at least 20 units of activity to be detected. This device served to exclude all the non-seminal substances tested in the study which could have given a "false positive" result. Riisfeldt was convinced that the method was specific for semen, and believed that when it yielded positive results, one could safely conclude that semen was present. Situations did occur, however, in which the AP test was negative but sperm cells were still found. In the case of a negative AP test, therefore, the search for cells had to be undertaken. If no enzyme was present, and no spermatozoa found, the conclusion that semen was absent would be warranted.

In 1947, Kaye recommended the acid phosphatase test for seminal stain identification. He employed the Gutman and Gutman (1938) technique, as modified by Benotti *et al.* (1946) with phenylphosphate as substrate. Seminal fluid stains would contain a minimum of 30 King Armstrong units of AP activity, he said, and any stain containing that level of activity or higher could be considered as being of seminal origin. He recommended that a search for sperm cells be conducted as well. In 1949, Kaye reported that a wide variety of substances, including vaginal secretions, urine, serum, blood, menstrual blood, saliva, perspiration, pus, nasal mucus, gastric juice, feces and a number of foods and beverages contained less than 5 King Armstrong units of activity

per mℓ, or per cm^2 of stained cloth. Any value in excess of 25 K-A units/cm^2 stain, he thought, could be considered positive for semen. Stains up to 6 months old gave strongly positive reactions. The only possibility of obtaining an inordinately high value for AP from another body fluid would be in serum from a patient suffering from metastasizing prostatic carcinoma. In 1951, Kaye noted that stains kept at room temperature for up to 3 years still gave a strongly positive AP test, well in excess of 25 K-A units/cm^2 stained cloth. Faulds (1951) said that he regarded 5 K-A units/cm^2 stain as suspect, and that 10 K-A units/cm^2 definitely indicated that the stain was seminal. Fisher (1949) advocated the test, saying that the finding of $\geqq$ 100 units AP/mℓ original fluid volume was a positive test. A unit was that amount of enzyme which liberated 1 mg of phenol from phenylphosphate per hr at 37° at pH 4.9, and the area of a stain could be related to the original volume of fluid by an empirically determined factor.

In 1950, Lundquist reviewed the experience of the University Institute of Legal Medicine in Copenhagen with the AP test. More than 2000 stains from 346 cases were examined over a period of several years. Careful examinations for spermatozoa were conducted in many of the cases, in addition to the acid phosphatase test. It was found that the AP test could be negative even when sperm were present. Similarly, the test was sometimes positive in the absence of sperm cells. The test, therefore, has no useful negative value, but Lunquist said that appropriately controlled positive AP tests leave no doubt about the presence of semen, even if no sperm cells be found.

It will be noted that phenylphosphate was used as substrate in the phosphatase assays in many of the above studies. The liberated phenol was then determined colorimetrically by means of phenol reagent (Folin-Ciocalteu reagent) (Folin and Ciocalteu, 1927). In 1948 and 1949, a somewhat different assay, or histochemical demonstration technique, for phosphatases was introduced (Manheimer and Seligman, 1948; Seligman and Manheimer, 1949). The principle of the method lay in the use of naphthylphosphate substrates, and subsequent coupling of the naphthol liberated in the reaction with a diazonium compound to form an insoluble colored product (see in Table 5.3). The original method employed a freshly prepared α-naphthyldiazonium chloride reagent, but it was soon found that a stabilized α-naphthyldiazonium derivative could be prepared, which was stable for months, and which reacted readily with β-naphthol to form a purple-red insoluble product (Fig. 10.1). The stabilized diazonium compound in Fig. 10.1 is also known as Fast Garnet B. Similarly, a stabilized diazonium compound was described which reacted with the α-isomer (Fig. 10.2). The stabilized diazonium compound in Fig. 10.2 was commercially available as Naphthanil Diazo Red AL, and diazonium compounds of o-dianisidine and 2-amino-4-chloroanisole were commercially available as Naphthanil Diazo Blue B and Naphthanil Diazo Red RC, respectively (See Fig. 10.3 and 10.4). There are a variety of other stabilized diazonium coupling salts available as well.

In 1950, Walker applied this technique to medico-legal identification of seminal stains. The test could be performed directly on fabric, or on filter paper to which stained material had been transferred. Blood was also colored by the reagents, but the color was very different, and the characteristic seminal AP color soon replaced the blood color in blood-semen stains. Walker noted further that the test reagents did not stain spermatozoa, and in no way interfered with their detection using a carbolfuchsin dye. The phosphatase activity of stains was observed to be concentrated more in the periphery, whereas the sperm cells tend to concentrate more in the center of the stain (see Section 10.1.1). This fact was quite clearly established by Leithoff and Leithoff (1965b). The use of α-naphthylphosphate substrate and a coupling diazonium salt for the acid phosphatase was soon adopted in the medico-legal AP tests for seminal stains and semen. Berg (1955) recommended this method, incorporating aqueous lauryl sulfonate into the reagent. He said (1957) that only the blue-purple color characteristic of seminal AP should be used to judge the result, and that he had always obtained the test when spermatozoa were found, but never in their absence. Several minor modifications of Berg's method appeared (Schiff, 1969; Ponsold, 1957), and Kempe (1958) said that he found no false positives with urine or with vaginal smears from pregnant or non-pregnant women, provided only the deep blue color was used to judge whether the reaction was positive. Kind (1958) described stable acid phosphatase test papers based on the azo dye coupling principle. Hazen (1955) used an inorganic phosphate assay to estimate AP activity with β-glycerophosphate as substrate, and said that values in excess of 18μg P_i released/mℓ extract could be regarded as being of seminal stain origin. Boltz and Ploberger (1956) recommended a technique using the complex salt of phenolphthalein diphosphate with pyridine. The method was recommended for darkly colored fabrics or fabrics whose weave did not readily allow a direct test. The stained material was pressed with moistened filter paper, and the filter paper then tested. Gültingen (1961) got good results with that technique in relatively fresh stains, but the test was sometimes negative with older stains whereas Berg's (1954) method with Ca α-naphthyl phosphate as substrate gave almost uniformly positive results. Phenolphthalein diphosphate was first proposed as a substrate for AP by Huggins and Talalay (1945) (see Section 10.2.4).

Not all authorities have agreed that a positive acid phosphatase test alone, in the absence of other evidence, should be taken as conclusive proof of the presence of semen. Hauck and Leithoff (1959) reviewed this subject at length, concluding that an AP test alone should not be considered conclusive for semen. They showed that a number of materials of plant origin gave high AP values. The value of the test could be improved not only by carrying out the test quantitatively, but at several different pH as well, and comparing the pH-activity curves of unknown materials with those of semen and various potentially interfering substances. The search for sperm cells should be conducted, they said, regardless of whether the test gives positive or negative results. Kind

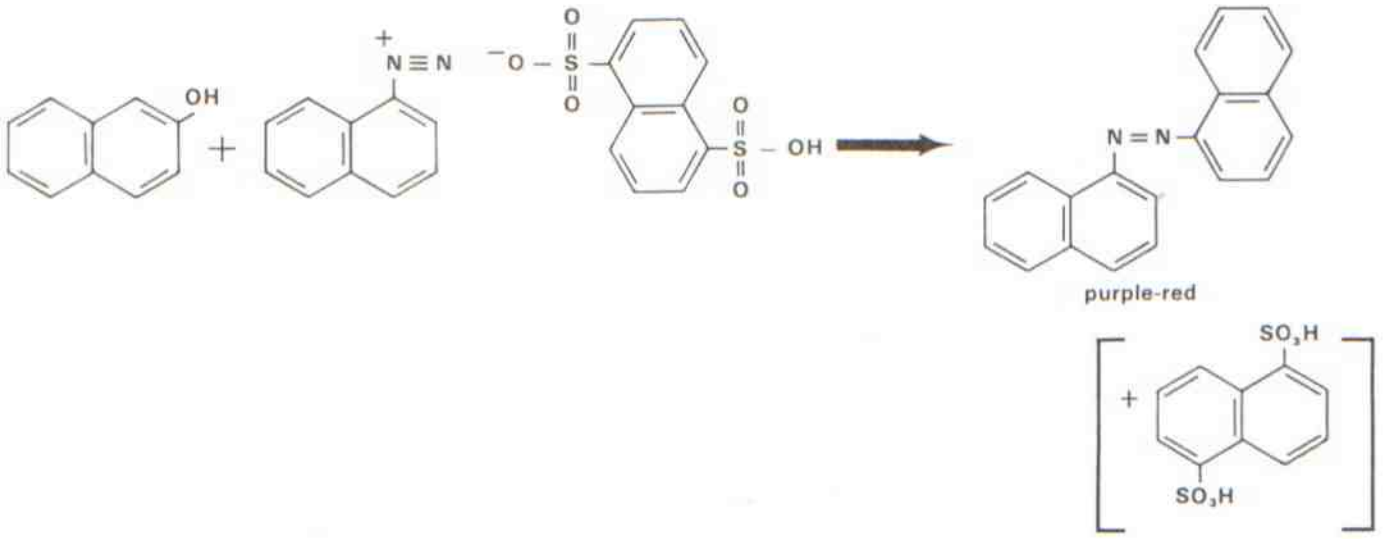

Figure 10.1 Reaction of β-Naphthol with Stabilized Diazonium Compound (Fast Garnet B)

(1964) reviewed the acid phosphatase test as well, and agreed in part with Hauck and Leithoff. Some of the objections to the test employing azo dye coupling technique were based on the fact that a number of amines and phenolic compounds would react with the azo dye to yield colored products. Not too many compounds, however, will react at pH 5, and fewer still would yield exactly the same color as α-naphthol. In any case, such interference can readily be diagnosed by applying the azo dye reagent separately from the phosphatase substrate reagent (Kind, 1964; Brackett, 1957). Formation of colored products in the absence of phosphatase substrate is an immediate indication of contamination by a compound capable of reacting with the azo dye. Such a two-step procedure is quite analogous to the use of a two-step procedure in the catalytic tests for blood, in which application of the reagent in the absence of H_2O_2 detects interfering oxidizing agents (see section 6). There are materials of plant origin, however, such as cauliflower juice and, as Kind established, extracts of gorse (*Ulex europaeus*) seeds, which have relatively high AP levels. Kind (1964) described the application of the phosphatase assay using p-nitrophenylphosphate as substrate, first reported by Ohmori (1937). In this technique, the p-nitrophenylate anion, which is resonance-stabilized in basic solution, is determined by its absorption at 400 nm. Kind also discussed the AP test reaction as a searching tool, for locating seminal stain on garments and surfaces, and Konzak (1977) recommended this technique as a useful one.

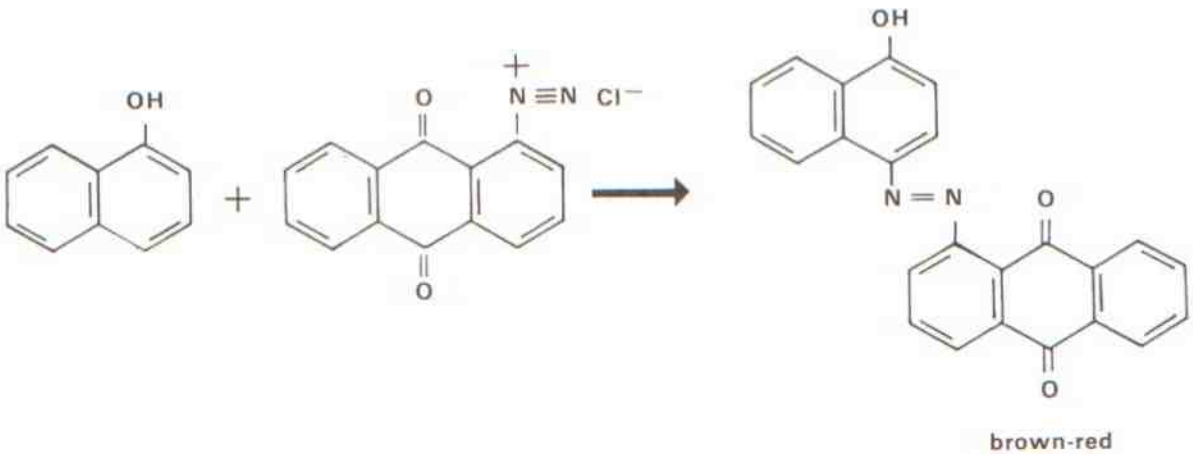

Figure 10.2 Reaction of α-Naphthol with Stabilized Diazonium Compound (Fast Red AL)

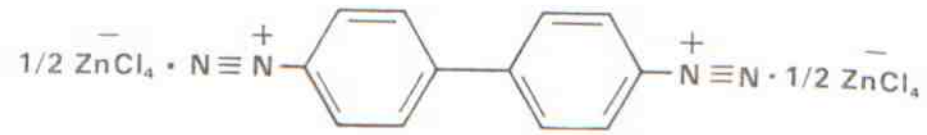

Figure 10.3 $ZnCl_2$ Double Salt of the Tetrazonium Chloride of Diazo Blue B (Fast Blue B; Dianisidine Blue)

A number of authorities have advocated quantitative AP assays for medico-legal determinations of semen and seminal stains, insisting that it is the large amount of AP and not its mere presence that characterizes seminal plasma (Rasmussen, 1945; Hansen, 1946; Riisfeldt, 1946; Hauck and Leithoff, 1959; Kind, 1964; Nakamura *et al.*, 1959; Kaye, 1947; Hazen, 1955; Walther and Höhn, 1971; Gomez *et al.*, 1975; Davis and Gomez, 1975). A number of investigators who have employed quantitative assays have recommended "cut-off" values, in units per volume, or area; samples which contained AP values in excess of these values, they thought, could definitely be regarded as being of seminal origin. In many cases, these values were empirically established by measuring the AP content of a large number of substances, and choosing values above those observed for substances which were non-seminal. Some of the recommendations are given in Table 10.1.

Walther and Höhn (1971) measured quantities of AP in some plant materials, with o-carboxyphosphate as substrate, which were as high as in some samples of semen. The AP enzymes could be differentiated by disc polyacrylamide gel electrophoresis, and this procedure would be necessary to eliminate the possibility of adventitious AP (Walther, 1971). Pinto (1959) had suggested that a control sample, heated to 145° for 30 min could be incorporated in answer to this problem, at least for the bacterial phosphatases, since the prostatic enzyme is quite heat-labile while the bacterial enzymes are not. Kind (1964) said that he would not report the presence of semen in a stain based on an acid phosphatase alone; corroboration of identification by finding sperm cells, or a positive Florence test would be required. Pinto (1959) said that he would report a high AP value even in the absence of spermatozoa or corroboration, with a suitable explanation, and let the prosecutorial or judicial authority judge its merit. Schiff (1978) disagreed with this idea. He thought that the expert witness should give an opinion on a scientific measurement which he thought would be too complex for nonscientists to be able to evaluate properly. He reviewed the AP test, and was convinced of its reliability as an indicator of the presence of semen, even in the absence of sperm. He favored a qualitative test in the hands of an experienced worker. If a quantitative test is used, he said, more time is required and an arbitrary "cut off" point must be chosen. He said that his experience with the test over many years had indicated that it was a reliable one for azoospermic semen.

Brown and Brown (1974) tested a large number of douche preparations, creams and foams used to treat vaginal infections, contraceptive creams and foams, vaginal deodorant sprays and foams, diaphragm lubricants, a number of detergents and several miscellaneous materials for positive AP reactions using two commercially available test kits, one by American Monitor and Warner-Chilcott's Phosphatabs-Acid. They could show that four products, Triva douche powder, V.A. douche powder, Langeen vaginal jelly (spermicidal) and Acinjel Therapeutic Vaginal Cream, gave positive reactions with the Warner-Chilcott Phosphatabs-Acid kit. Quantitative retesting could eliminate the false positive reactions but some problems were encountered (precipitation). The American Monitor kit reacted only with dilute Clorox solution, but not with fabric soaked in dilute Clorox

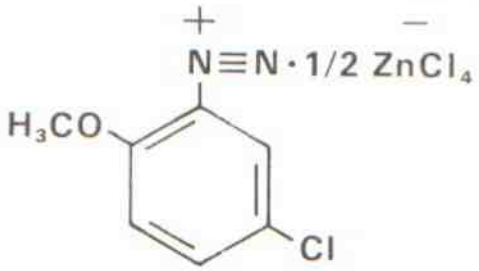

Figure 10.4 $ZnCl_2$ Double Salt of Diazo Red RC (Fast Red RC)

Table 10.1 Recommended Concentrations of Acid Phosphatase to be Observed for the Diagnosis of Semen

Acid Phosphatase Concentration	Units Used ★	Reference
2 units/cm²	King-Armstrong	Hansen, 1946
20 units/mℓ original material	King-Armstrong	Riisfeldt, 1946
30 units/cm²	King-Armstrong	Kaye, 1947
25 units/cm²	King-Armstrong	Kaye, 1949
20 units/mℓ original material	King-Armstrong	Fisher, 1949
10 units/cm²	King-Armstrong	Faulds, 1951
2 units/mg difference in dry weight before & after extraction	—	Gilli & Fallani, 1952
20 units/cm²	—	Perez de Petinto y Alfonzo Martinez, 1953
18 units/mℓ extract	Modified Bodansky	Hazen, 1955
50 units/mℓ vaginal aspirate	King-Armstrong	Breen et al., 1972
300 units/dℓ (swab in 3 mℓ saline)	King-Armstrong	Schumann et al., 1976
300 units/ℓ (swab in 3 mℓ saline)	International	
300 units/ℓ (swab in 2 mℓ saline)	International	Findley, 1977

★ Note that not all units are necessarily identical because of variations in pH, substrate concentration, etc. (See Section 10.2.4)

and allowed to dry. Walther (1967) said that stains which had been washed in detergents and water hotter than 40° no longer showed AP activity.

Owen & Smalldon (1975) raised the issue of the evidentiary meaning of positive AP test findings. This question was discussed in Section 6.8 in connection with catalytic tests for blood. In examining 100 men's jackets and 100 pairs of men's trousers selected at random from a dry cleaner, it was noted that 44 pairs of the trousers showed areas of significant AP activity. 37 of them were sufficiently high to indicate possible prostatic origin. This sort of finding goes to the question of interpreting the evidentiary value of positive results, rather than to any technical considerations involved in actually obtaining them.

It should be noted that negative results can have value in using the AP test as well. In the absence of any acid phosphatase activity, the probability of semen being present may not be very great, and this fact should not be overlooked.

10.3.3 Persistence of acid phosphatase

There are two separate issues to be considered under this heading: (1) the persistence of AP activity in the vagina as a function of elapsed time after semen deposition; and (2) the persistence of the enzyme in a dried seminal stain.

As with sperm cells, there has been an interest in establishing the time of persistence of AP activity in the vagina following coitus, and in relating the residual activity to the elapsed time if possible. Pinto (1959), using the α-naphthylphosphate-Naphthanil Diazo Red AL assay method, constructed a color chart which showed that color intensity gradually decreased as a function of elapsed time since intercourse from 6 to 48 hrs. The chart could be used to obtain an estimate of elapsed time in unknown samples. In a series of 34 specimens, in which the time estimate from the color chart was compared with the elapsed time reported by the examining physician, 15 were in close agreement. In 16 cases, there were moderate discrepancies amounting to a few hours. In 12 of these 16 samples, the color chart overesti-

mated the elapsed time, while in 4 others, it underestimated the time. In 3 of the cases, there were large discrepancies, amounting to a matter of days. In these cases the chart greatly underestimated the elapsed time since coitus. Pinto recognized that this approach provided only a crude estimate in some cases, and said that a quantitative assay correlation with time might be more informative. Rupp (1969) noted that AP activity survived well beyond 24 hrs postcoitus. Davies and Wilson (1974) noted that AP activity may be present up to 3 days following semen deposition, but that the test is most useful on swabs taken within one day, and rarely useful after two days. McCloskey *et al.* (1975) said that all specimens which showed at least 25 K-A units of activity were taken within 48 hrs of intercourse. Samples showing at least 50 K-A units were all collected within 24 hrs. Some samples collected within the first 24 hrs following coitus, however, showed activities of less than 25 K-A units. If 25 K-A units were used as a "cut-off" value, therefore, some of these latter samples would have been called "negative". Brown (1977) reported that AP was detectable on swabs up to 24 hrs following intercourse.

Gomez *et al.* (1975) assayed AP activity in vaginal washings from 41 women, using several different methods. Two of the methods were qualitative, and were based on the azo dye coupling assay with α-naphthylphosphate as substrate. One of these made use of the General Diagnostics Phosphatabs-Acid kit, while the other was a modification of several published assays using α-naphthylphosphate as substrate in citrate buffers at pH 5.6 with tetrazotized o-dianisidine as coupling dye (Fig. 10.3) Two quantitative assays were used as well. One employed α-naphthylphosphate as substrate and diazotized 5-nitro-o-anisidine (Fast Red Salt B), and was described by Babson and Phillips (1966). The other employed thymolphthalein-phosphate as substrate, which generates its own chromophore upon addition of base. Thymophthalein can be determined at 590 nm, and the procedure was initially described by Roy *et al.* (1971). Patients were divided into two groups: those with no history of recent intercourse, and in whose washings no sperm were found, and those with histories of recent coitus and/or in whose washings sperm could be identified. It could be shown that the Phosphatabs quantitative test was more sensitive than the author's modified qualitative test. The highest values measured quantitatively in the first group of patients (no history of recent coitus) overlapped with the lowest values in the second group (recent coitus). For example, one person whose washings were sperm positive, and who was alleged to have been raped 5 hrs previously, showed a lower AP activity than another patient who said she had not had intercourse for 60 hrs. The qualitative test would have been negative in both cases. There was a fairly good correlation between the qualitative and quantitative tests in most cases. There was sufficient endogenous vaginal AP activity and sufficient individual variation in its levels, even in the few people studied here, to suggest that errors would sometimes result using a qualitative test or a quantitative test in which a particular "cut-off" point had been chosen. In many cases, when an enormous amount of enzyme is present, there will be no doubt that vaginal AP can be excluded as a possible source. But it would be necessary to be employing a quantitative test to know that a relatively large amount of enzyme was present.

Enos *et al.* (1963) reported their results in 36 cases of sexual assault. They used so-called Bodansky units for quantitation of the AP activity. A Bodansky unit was originally defined as that amount of enzyme which liberated 1 mg P_i from glycerophosphate per hour at 37° (Bodansky, 1933). In samples taken from 1½ to 5 hrs postcoitus, the activity varied from 8.2 to 78 units. In several control patients, it was noted that the AP activity declined to zero activity within 12 hrs of coitus. Enos and Bayer (1977) correlated the number of Bodansky units found in vaginal material with the elapsed time since intercourse. 100 units corresponded to 1 hr, 30–50 units to 2–3 hrs, 10 unit to 6 hrs, 5 units to 12 hrs and zero units to $\geqq$24 hrs. Rupp (1969) said that AP activity could be detected in vaginal aspirates for periods exceeding 24 hrs after intercourse. Schumann *et al.* (1976) studied the time decay characteristics of AP in a number of subjects, using a manual quantitative assay with phenyl phosphate as substrate and an automated assay with thymolphthalein phosphate as substrate. They were of the opinion that a definite cut-off point could be established for determining positively the presence of semen (see Table 10.1), and they found the AP measure to be more certain than the finding of sperm cells. The decay of activity could be used as a fair measure of time elapsed since coitus. Findley (1977) did very similar studies and came to similar conclusions. His threshold value for interpretation of the presence of semen is slightly lower than that of Schumann *et al.*, however, because of dilution factors (see Table 10.1). Davies (1978) and Allard and Davies (1979) presented their studies on a large number of cases, in which acid phosphatase activity had been determined on vaginal swabs quantitatively using p-nitrophenyl phosphate as substrate. These workers thought that measured levels of AP not very much above 20 Sigma units per swab provided quite convincing evidence that semen was present.

Dahlke *et al.* (1977) and Duenhoelter *et al.* (1978) reported the presence or absence of acid phosphatase in the series of materials they examined. A qualitative test was used by Duenhoelter *et al.* (1978). They reported the surprising result that AP was undetected in a higher percentage of the cases examined within two hours than in those examined in the 3 to 12 hour interval. Dahlke *et al.* (1977) employed a commercially available assay kit, based on the thymolphthalein phosphate method of Roy *et al.* (1971). Their data indicated that values in excess of 50 units per liter vaginal eluate were suggestive of semen. This level was seen in 40% of the study population.

Sensabaugh (1979) carried out a detailed statistical analysis of endogenous and post-coital AP levels using his own data, and a number of sets of published data. Endogenous

AP levels were found to be lognormally distributed. The percentage of postcoital AP values which would fall into the "endogenous" range increased with time after semen deposition. This complex but important paper should be read by those interested in the interpretation of quantitative AP results.

Standeffer and Street (1977) determined the post-mortem stability of AP activity. They said that samples collected at autopsy should be analyzed within 48 hrs, and stored at 4° in the interim. Longer storage was possible if samples were kept at −20°. AP activity could be detected for up to 7 days postmortem in vaginal samples, for up to 36 hrs in the oral cavity, and for up to 24 hrs in the rectum. They noted that temperature could be a major variable in postmortem survival, the enzyme tending to be more stable in a body that was in a colder environment. AP was determined quantitatively in these studies.

The other issue which is of importance here is the survival of AP activity in stains as a function of drying and elapsed time. Faulds (1951) studied this matter in some detail. Stains were prepared and kept at different temperatures for a number of months. Over the course of about 5 months, the stains declined in AP activity by as much as 78% and by as little as 48%. The decline in activity was as great in the stain kept at −14° as in the stain kept at 37°. He also noted that the semen could lose up to 50% of its AP activity simply upon drying. Perez de Petinto y Alfonso Martinez (1953) indicated that semen having about 2,000 units of activity/mℓ showed only about 90 units/cm² in 6 hr old stains. At 3 months of age, the remaining activity was 20 to 25 units/cm². As noted above, Kaye (1951) showed that stains kept at room temperature for 3 years retained AP activity. Schiff (1975) agreed with this estimate. Kerek (1972) indicated that seminal stains kept at room temperature retained activity for at least 14 months, and could still be active after 4½ years if stored at −20°.

10.3.4 Acid phosphatase assay techniques and activity units

It will be clear from the foregoing discussion that a number of different substrates and assay techniques have been employed in studies on phosphatases, and that different authors have made use of many different activity units. Naturally, the enzyme is not expected to exhibit the same affinity for all substrates so the sensitivity of assays using different substrates will be different. Many of the apparent discrepancies in estimates of survival can probably be explained in this way. Moreover, because different units tend to be defined for different substrates, it is often impossible to compare the results of one author with those of another. And even if comparable units have been used, they are not always related to the material being assayed in the same way. For example, one author might relate units of activity to stain area, while another relates the same units to the protein concentration in the stain extract. The relationship between a cm² of stain area and a particular protein concentration will be neither obvious nor comparable. A brief discussion of phosphatase assay methods, and expressions of activity units, has therefore been included here.

The acid phosphatase enzyme, as noted, is nonspecific, i.e., it will hydrolyze a variety of phosphate esters. Substrates that have been used for AP determinations include α- and β-glycerophosphate, phenylphosphate, p-nitro-phenylphosphate, α- and β-naphthylphosphates, phenolphthaleinphosphate, thymolphthalein phosphate, phosphate esters of 3-hydroxy-2-naphthanilides, flavone-3-diphosphate and 4-methylumbellifrylphosphate. Since the reaction has two products, either one of them could be determined in assessing the progress of the reaction. Substrate disappearance could be used as well, but has not been common. Phosphate determinations have been used by a number of authors, this method being more popular some years ago than it is now. Most methods of P_i determination are based on the fact that orthophosphate will form a complex with ammonium molybdate in strong (10N) H_2SO_4 and this complex may be reduced to yield a blue chromophore (Taussky and Schorr, 1953). The optical density may be read in the 700 nm region of the visible spectrum. There are, in addition, extraction procedures for separating P_i from organic phosphates in the reaction mixture prior to assay (Lindberg and Ernster, 1956). Since any substrate yields orthophosphate as one of the products, this method can be employed with all of them.

Most assay procedures have concentrated on the other product. Substrates have been chosen because they generate a chromophore upon hydrolysis, or else a compound that is readily convertible to a colorimetrically determinable chromophore. Phenylphosphate yields phenol, which is determined with phenol reagent (Folin and Ciocalteu, 1927). Para-nitrophenylphosphate generates p-nitrophenol. This method was first employed by Ohmori (1937). The p-nitrophenol readily forms a resonance-stabilized p-nitrophenylate anion in basic solution which absorbs at around 400 nm. This substrate is hydrolyzed a little faster than phenylphosphate (King and Delory, 1939) and has been used to assay serum phosphatases (Bessey *et al.*, 1946; Andersch and Szczypinski, 1947) and seminal AP (Kind, 1964). The α- and β-naphthylphosphates have enjoyed extensive use as substrates. The naphthols may be determined directly by their absorption at 335 nm (α) and 345 nm (β) (Moss, 1966). The α-compound has a higher extinction coefficient than does the β-compound. Alternatively, the naphthols may be allowed to react with a variety of azo coupling dyes. These methods are especially valuable for qualitative assays, histochemical localization of the enzyme (Kupcsulik *et al.*, 1970), and for detecting enzymes in gel electrophoretic media, since the brightly colored products are frequently insoluble. Quantitative assays can be carried out using these methods, however, by selecting azo compounds which form soluble colored products (Babson and Phillips, 1966). Phenolphthaleinphosphate (Huggins and Talalay, 1945) and thymolphthaleinphosphate (Roy *et al.*, 1971) generate their own chromophores upon hydrolysis if the solution be made basic. The latter was employed by Gomez *et al.* (1975) and Konzak (1977), and Willott (1975) said that he found the

sensitivity to be of the same order as that of p-nitrophenylphosphate.

Fluorimetric procedures have been employed to increase the sensitivity of the assays. Both α- and β-naphthol fluoresce and their phosphate esters may be used as fluorogenic substrates in solution (Campbell and Moss, 1961), or for localizing enzymes in gels with UV light (Moss *et al.*, 1961). Vaughan *et al.* (1971) studied a series of so-called naphthol AS phosphates as fluorogenic substrates for acid and alkaline phosphatases: Naphthol AS (3-hydroxy-2-naphthanilide); Naphthol AS-BI (6-bromo-3-hydroxy-2-naphthyl-o-anisidine); Naphthol AS-D (3-hydroxy-2-naphthyl-o-toluidine); Naphthol AS-GR (3-hydoxy-2-anthryl-o-toluidine); Naphthol AS-LC (4′-chloro-3-hydroxy-2′,5′-dimethoxy-2-naphthanilide); Naphthol AS-MX (2′,4′-dimethyl-3-hydroxy-2-naphthanilide) and Naphthol AS-TR (4′-chloro-3-hydroxy-2-naphthyl-o-toluidine). Naphthol AS-BI-phosphate was found to be the best substrate for phosphatases. It had a K_m for alkaline phosphatase of 2.2×10^{-5}M and detected alkaline phosphatase at a concentration of 5×10^{-5} units, where a unit was 1 μmole substrate hydrolyzed/min at 25°. Land and Jackson (1966) used flavone-3-diphosphate as a substrate, and noted that it was more stable than 3-O-methylfluoresceinphosphate, and more sensitive than β-naphthylphosphate. Ortho-carboxyphosphate may be used as a fluorogenic substrate (Brandenberger *et al.*, 1967), the reaction product being salicylic acid. It can also be employed as a substrate and salicylate detected by its absorption properties, as was done by Walther and Höhn (1971). Neumann (1948) described three fluorogenic substrates for phosphatases: fluoresceinphosphate, eosinphosphate and 4-methyl-7-oxo-coumarinphosphate, the last of which is better known as 4-methylumbelliferylphosphate, and has been used by Adams and Wraxall (1974) to detect seminal, vaginal and fecal AP in acrylamide gels following electrophoresis. The substrate is in use in a number of laboratories for detection of the isoenzymes of erythrocyte AP in gels as well (Wraxall and Emes, 1976). For a review of fluorimetric enzyme assays, including the assay of phosphatases, see Roth (1969).

The other matter is that of units of activity. Units are always defined as mass units of substrate hydrolyzed (or product formed) per unit time at some defined temperature and pH. Some of the unit definitions in the phosphatase field have acquired the names of their definers, and may still be encountered in the literature in those terms. Some unit definitions that have appeared over the years are summarized in Table 10.2. Since the enzyme exhibits different affinity for, and different rates of hydrolysis with the various substrates, the units are not always readily comparable. The international unit is based on the recommendations of the International Union of Biochemistry (IUB) for all enzyme units. In expressing enzyme concentration, the IUB recommended the use of units/mℓ solution. In clinical chemistry, it is sometimes preferred to express concentrations in units/ℓ. The international unit is denoted by the symbol "U". In order to obtain convenient numbers, it is permissible to express international units in logarithmic multiples using the usual metric prefixes, e.g. milli-units, mU; kilounits, kU; etc. It was common in the older literature to express serum phosphatase concentrations in terms of units/100 mℓ serum. Babson and Read (1966) said that 1 μmole α-naphthol/min (international unit) was equivalent to 0.865 Babson-Read units. A Babson-Read unit is equivalent to about 0.18 Bodansky units, and a King-Armstrong unit/100 mℓ is equivalent to 1.8 I.U./ℓ at the same temperature and pH (Bowers and McComb, 1970).

10.3.5 Specificity of the acid phosphatase test and the problem of vaginal acid phosphatase

In sexual assaults, the seminal stains may well be contaminated with vaginal secretions. In swabs taken from victims, there is no question that they are so contaminated. Because vaginal secretions contain endogenous, though variable, acid phosphatase activity, and because seminal samples may show low AP activity, there has been some concern about the possibility of vaginal AP leading to "false positive" reactions. As was discussed above, it matters little to this problem whether qualitative or quantitative tests are being used. In a quantitative test, some judment has to be made as to where the "cut-off" point is going to be, in terms of units per volume or area, and above which the material will be regarded as being of seminal origin. A qualitative test has an internal, or built-in, "cut-off" point, as it were, namely the lower limit of the sensitivity of the assay. The problem has been one of attempting to discover whether the upper limits of endogenous vaginal acid phosphatase (VAP) activity may overlap the lower limits of seminal acid phosphatase (SAP) activity.

The studies of Gomez *et al.* (1975) indicated that endogenous VAP activity could be higher than some examples of SAP activity measured in vaginal washings, and that misinterpretation was therefore possible using AP alone as a criterion for the presence of semen. The meaning of the term "vaginal secretions" is not very clear, and the term does not describe a well-defined bodily secretion whose origins are known. In medico-legal discussions, the term is usually taken to refer collectively to any material which is recovered on vaginal swabs or in vaginal washings in the absence of semen. As such, it may consist of substances from the vaginal mucosa, cervix, endometrium, bacterial flora, lymph or tissue fluids, or even urine. In pregnancy, substances from gestational tissues might be found. Moreover, if an infection is present, substances or cells characteristic of yeast, fungi, gonorrheal or leucorrheal discharge may be present. No one would suggest that materials characteristic of vaginal pathologies constitute "vaginal secretions", but the fact is that unless a pathological condition has been diagnosed by the examining physician, and the evidence examiner informed, these types of adventituous substances might be present in the sample and no tests conducted to determine their possi-

Table 10.2 Some Units of Phosphatase Activity

Name of unit	Substrate	pH	Temperature	Definition of Unit	Reference
King-Armstrong (for alkaline phosphatase)	phenylphosphate	9.1	37.5	1 mg phenol/30 min	King & Armstrong (1934)
King-Armstrong (for acid phosphatase)	phenylphosphate	4.9	37	1 mg phenol/hr	Gutman & Gutman (1938), Riisfeldt (1946)
	phenylphosphate	5.9	37	1 mg phenol/hr	Rasmussen (1945), Hansen (1946)
Bodansky (for alkaline phosphatase)	β-glycerophosphate	8.6	37	1 mg P_i/hr	Bodansky (1933)
Bodansky (for acid phosphatase)	β-glycerophosphate	5.0	37	1 mg P_i/hr	Bodansky (1972), Enos et al. (1963) Enos & Bayer (1977)
	β-glycerophosphate	6.0	37	1 mg P_i/30 min	Hazen (1955)
Babson-Read	α-naphthylphosphate	5.2	37	1 mg α-naphthol/hr	Babson & Read (1959)
Huggins-Talalay	phenolphthalein-diphosphate	5.5-6.0 (acid phosphatase)	37	0.1 mg phenolphthalein/hr	Huggins & Talalay (1945)
		9.1-9.6 (alkaline phosphatase)		0.1 mg phenolphthalein/hr	
International (I.U.)	any	defined	defined	1 μmole substrate/min	Bowers & McComb (1970)

Fishman and Mitchell (1959) showed by histochemical techniques that AP is present in the middle and superficial layers of vaginal epithelium. Moursi *et al.* (1971), studying exfoliated vaginal mucosal cells by histochemical techniques, indicated that AP was present in cytoplasmic granules, but not in nuclei. Activity was lower in most patients in the follicular phase of the menstrual cycle than in the luteal phase. Gregoire *et al.* (1972) measured the AP activity of cervical mucus during different cycle phases in patients using no contraceptive devices as well as in patients using an IUD or hormonal contraceptive therapy. AP activity was expressed in units/100 mg protein where a unit of activity was defined as that amount of enzyme liberating 1 mmole p-nitrophenol from p-nitrophenylphosphate in 30 min at 37°. With no contraceptives, AP was relatively low in the proliferative phase (roughly the same as the follicular phase), increased slightly in the ovulatory phase (midcycle), and then increased dramatically in secretory phase (roughly corresponding to the luteal phase). With an IUD, AP was highest in proliferative phase, decreased significantly (by about 80%) at midcycle, and then increased again in secretory phase, but only to about 60% of the initial (proliferative phase) value. With hormonal contraceptives, proliferative and ovulatory phases showed similar activities, but they were of the same order of magnitude as the highest values seen in the other two groups. A slight increase was observed in secretory phase. The actual values observed ranged from 3.0 to 20.7 units/100 mg protein. The only relatively comparable data on SAP is that of Kind (1964) who used a similar assay, but whose units were defined as μg p-nitrophenol liberated/min at 37°. There would be no difficulty in converting mmoles to μg, but Kind expressed the concentration of the enzyme not in terms of a mass value of protein, but in terms of that amount of seminal stain extract which gave an OD of 1.0 at 270 nm with a 1 cm path length. The OD_{270} is a rough measure of protein concentration to be sure, although it is more usual to determine either OD_{280} or the ratio of absorbancies at 260 and 280 nm. The trouble is that every protein has a different extinction coefficient, and Kind did not say what this was in his paper.

A quite definitive study of the problem was carried out by Godwin and Seitz (1970), using a commercial kit, based on the assay of Babson and Read (1959), for quantitative AP determination. Measurements were made on swabbed material eluted into 1.5 mℓ saline. The highest value seen in a non-pregnant patient in the absence of semen was 64 units, while in a pregnant patient, it was 85. Where intercourse had occurred within 12 hours, the AP activity was usually >300 units, and spermatozoa were usually found. In one patient, AP activity was 905 units, but no sperm cells could be found 11 hours after coitus. In 9 cases in which sperm were found, however, the AP activity was 85 units. In samples collected up to 36 hours after intercourse overall, AP activity varied from 10 units to 4,100 units. Godwin and Seitz concluded that the AP activity was not as reliable an indicator of semen as the presence of sperm cells, as is evident from the data, but that AP activities might be useful in diagnosing azoospermic semen, presumably where the activity is very high, and well above any endogenous VAP level that has been observed. Sensabaugh (1977) has shown that the total acid phosphatase activity in 8–12 hour postcoital samples of the vaginal pool is only about 1% of that present in the ejaculate. In addition, the activity in the recovered samples varied from 0.16 to 32.4 units, where a unit was 1 μmol p-nitrophenyl phosphate hydrolyzed/min at 22°. Endogenous VAP activity varied from 0.12 to 0.68 in the same units, a significant overlap between the range of endogenous activity and the range of the activity in samples recovered 8–12 hours post coitus in the undoubted presence of semen. Sensabaugh (1979) has extended his analysis of this problem, using data collected in a number of different laboratories. The paper, which must be read by those interested in the significance of quantitative AP results, places the entire problem into a statistical perspective.

It is quite clear that some other approach is required if the AP test by itself is to be rendered specific for semen. Quantitative determinations alone do not necessarily exclude VAP, although most authorities agree that they are preferable to a qualitative test. One way of avoiding the difficulty, of course, is simply to side-step the issue and regard the AP test as presumptive. In the absence of a corroborative finding (sperm, Florence test, immunological test, etc.), therefore, the results would be reported as "negative for semen", or as "negative for spermatozoa, but with such-and-such an AP result", depending on the individual expert's feelings about the matter. There are, however, other approaches which may be taken to meet the problem. Two of these, which have been tried, are: differential inhibition, in which an inhibitor is used which inhibits either SAP or VAP, but not the other; and separation of SAP from VAP by a protein separation technique, such as electrophoresis, isoelectric focusing, etc.

In 1970, Sivaram reported that he could take advantage of the fact the L-tartrate inhibited SAP to distinguish this enzyme from other acid phosphatases in a qualitative test using α-naphthylphosphate as substrate and Brentamine Fast Blue B as the coupling dye (Table 5.3). In 1971, Sivaram and Bami enlarged these studies, carrying out a quantitative AP assay with phenylphosphate as substrate, and showing that L-tartrate completely inhibited SAP at a concentration of 0.04M. That L-tartrate inhibits prostatic AP had been known since the work of Abul-Fadl and King (1949), who noted that L-tartrate did not inhibit erythrocytic AP. This fact was soon put to use in clinical chemistry, because in assaying serum AP, the circulating enzyme of prostatic origin could be assessed based on the fraction of "tartrate-inhibitable" AP present (King and Jegatheesan, 1959; Fishman and Lerner, 1953; and many others). King and Jegatheesan (1959) observed complete inhibition of the prostatic enzyme in serum by 0.025M tartrate. Unfortunately, the possibility that an inhibitor had been found in L-tartrate which could differentiate between SAP and VAP was soon laid to rest by Willott (1972). He showed that

L-tartrate inhibited endogenous VAP to the same extent as it did SAP at the same concentrations. Gomez *et al.* (1975) fully confirmed Willott's findings, as did Brown (1977). Thus, while L-tartrate inhibition may be useful in differentiating SAP from plant, vegetable or other adventitious acid phosphatases, it will not differentiate SAP from VAP.

The other approach, that of devising a separation method for SAP and VAP, was much more successful. Walther and Höhn (1970) using polyacrylamide disc gel electrophoresis had noted two bands of AP activity were observed with semen, but only one with vaginal secretions. Höhn *et al.* (1971) confirmed that two AP active bands could be separated by polyacrylamide disc gel electrophoresis of semen, and that a "storage band" developed after about 4 weeks of storage. VAP was not studied, but the system was useful in differentiating SAP from the AP of plant origin. The discreet banding patterns of the plant source AP enzymes tended to become diffuse, however, after relatively short storage periods.

In 1964, Anzai showed that SAP and VAP could be separated electrophoretically on agar gels in veronal buffers at pH 8.5, the SAP migrating anodically while the VAP migrated cathodically. This observation clearly established that SAP and VAP differ at least in respect to net charge at pH 8.5. Not much notice appears to have been taken of this work in the Western literature for nearly 10 years, perhaps because the paper was published in a rather obscure journal. In 1974, Adams and Wraxall drew attention to this work in proposing a somewhat different electrophoretic method for the separation of SAP from VAP. Their method was carried out on 1 mm polyacrylamide gels with a starch gel insert (Parkin, 1971) with separation taking place in veronal buffers at pH 8.5. Sites of activity were detected using 4-methylumbelliferylphosphate (Cf Section 10.2.4). SAP and VAP could be differentiated from one another using this method, and from fecal AP, buccal epithelial AP, several animal seminal AP, and a number of AP enzymes of plant origin including vaginal yeast type organisms, as well. The activity could be detected in swabs up to 72 hrs post coitus, provided that the swabs were kept in a dry storage environment. Stolorow *et al.* (1976) confirmed the value of this technique. They showed, however, that seminal and fecal enzyme could not always be readily distinguished, particularly in cases where SAP activity was relatively weak. The substitution of α-naphthylphosphate for 4-methylumbelliferylphosphate as substrate in the system did not substantially improve the results. They cautioned, therefore, that great care should be exercised in the diagnosis of azoospermic semen on anal swabs by this technique. In 1975, Sutton and Whitehead reported that seminal, vaginal and fecal AP could be separated by isoelectric focusing in a pH 5-7 gradient on polyacrylamide gels. Seminal AP could be separated readily from either vaginal or fecal AP by this method, but because the isoelectric points of the fecal and vaginal AP bands were very close or identical to one another, fecal and vaginal AP activities could not be distinguished from one another. It was said that this technique had the advantage of speed in comparison to the polyacrylamide gel technique. Isoelectric focusing required only about 3 hrs whereas the polyacrylamide gel system was electrophoresed for 17 hrs. Linde and Molnar (1980) have recently described a procedure in which SAP and VAP can be electrophoretically discriminated, and the PGM_1 isozymes can be typed simultaneously. PGM typing is discussed in section 27.

There is little question that the separation and identification of SAP and VAP presently represent the best method available for assessing vaginal secretion contamination of seminal fluid containing material, and for ensuring that the AP activity in an unknown sample is in fact due to the presence of seminal material.

Blake, Lofgren, Inman and Sensabaugh (see in Sensabaugh, 1977) have made the very important point that any realistic expectation of devising methods for the differentiation of SAP from vaginal and other tissue AP must be based ultimately on molecular specificity. The answer to this problem rests finally on a determination of the relationships between the genetic loci which code for the various enzymes. In comparative biochemical studies, they could show that the catalytic properties and MW of SAP and VAP were similar to one another and to those of kidney or liver lysosomal AP. Further, antisera prepared against SAP cross-reacted not only with VAP, but with the A, B & C isoenzymes of placental acid phosphatase as well. These isoenzymes are discussed in Unit VI, but the results are consistent with SAP and two of the tissue isoenzymes being coded for at a common genetic locus (see in section 29.3). If this turns out to be the case in fact, theoretical limitations would be imposed on the degree of specificity which can be expected from any AP test. Blake *et al.* (see in Sensabaugh, 1977) could show that neuraminidase treatment of SAP and VAP resulted in a change in their electrophoretic mobility on acrylamide gels, treated SAP running with the same mobility as untreated VAP. The result suggests that the two may differ only in respect to sialic acid residues attached to the protein. It could also be shown that vaginal mucus from women using IUD contraceptive therapy frequently showed multiple AP bands on acrylamide, perhaps related to uterine leucocyte build-up in these patients and the leakage of white cell AP into the vaginal pool.

10.12 Seminal Stain Fluorescence

In 1927, Ito reported that a number of body fluid stains, including seminal stains, fluoresce under UV light. Since that time, the examination of articles submitted for blood and/or body fluid analysis under UV light has become virtually routine in most places. Many authors have mentioned this fact, including Thomas (1937), Pollack (1943), Kirk (1953) and Nickolls (1956) and many others. Characteristic fluorescence under UV light in no way establishes the presence of semen, but is an excellent, simple, non-destructive screening technique. Garbutt and Sensabaugh (see in Sensabaugh, 1977) have looked into the mechanism of seminal stain fluorescence. The fluorescence spectrum of stains is rather different from that of liquid semen. Studies on fractionated seminal fluid components dried out on substrata indicated that the visible fluorescence that develops in stains is the result of the conversion of one or more non-proteinaceous precursors in semen to several fluorescent products. These have not as yet been characterized. Liquid semen was shown to develop a yellow color upon standing, and this could be shown to be the result of the development of fluorescent compounds in the material. Two distinct fluorescence spectra could be observed: one, having an excitation maximum of about 400 nm and an emission maximum of 460 nm, was called the 400/460 fluorescence; the other had an absorption maximum of 420 nm and an emission maximum of 500 nm and was called 420/480. Preliminary experiments indicated that the 400/460 fluorescence developed first, and sometimes converted to the 420/480 fluorescence. At least five non-proteinaceous compounds were responsible for this fluorescence behavior. While the compounds could not be characterized, it could be shown that the fluorescence developed solely as a result of contamination of the samples with a strain of the bacterium *Pseudomonas fluorescens*. It is not inconceivable that this property could be used to develop a fluorescence test for seminal fluid, although the bacterium is known to interact with other body fluids to produce fluorescent products with different fluorescence spectra, Garbutt and Sensabaugh said.

Calloway *et al.* (1973) reported a low temperature phosphorescence technique which was non-destructive and could indicate the order of deposition of mixed blood and seminal stains.