UNIT III
IDENTIFICATION OF BODY FLUIDS

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 ? 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 60×10^6/mℓ 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

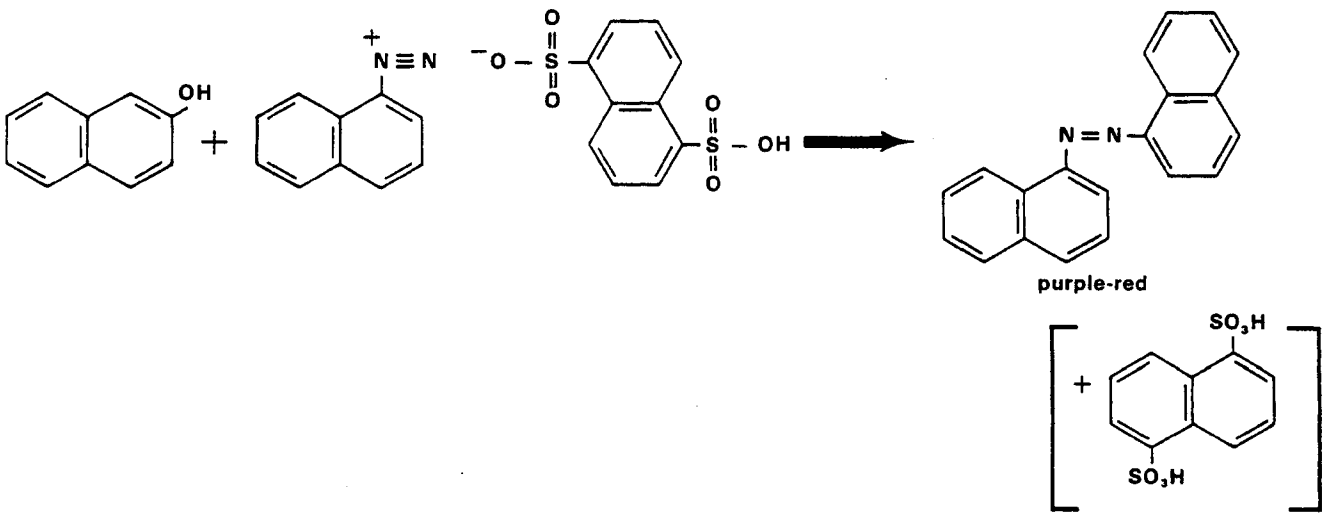


Figure 10.1 Reaction of β-Naphthol with Stabilized Diazonium Compound (Fast Garnet B)

(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.

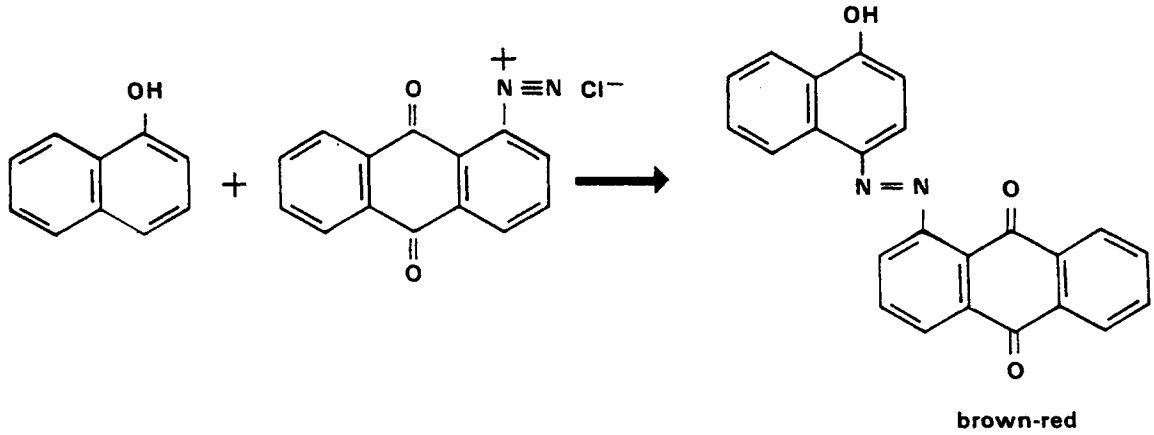


Figure 10.2 Reaction of α-Naphthol with Stabilized Diazonium Compound (Fast Red AL)

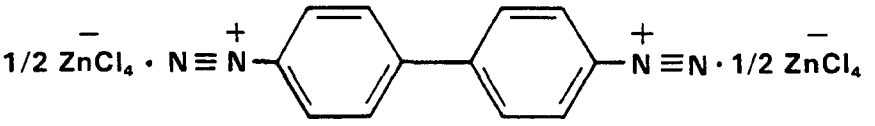


Figure 10.3 $ZnCl_2$ Double Salt of the Tetrazonium Chloride of Diazo Blue B (Fast Blue B; Dianisidine Blue)

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

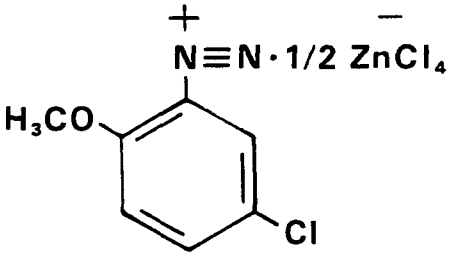


Figure 10.4 $ZnCl_2$ Double Salt of Diazo Red RC (Fast Red RC)

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/cm^2 in 6 hr old stains. At 3 months of age, the remaining activity was 20 to 25 units/cm^2. 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 cm^2 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 possible presence.

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.

10.3.6 Purification, properties and molecular heterogeneity of prostatic acid phosphatase

The prostatic (seminal) acid phosphatase is systematically called an orthophosphoric monoester phosphohydrolase (EC 3.1.3.2), and has been studied extensively. In the biochemical literature it is often referred to as acid phosphomonoesterase, and abbreviated as "acid PME". In 1946, Lundquist presented preliminary evidence that phosphorylcholine was the natural substrate for SAP. He established this fact firmly the following year (1947a), and carried out a number of biochemical studies on the enzyme as well (1947b). Fluoride, Zn^{++}, oxalate and maleate inhibited SAP to varying degrees, while citrate acted as an activator with phosphorylcholine or glycerophosphate as substrate in

the absence of inhibitors. Systematic studies on the enzyme were carried out by Abul-Fadl and King (1949). They showed, among other things, that the enzyme is not activated by a variety of metal ions, while Fe^{3+} strongly inhibits the enzyme in acetate buffers, but not in citrate buffers. Glycine, alanine and stilbestrol had no effect, whereas cysteine slightly activated and L-tartrate strongly inhibited the enzyme. London and Hudson (1953) purified the enzyme about 5000-fold. Davidson and Fishman (1959) purified the enzyme by different techniques, taking advantage of its stability in Tris-ammonium sulfate solutions at acid pH. An approximately 50-fold purification could be obtained from filtrates of homogenized frozen prostate glands. Nigam *et al.* (1959) studied the properties of the enzyme purified in this way. The K_m for phenylphosphate, p-nitrophenylphosphate and glycerophosphate were found to be different and were not the same in acetate as in citrate buffers. The K_i for tartrate in acetate buffers was 9.5×10^{-6}M with phenylphosphate, 4.5×10^{-5}M with p-nitrophenylphosphate and 2.4×10^{-5}M with glycerophosphate. Oxalate inhibited the enzyme with any substrate, but pyruvate, maleate, glutamate, malonate and glucuronate inhibited only with β-glycerophosphate as substrate. In 1961, Ostrowski and Tsugita reported a purification procedure which yielded a prostatic AP preparation devoid of appreciable phosphodiesterase activity. Kuciel and Ostrowski later (1970) purified the prostatic phosphodiesterase and examined its properties. Ostrowski and Rybarska (1965) reported that the purified prostatic acid phosphatase enzyme had a MW of 95,000. Greenberg and Nachmanson (1965) studied the effect of DIFP on the protein, and could show that it did not irreversibly inhibit the activity. These data, along with the results of the ^{32}P incorporation studies, indicated that, unlike the situation in other phosphatases, O-phosphorylserine did not form as an intermediate in the reaction, and that an amino acid other than serine must be involved in the active site. Ostrowski and Barnard (1971 and 1973) confirmed these results, but said that their kinetic and ^{32}P incorporation studies indicated that a phosphoryl-enzyme intermediate did form. It was of a quite different nature than the intermediate formed by alkaline phosphatase (i.e. O-phosphorylserine), however.

The first suggestion that prostatic AP might exhibit molecular heterogeneity came in 1962, when Sur *et al.* reported that prostatic tissue extract could be separated into multiple bands with AP activity by starch gel electrophoresis. That there are indeed a number of molecular species of prostatic AP came to be recognized through several different lines of evidence, both biochemical and immunological. In 1964, Shulman *et al.*, employing immunoelectophoresis and a specific anti-prostatic AP serum, found that there were two bands with AP activity. The bands could not be observed in extracts of a number of other organs and tissues, except in some sera from patients with prostatic carcinoma. In 1970, Pfeiffer *et al.* confirmed that rabbit immune anti-prostatic tissue extract serum reacted with the serum of patients with prostatic tumors, malignant or benign, and that the precipitate complex had AP activity. Vernon *et al.* (1965) isolated and partially purified two different AP enzymes from prostatic tissue homogenates, and called them P_1 and P_2. Their apparent molecular weights were similar, and were estimated to be about 120,000 to 150,000 by sedimentation equilibrium, and 110,000 to 130,000 by calibrated Sephadex columns. Two enzyme activities could be isolated from seminal plasma as well, but their apparent molecular weights were not only different from one another, but considerably smaller than the prostatic tissue enzymes. Lundin and Allison (1966a and 1966b), in their studies of tissue acid phosphatases from a number of different species including human, noted multiple AP bands from human prostate tissue using polyacrylamide disc gel electrophoresis. Ostrowski and Rybarska (1965) had noted that two fractions of AP activity could be observed from prostatic tissue by CM-cellulose chromatography. In 1968, Smith and Whitby purified two AP activities from prostate gland on DEAE-cellulose and noted that the biochemical properties of the two enzymes were identical. Treatment of the material with neuraminidase abolished the apparent molecular heterogeneity of the enzyme, and indicated that the different fractions of activity being observed were due to the attachment of different numbers of sialic acid residues to one and the same protein. Ostrowski *et al.* (1970) confirmed these results using isoelectric focusing. However, the two fractions obtainable by chromatography had different kinetic and biochemical characteristics, and the "desialo" enzyme, obtained by neuraminidase treatment, had somewhat different characteristics than either of the initial fractions. Among other things, the "desialo" enzyme has a greater substrate affinity, and was more strongly inhibited by L-tartrate (Ostrowski, 1971, Dziembor *et al.*, 1971). Derechin *et al.* (1971) purified to a high degree one of the two fractions obtained from DEAE-cellulose chromatography of prostatic tissue homogenate. The enzyme had a MW of 102,000 and was found to be dissociable into two subunits without disulfide bond cleavage. It was found to contain 13 neutral sugar residues, 10 hexosamine residues and 6 N-acetylneuraminic acid residues per molecule. Lam *et al.* (1973) estimated the MW of what was probably the same enzyme from prostatic tissue as 100,000. Either of the two fractions obtained from DEAE-Cellulose chromatography exhibits a number of bands of activity upon polyacrylamide gel electrophoresis or isoelectric focusing (Ostrowski, 1971).

In 1973, Ueno and Yoshida said that they could obtain multiple molecular patterns of AP activity on starch gels from seminal plasma which were reproducible from a given person. Three different patterns were apparent in 95 subjects. Vaginal acid phosphatase showed no heterogeneity in the system. An underlying genetic mechanism was implied, but no genetical hypothesis was presented, nor confirmatory family studies carried out. Pfeister *et al.* (1975) looked at 75 seminal samples, and confirmed that there was molecular heterogeneity, but they observed considerable individual variation and said that they could not confirm Ueno and Yoshida's observations. Suyama *et al.* (1976a and 1976b)

fractionated seminal plasma by Sephadex chromatography and by polyacrylamide disc gel electrophoresis, obtaining two fractions of AP activity. From their results, it is pretty clear that these differed in carbohydrate content, although the neuraminidase experiment was not done. It was worthy of note, however, that only two fractions were obtained from seminal plasma, whereas multiple bands had been obtained by others from prostatic tissue homogenate or extract (Lundin and Allison 1966a and 1966b; Smith and Whitby, 1968; Paris *et al.*, 1974). It would appear that seminal plasma may not contain as many molecular species of AP as does the parent prostate gland. Ohya *et al.* (1976) showed that multiple AP bands could be detected in seminal fluid by isoelectric focusing. There were three distinct patterns in 37 different people, very likely corresponding to Ueno and Yoshida's (1973) results. Following neuraminidase treatment, however, AP migrated to a single isoelectric point indicating that the differences could be fully accounted for on the basis of variable numbers of sialic acid residues on the protein.

The immunogenicity of prostatic AP has been studied by a number of workers, and deserves brief mention. As previously discussed, Shulman *et al.* (1964) utilized an antiserum to the enzyme in their immunoelectrophoretic studies, which suggested the presence of more than one enzymatically active molecular entity. Abe (1968) noted that some preparations of anti-AP cross-reacted with serum, and that the cross-reacting serum protein was transferrin. Some transferrin contamination was apparently present in the "purified" acid phosphatase which had been used for immunization. Repurification of the enzyme to remove the contaminant, or absorption of the cross-reacting antisera with partially purified transferrin, rendered the antiserum specific. Mukherjee and Ghosh (1970) showed conclusively that the antigenic determinant and the active site of the enzyme were independent. The antibody from rabbit or guinea pig did not inhibit enzyme activity at all. Kulhanek and Pernicova (1971) confirmed these findings. The fact that the enzyme is still active in an antigen-antibody complex was clear from the work of Shulman *et al.* (1964). If antibody had inhibited the enzyme activity, the precipitin bands could obviously not have been "activity stained". Milisauskas and Rose (1972) could prepare active anti-AP from ammonium sulfate fractions of urine. Moncure and Prout (1970) prepared antibodies in goats to azoospermic semen and to hyperplastic prostatic tissue extracts. By suitable absorption, the antisera could be rendered specific for prostatic proteins. Four prostatic-specific precipitin lines were obtained upon immunoelectrophoresis, two of which had acid phosphatase activity.

10.3.7 Identification of vaginal secretions

It was previously mentioned that "vaginal secretions" do not constitute a well defined body fluid. A brief discussion of the biology and physiology of the vagina, cervix and uterus is included here because the composition of the secretions of these tissues and organs and their cyclic variations have a bearing upon the tests for identifying menstrual blood (section 8.1.1) and on the assessment of results obtained when examining vaginal swabs in cases of sexual assault (sec-10.3.5). Vaginal acid phosphatase has already been discussed in connection with the acid phosphatase test for semen (section 10.3.5).

One of the techniques of identyfying menstrual blood (section 8.1) is based on the identification of glycogen containing epithelial cells (Strassman, 1921; Merkel, 1924). The presence of glycogen in the vaginal epithelium is closely related to ovarian endocrine activity. This relationship has been clear at least since the extensive studies of Cruickshank and Sharman in 1934. Glycogen is present in the vaginal epithelial cells from menarche to menopause. Cruickshank and Sharman suggested (1934a) that glycogen deposition is under the control of estrogens. The fact that glycogen-containing epithelial cells can be found in newborns up to about 3–4 weeks of age is explained by the hormonal influences of maternal circulation. Rakoff *et al,* (1944) agreed with this view. They could also show that the glycogen content of vaginal epithelial cells was cycle-dependent, increasing in the superficial epithelial layers throughout the cycle until just prior to menstruation, when it decreased. Maeyama *et al.* (1977) showed by means of a sensitive enzymatic assay that a similar pattern could be observed in endometrial tissue. Furthermore, fertile patients showed a large increase in endometrial glycogen (about 5-fold) at midcycle, whereas infertile patients showed a much smaller increase (about 1.7-fold). Willson and Goforth (1942) showed that some glycogen was present in the vaginal epithelial cells of postmenopausal patients, and that dietary carbohydrate had no effect on the amount. Administration of 1 mg doses of diethylstilbestrol for 12 days led to significant increases in glycogen levels in these patients. Diethylstilbestrol is used for therapeutic estrogen replacement.

The glycogen content of epithelial cells is closely related to the presence of the natural vaginal flora, which are found during the fertile period of life. The flora consist of a form of bacterial life known for a long time as Döderlein's vaginal bacillus, after Döderlein (1892), who did extensive studies on it. The presence of Döderlein's bacillus follows the same pattern as the presence of glycogen in epithelial cells throughout life (Cruickshank and Sharman, 1934b). The bacteria ferment the glycogen to lactic acid (Cruickshank, 1934) which, in turn, accounts in large part for vaginal acidity. Rakoff *et al.* (1944) showed that there tends not only to be an intravaginal pH gradient, the pH being lower closest to the vaginal orifice and about the same as that of vaginal secretions in the mid-vaginal region, but that there is cyclic variation of the pH as well. Intravaginal pH is lowest at midcycle, and highest at the premenstrual stage. Moreover, the pH determines the type of bacteria which characterize the vaginal flora. Lower pH tends to favor Döderlein's bacillus as the exclusive resident flora, while higher pH (around 5) allows the growth of various staphylococci, streptococci, coliforms, yeast and yeast-like organisms. pH values of 5.5 and upward tend to inhibit

Döderlein's bacillus, and favor the other organisms (Rakoff *et al.*, 1944). More recently, it has become clear that Döderlein's vaginal bacillus is not a single organism, but a mixture of a number of different strains of *Lactobacillus acidophilus* (Hunter *et al.*, 1959). Yeasts and yeast like fungi which may be cultured from the vagina consist of species of *Saccharomyces, Cryptococcus* and *Candida* (Monilia). *Saccharomyces* infections are relatively infrequent, while *Crytococcus* infections are more common and *Candida albicans* is a common cause of mycotic vaginitis (Carter *et al.*, 1959). The gram-negative *Hemophilus vaginalis* has been linked to non-specific vaginitis (Heltai *et al.*, 1959) but not everyone agrees that it is the specific etiological agent (Gardner and Dukes, 1959). The incidence of mycotic infection is related to ovarian hormone function, hormone administration and pregnancy (Carter *et al.*, 1959) as is so-called leucorrhea of non-infective origin (Cruickshank and Sharman, 1934a).

Cervical mucus may be a component of "vaginal secretions", depending upon how the sample is collected. The properties of cervical mucus undergo cyclic changes, which are believed to be related to sperm penetrability and survival, and ultimately, therefore, to fertility. The quantity of cervical mucus secretion increases from the beginning of the menstrual cycle to midcycle, and then decreases again steadily in the secretory phase to menstruation.

Cervical mucus viscosity is more or less inversely proportional to quantity, midcycle mucus being least viscous (Moghissi, 1967). Cervical mucus can be fractionated into two major components, one of which is largely glycoprotein and is responsible for the rheological properties. This material, sometimes called the "high viscosity component" (Davajan and Nakamura, 1975) is thought to consist of a polypeptide backbone with various carbohydrate residues, including N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid, attached by way of O-seryl and/or O-threonyl linkages (Gibbons and Mattner, 1967). The other component, sometimes called the "low viscosity component" (Davajan and Nakamura, 1975), consists in part of serum proteins, albumin and γ-globulins which exhibit cyclic variations as well (Moghissi, 1967). As previously noted (section 4.2.5), cervical mucus contains an endogenous AP activity which exhibits cyclic variation, and the variation is altered by hormonal or intrauterine contraceptive therapy (Gregoire *et al.*, 1972).

Vaginal secretions may also contain esterase, alkaline phosphatase, β-glucuronidase and DPNH-diaphorase which originate from the epithelial linings (Fishman and Mitchell, 1959) and smaller molecular weight carbohydrates, such as glucose, maltose and maltotriose (Sumawong *et al.*, 1962). Recently, Raffi *et al.* (1977) identified albumin, α-antitrypsin, α_2-haptoglobin, α_2-macroglobulin, β-lipoprotein, orosomucoid, ceruloplasmin, γ-chains, γG.K. (Bence-Jones) and IgG, IgA and IgM in vaginal fluid as well as in cervical mucus. The absence of α_2-haptoglobin, α_2-macroglobulin, β-lipoprotein, orosomucoid and IgM in a population of hysterectomized women led to speculation that the presence of these proteins in the vaginal fluid of normal women may in fact be due to cervical mucus contamination. Not all the proteins were found in all the normal subjects with the single exception of albumin, which was universally present.

It is worthy of note that Thomas and Van Hecke (1963) proposed that recent intercourse by males could sometimes be diagnosed by demonstrating vaginal epithelial cells on the glans penis. The iodine vapor method for glycogen containing epithelial cells was employed (section 8.1.1). obviously, the suspect man would have to be examined quite soon after coitus, and prior to any cleansing, for this method to be of value. And too, Popielski's findings would have to be considered before interpreting the results (section 8.1.1). Rothwell and Harvey (1978) have recently questioned the validity of "vaginal" epithelial cells being identified on penile swabs. Their studies indicated that false positive findings can be obtained, and that this approach to determining whether a male has recently had intercourse may not be a reliable one.

10.4 Immunological Methods for the Identification of Semen

10.4.1 Precipitin tests

The development of an immunological test for semen identification followed closely upon the development of the immunological test for species determination of blood and bloodstains by Dr. Uhlenhuth. This latter material is discussed in Unit IV (section 16.1.1). Once it became recognized that more or less specific antibodies could be prepared against blood, organ and body fluid proteins, the preparation of anti-seminal plasma antisera and the exploration of their applicability to medico-legal evidence logically followed. It must be kept in mind too that, at the time, around 1900, essentially the only medico-legal tests available for the identification of semen were the finding of spermatozoa and the Florence test (section 10.5.1). There was not universal agreement as to the proof value of the crystal test, and the unequivocal identification of azoospermic semen in forensic cases was a significant problem. What is now generally called the "precipitin test" was then often referred to as the "biological test" or the "serological test". The term "serological test" would be less preferred today, since it could easily be confused with tests designed to determine the ABH groups in semen. The blood groups were only discovered in 1900, however, and it was not until 1926 that the presence of soluble ABH substances in semen came to be recognized.

Farnum (1901) was the first investigator to prepare antibodies to human, bull and dog semen and to testicular extracts. The antisera were species-specific within the domain of the species under investigation, and the anti-bull semen did not cross react with goat semen. The anti-human semen serum reacted with the filtered extract of a 34 day old human seminal stain, and did not cross react with human serum. Farnum suggested that the medico-legal applicability of such antisera be pursued. Strube (1902) prepared antisera to human semen and testicular extracts in rabbits, but there was some cross reaction with serum. Pfeiffer (1905) looked

at the immunogenicity of bull semen, and found that the crude antiserum cross reacted with homologous serum and organ extracts, but that it could be absorbed by the cross-reacting materials and rendered specific. Dunbar (1910) studied immunological reactions of antisera prepared against the pollen of various plants, and the semen of several species of fishes. Dervieux (1921 and 1923) prepared anti-human semen serum, and found that it was species-specific. Antisera could be rendered semen-specific by suitable absorption. Dervieux thought that the antiserum cross-reacted more strongly with male than with female serum, and that this fact might be useful in sexing bloodstains. He said further that the reaction was stronger with the semen of the individual which had been used for immunization, and that the antiserum might, therefore, be individual-specific. Although the presence of soluble ABH substances in seminal plasma had not yet been recognized, it is possible that the stronger precipitin reaction he obtained with the semen against which the antiserum was actually prepared, as compared with that from different individuals, could be explained on this basis (see in Section 19.8.1).

In 1922, Hektoen carried out extensive experiments on the preparation and applicability of antisera against seminal plasma to human semen detection. The antisera obtained reacted with semen as well as with serum, though absorption by serum rendered the antibody preparation semen-specific. Absorbed antisera had titers of about 1:256 against the supernatant liquor obtained by centrifuging seminal plasma, although with different samples of semen, it could be as low as 1:8. Seminal and prostate extract stains reacted, but not stains of blood, serum, saliva or pus. Extracts of stains soaked in soap solutions gave non-specific precipitin reactions. Antisera to several animal semens were prepared and tested as well. Hektoen did not agree with Dervieux's assertion that the antisera possessed any individualizing specificity, nor with his finding that anti-serum serum did not cross react with homologous semen. Hektoen and McNally (1923) regarded the applicability of the precipitin test to forensic cases for semen stain identificaiton as being promising, but not clearly demonstrated. In 1928, Hektoen and Rukstinat noted that their anti-human serum serum, which had a titer of about 1:10,000 against serum, had a titer of about 1:20 against semen. Conversely, they could prepare anti-semen serum with a titer of about 1:1000 against semen, and this reacted with serum to dilutions of around 1:20.

There does not seem to have been a great deal of interest in the subject for a number of years following Hektoen's investigations, although the immunological test was mentioned in textbooks, and was certainly in use in some laboratories. Some authorities indicated that the technique was primarily of value for species determination, in cases where there was suspicion that non-human semen might be involved, rather than for the identification of semen as such.

In 1963, Coombs *et al.* prepared rabbit antiserum to human seminal plasma, which could be rendered semen-specific by absorption with serum and with boiled saliva. The antiserum did not cross react with extracts of stains of sweat, blood, or several animal semens. There was a weak cross reaction with pig semen, which could be removed by absorption. This work, which brought about a renewed interest in the subject, was apparently prompted by Hermann's immunoelectrophoretic studies (1964) of human seminal plasma, showing that while semen shared some proteins in common with serum, there were a number of seminal-specific proteins as well. Mischler and Reineke (1966) recommended the immunological method. They could show that the reaction occurred with seminal stain extracts even after the stains had been washed with soapy tap water or heated. Culliford (1964 and 1967) confirmed many of the findings of Coombs *et al.*, noting the importance of using antisera prepared against the specific body fluid one wants to identify in any immunological identification test. He also showed that the test could be carried out using crossed over electrophoresis. The antihuman semen serum, prepared according to Coombs *et al.* (1963), could have a titer as high as 1:8000 against human seminal plasma. Černov (1971) reported preparation of an anti-human semen serum in rabbits which did not cross react with capillary or menstrual blood, saliva, urine, nasal mucus, horse radish extract or vaginal secretions. It was species-specific as well. The problem of cross reactivity with vaginal mucus is obviously very critical if the antiserum is to be used for the identification of semen. Kerek (1972) got positive immunological reactions with seminal stain extracts after storage of the stains at room temperature for up to 14 months and at -20° for up to 4-1/2 years. Thornton and Dillon (1968) showed that the immunological test for semen could be carried out by immunodiffusion of cellulose acetate membranes. They got no cross reactions in this system with blood, saliva, urine or vaginal secretions using a commercial anti-human semen serum. Tröger and Jungwirth (1974) said crossed over electrophoresis was more sensitive than immunodiffusion (in gels), an antiserum they tested being able to detect a 1:1500 dilution of whole semen by the former, and only a 1:600 dilution by the latter method. In 1963, Suyama and Sawada reported that they had prepared a specific anti-seminal acid phosphatase antibody by immunization of rabbits with prostatic tissue homogenates and subsequent absorption with serum. The antiserum could be used to identify stains up to 4 years, 2 months old in an immunodiffusion test, but was negative with a 9 year, 4 month old stain. The antibody did not react with saliva, nasal mucus, urine or plant acid phosphatases, and with serum only in patients suffering from metastasizing prostatic carcinoma.

It is to be noted that most of the procedures mentioned in the foregoing discussion relied upon antisera raised against pooled human seminal plasma. Studies on the antigenic composition of human semen have indicated that the fluid may in fact contain several specific antigenic proteins. This matter is discussed in detail in section 10.13.2. If a seminal plasma-specific protein could be isolated, and specific antisera raised against it, the basis would be provided for a

specific immunological test for the identification of the material. This approach has been taken by the Japanese workers in their studies on γ-seminoprotein (see section 10.10) and by Sensabaugh (1977) in his studies on the p30 protein (see section 10.13.2). In the latter study, it could be shown that several commercial anti-human semen reagents contained a preponderance of antibodies against lactoferrin (see section 10.13.1) which occurs in milk, sweat and tears as well.

10.4.2 Other immunological tests

Weil *et al.* (1959) utilized an immunological test different from the precipitin reaction for detecting seminal fluid residues on female genital swabs. The test relied on the detection of the reaction between seminal plasma antigens and specific antibodies, but a complement fixation assay was employed. Vaginal swabs were soaked in saline to elute any seminal antigens. After centrifugation, serial dilutions of the supernatant fluid were incubated with antiserum and complement for a time, and the solutions then tested with sheep red cells and anti-sheep red cell homolysin. Absence of hemolysis indicated that complement had been fixed, and that the swab had contained seminal antigens. Inhibition of hemolysis at a 1:4 dilution of vaginal swab eluate was required before the test was regarded as being positive, and an antiserum had to be used which did not cross react with vaginal mucus serum proteins. While the test is exceedingly sensitive, it does not appear to have been very widely adopted.

Baxter (1973) tested a number of immunological techniques for detecting the antigen-antibody reaction, and their applicability to semen identification. A pooled, unabsorbed, hyperimmune serum was found to be species specific, but to be cross reacting with a number of other body fluids, including vaginal secretions. One cross reacting antigen was common to semen, tears and saliva, two others to tears and semen, and at least two more to semen and vaginal secretions. The serum contained an anti-A activity as well. No practical means could be found to remove all the unwanted cross reactivities. Attention was turned, therefore, to the anti-acid phosphatase activity in the antiserum. As noted previously (Section 10.3.6), Shulman *et al.* (1964) had shown that the catalytic site and the antigenic determinant of acid phosphatase were independent, by virtue of the fact that the antigen-antibody complex still showed acid phosphatase activity. Mukherjee and Ghosh (1970) had unequivocally established the independence of the two sites. Baxter (1973) took advantage of this property in his studies, and found that the anti-AP activity of the anti-semen serum cross reacted with the AP of vaginal mucus. Studies using electroimmunodiffusion (see section 2.4.3) were then conducted to quantitate acid phosphatase in these fluids. In this technique, an antiserum is incorporated into a gel (agar or agarose) support medium, and antigen-containing samples subjected to electrophoresis in the gel. As the antigen moves, the antigen-antibody reaction occurs along the path of migration, forming rocket-shaped peaks, which can be detected with acid phosphatase staining reagents. Peak height is proportional to antigen (i.e. acid phosphatase in this case) concentration in the sample (and inversely proportional to antibody concentration). SAP and VAP were found to have different mobilities in the system, and could be distinguished on this basis. SAP migrated anodally, while VAP showed an apparent cathodal and diffuse pattern, probably the result of electroendosmosis. Blind trial studies showed that false positives were not obtained for SAP in this system, but that the test could be negative in the presence of semen. The test became negative on vaginal swabs at 7–9 days postcoitus. A 6-month old stain extract could be detected. It was noted that the sensitivity of the method could be adjusted by varying the concentration of antiserum in the gel. Baxter said that the method had the advantages of speed and ease of manipulation in routine casework. Gdowski (1977) reported that she had successfully employed this procedure. It is to be noted that the technique is in some ways more closely related to the SAP/VAP separation procedures discussed in section 10.3.5 than to the other immunological techniques.

As a matter of historical interest, it should be noted that a number of workers looked into the applicability of anaphylaxis as an immunological method for the detection and species determination of seminal plasma. Any method employing immunological phenomena as a basis could theoretically be adapted to the needs and requirements of medico-legal tests. Once an antiserum has been obtained which exhibits appropriate specificity, any of a variety of immunological methods are available for detecting the antigen-antibody reaction, including precipitation, agglutination reactions, complement fixation and anaphylaxis. Applications of all these techniques have been employed at various times by various workers. More work has been carried out on immunological determination of species of origin of bloodstains than for any other purpose in forensic immunology. These studies are discussed in Unit IV. For various reasons, most workers have preferred precipitin reactions to detect antigen-antibody reactions, and still do at the present time.

Anaphylactic shock is perhaps the most dramatic indication that an antigen-antibody reaction has occurred. The guinea pig responds quickly and characteristically and has commonly been used in anaphylaxis experiments. In principle, the technique consists of injecting the test animal with a foreign protein (the sensitizing injection). After a suitable interval of time, during which there are no harmful effects, a further injection of antigen (the shocking or toxic injection) then causes the onset of anaphylactic shock. In the guinea pig, this reaction is systemic, and takes the form of restlessness, chewing, dyspnea, convulsions and usually death. The anaphylaxis reaction as a means of determining that an antigen-antibody reaction has occurred is generally as sensitive and specific as other methods.

Pfeiffer (1910) conducted anaphylaxis experiments with cattle semen, seminal proteins and blood and thought that the reaction was organ specific, i.e., shock was not induced by seminal protein if serum had been used for sensitization.

Minet and Leclercq (1911c and 1911d) first indicated that anaphylaxis could be used as a means of detecting semen-anti-semen reactions. These studies were based on their earlier experiments on the test for species determination in bloodstains (1911a and 1911b) (see Section 16.6.2). They found that the method was species-specific and specific for semen as well. Verger (1911) confirmed these results, but noted that there was some variability in the reactions of individual animals, and that a number of animals (about 10) should always be used in the test to compensate for the possibility of occasional negative reactions. Alvarez de Toledo y Valero (1915) conducted experiments on the method, but could not confirm the seminal specificity. Serum induced shock in animals sensitized with semen, and conversely. In his review in 1918, Lecha-Marzo said that the method appeared to require further evaluation and study as to its medico-legal applicability. Hektoen and McNally (1923) agreed with Lecha-Marzo. The test does not appear to have enjoyed very wide practical use, as it is obviously somewhat impractical *per se,* and probably hopelessly so for a laboratory conducting many examinations on a routine basis.

10.5 Crystal Tests

Crystal tests were the first non-morphological tests for semen to be proposed which have persisted until relatively recent times (see section 10.1). The first paper on the subject appeared in 1896, and the tests are still used in some laboratories. Any number of modifications have been proposed, and other crystal tests, based on other active constituents of the seminal plasma have been reported. The initial report of a crystal test for semen created something of a flourish of activity in the medico-legal community, everyone being anxious to have a reliable non-morphological test at their disposal.

10.5.1 The Florence Test

In 1895 and 1896, Dr. Florence in Lyon published a series of papers recounting his studies on seminal fluid and its medico-legal identification. Much of the material dealt with isolation and recognition of sperm cells in seminal stain investigation, and a good review of older methods was given as well (see in Unit IX, Translations). In the third paper, the now-familiar Florence test was introduced. Florence did not make any particularly extravagant claims for his test, and appears to have considered it primarily a useful presumptive test that would save the time required to conduct a careful search for sperm cells in every suspected seminal stain. The reagent consisted of 1.65 g KI and 2.54 g iodine in 30 mℓ water. The amount of iodine could be halved without effect. The test was found to be quite sensitive, and was always obtained with seminal stains. The characteristic crystals were not formed by nasal or vaginal mucus, urine, sweat, saliva, tears, milk, cerebral fluid or leucorrheal discharge, nor with several animal semen samples tested. The seminal component giving the crystals was called *virispermine,* and Florence said that it was not identical to Poehl's spermin (Poehl, 1891a and 1891b) which latter, he said, did not give the test. He believed that the test was specific for semen, and that a positive result provided very strong evidence of its presence, even if no spermatozoa could be found. The possibility that other materials, not yet investigated, would give identical crystals was not discounted, however. Johnston (1896) confirmed Florence's results, and said that blood, pus, nasal and vaginal secretions and urethral secretion did not give the test. He got a test on a year old seminal stain. Johnston (1897a and 1897b) noted that he had made some effort to identify the active substance in semen but had been unable to do so. The test was usually carried out on a microscope slide, the sample being put in place, a cover slip added, and reagent allowed to diffuse under the cover slip. The crystals began to form at the interface of the liquids, and were identified visually by microscopical examination.

Richter (1897a) reported that saliva and nasal mucus did not give the test, but that he could get a positive test with vaginal and uterine mucus from dead bodies, as well as with prostatic fluid and various organ extracts from decomposing tissues. He said further that Poehl's spermin did give very similar crystals, but that he did not think it was the active principle in semen. He tested a number of substances to determine whether they would give Florence's crystals. Partly on the basis of the fact that lecithin gave the test, he thought choline might be involved and perhaps other things (1897b). He did think that seminal stains always gave the test, however, and that it had value as a sorting technique. Whitney (1897) showed that seminal stains 2½ years old gave a positive test. No reaction was seen with urine, saliva, milk, fecal matter or leucorrheal or gonorrheal discharges, but morphine, strychnine and a number of alkaloids gave the test. He considered a positive result, therefore, as presumptive evidence of semen. In the following year, Whitney indicated that he thought it was the choline from the lecithin in semen that was reacting with the halogens to form the crystals (Whitney, 1898a and 1898b). That choline was in fact the reactive constituent of semen was established by Bocarius in 1902. Duquenne (1900) conducted a number of experiments on the procedure. The test could be negative in stains that had been soaked in alkali soap water, alcohol and dilute HCl, and several other solvents. Some alkaloids and pyridine and methylpyridine gave the test, but nasal, vaginal and uterine mucus did not. Duquenne's results prompted the Editor of the *New York Medical Journal* to warn, in 1901, that the Florence test should be regarded as presumptive only, until more information about its actual specificity was available. In 1909 and 1910, Dervieux said that Florence's test had no medico-legal value, whether the results were positive or negative.

De Domincis (1912) proposed a modification of the test in which $AuBr_3$ was used in the reagent. He thought that this method was specific, and had medico-legal value. Welsch and Lecha-Marzo (1912) reviewed the subject, but did not accord the test the same proof value as had De Domincis. In 1907, Lecha-Marzo had said that the test was not specific for

human semen. Hektoen and McNally (1923) considered a positive Florence test to be presumptive, but a negative test to indicate the absence of semen unequivocally. Villamil (1934) proposed the use of AuI_3 as the crystallizing agent. This reagent was sensitive to up to a 1:500 dilution of whole semen, but he did not claim any more specificity for the test than would be attributed to Florence's original test. In 1939, Bagchi noted that he had observed many examples of negative Florence tests in the undoubted presence of semen, and that negative inferences could therefore not be drawn. He believed that the test was semen-specific, however, and that positive results were certain proof of semen. Forbes (1940) did not agree. A positive result was presumptive evidence in his view, and negative results did not necessarily establish the absence of semen. Depending on the individual sample, the test failed in Forbes' hands with dilutions of whole semen of from 1:3 to 1:40. Seminal vesicle contents gave crystals in the absence of prostatic secretion, and prostatic fluid alone gave negative results. These results were in disagreement with the contention of Kahane and Levy (1939) who had said in their paper on seminal choline that the choline was contained in semen in a phospholipid, and that a prostatic enzyme was needed to hydrolyze this material and liberate the free choline. Fletcher *et al.* (1935) showed that in rats spermatic fluid was the tissue with the richest choline content. Kaye (1947) reviewed the test and some of its modifications and noted that a positive result was presumptive, but that a negative result indicated that semen was absent. Palmieri (1955) made something of the same point about the semen crystal tests that Dalla Volta (1932) had made earlier with respect to the blood crystal tests (Section 4.2.1), namely that more careful, accurate, physicochemical characterization of the crystals should be carried out before using them as a basis for an identification. His contention applied to the Florence test, and to the other crystal tests which are discussed below.

Takemoto (1970) suggested a curious kind of acid phosphatase test using the Florence method. The stain extract was incubated with choline phosphate in pH 5 acetate buffer. In the presence of AP, choline was liberated upon the hydrolysis of the choline phosphate, and could be detected by means of Florence's reagent.

Kerek (1972) reported no difficulty in getting a positive Florence test in stains which had been kept up to 14 months. Kind (1964) noted that he very rarely got a positive Florence test with vaginal swabs. Davies and Wilson (1974) said that vaginal swabs were often negative. The swabs which did give positive results were all taken within a day of intercourse, and the majority within 14 hours.

It may be noted that Kahane and collaborators conducted a number of studies on the biochemistry, metabolism and tissue distribution of choline. Seminal choline was discussed by Kahane and Levy in 1937. Human semen is said to contain from 11.2 to 14.4 mg choline/100 mℓ semen (*Blood and Other Body Fluids,* 1961). Any tissue or biological material containing sufficiently high choline concentrations would give the Florence test.

10.5.2 The Barberio Test

In 1905, Barberio in Naples reported a different crystal test for seminal fluid. The original paper (1905a) in the Italian language was translated by Dr. A. W. Herzog, and published in the American literature with Dr. Barberio's concurrence (1905b). It was noted that the original claims of species and seminal fluid specificity made by Dr. Florence for his crystal test had not withstood the experimental scrutiny to which it had been subjected. The Barberio test employed a saturated aqueous solution of picric acid. Saturated solutions of picric acid in absolute ethanol served as well. The test was carried out on a microscope slide, and the resulting refractive, yellow crystals examined microscopically. The reaction was said to be very sensitive. Positive results could be obtained with semen, seminal stains and partially putrefied seminal material. Seminal stains which had been heated to 150° for an hour still gave crystals, as did a 3 year old stain. Exposure of the stain to temperatures of 200°, however, abolished the reaction. Vaginal mucus, nasal mucus and saliva did not give the test. Barberio thought that the substance in semen responsible for the reaction was organic, was contained in seminal plasma even in azoospermic specimens, and was different from the reactive substance in Florence's test.

Cevidalli (1906) proposed that the test be carried out with glycerin containing saturated solutions of picric acid in alcohol. He did not get the crystals with dog, horse or pig semen, and thought that the active principle in human semen which was reacting with the reagent might be protamine. Bokarius (1907) used picric acid in nitric acid solutions containing glacial acetic acid or cadmium iodide for the test. Posner (1907) said that the test was specific for human semen. Lecha-Marzo in his 1907 review discussed Barberio's procedure in some detail. He regarded it as more specific than Florence's test, and much preferred it. Littlejohn and Pirie (1908) said that in their experience the test had proven to be specific for semen. The test could, however, be negative in the presence of spermatozoa, but these results had been observed in urine specimens rather than in seminal ones. They noted that they obtained better results with the original reagent than with Cevidalli's modified solution. When positive, the result was considered to be a better indication of the presence of semen than was a positive Florence test. Dervieux (1909 and 1910) said that he put no confidence in the test whatever, regardless of whether positive or negative results were obtained. Lecha-Marzo covered the subject again in his 1918 review, noting that many other body fluids, including vaginal mucus and a number of vegetable extracts, gave negative results.

Baecchi (1913) suggested that the crystals might be spermine picrate. Rosenheim (1924) mentioned that Barberio's crystals were in fact spermine picrate. He was studying spermine phosphate crystals, which form spontaneously in semen if it is left sitting for a time. He noted that the spermine phosphate crystals were first observed and described by van Leeuwenhoek in 1678, and subsequently rediscovered twice,

by Vauquelin in 1791 and by Böttcher in 1865. Poehl (1891a and 1891b) had isolated these crystals, and so-called Poehl's spermin was actually spermine. Harrison (1932) established beyond doubt that Barberio's crystals were spermine picrate. He proposed a modification of the test as well. He said that the search for the crystals was made difficult by all the amorphous protein precipitated by the picric acid. If the stain was extracted with 2.5% trichloroacetic acid, and centrifuged to bring down the protein, the test could be more easily performed on the supernatant fluid, which contained the spermine. Blood, pus, plasma, serum, urine, feces, saliva, and cow's milk all gave negative results with the test. The structure of spermine is shown in Fig. 10.5, and that of choline in Fig. 10.6. Spermine occurs in human seminal

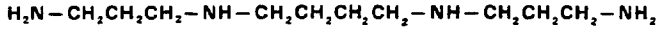


Figure 10.5 Spermine

plasma in concentrations ranging from 20 to 250 mg/100 mℓ semen. (*Blood and Other Body Fluids,* 1961). Fair *et al.* (1972) said that the spermine content of human semen positively correlated with sperm motility.

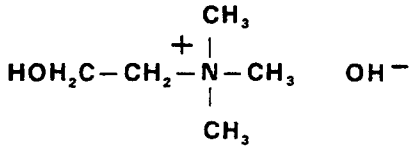


Figure 10.6 Choline

10.5.3 Puranen's Test

In 1936, Puranen proposed a microchemical test for semen using dinitronaphtholsulfonic acid, or Naphthol Yellow S, as reagent. This compound, like picric acid, reacts with spermine to form characteristic orange crystals. He thought that this method was specific for human semen. The structures of Naphthol Yellow S (Naphtholgelb S), which is the salt, and of the corresponding acid, flavianic acid, are shown in Figure 10.7.

Berg (1949) studied the reaction quite extensively. Blood, urine, feces, nasal mucus, saliva, human milk, cheese and vaginal and uterine mucus all gave negative results. Some animal semen reacted in the test, and Berg thought that the test was seminal specific but not human specific. Berg discussed the technique briefly in his 1954 paper as well.

10.5.4 Other crystal tests

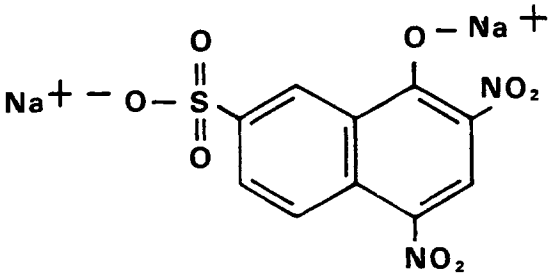
Two other tests have been proposed, but have not enjoyed very wide use. Niederland (1931) said that if seminal stain extracts are treated with dilute H_2SO_4, crystals will form. The test is not semen-specific, and is given by animal semen, vaginal secretions, serum, hen egg albumin and some other materials. It was suggested that the test had primarily negative value.

Peltzer (1931) noted that seminal stains would foam if treated with H_2O_2. If the stain material was treated with 2% eosin, long lance-like crystals, similar to Florence's, formed, and these could be converted to characteristic Florence crystals by the addition of iodine/KI solution.

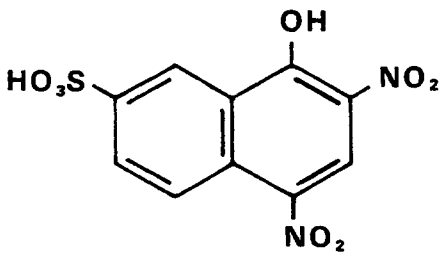
10.6 Chromatographic and Electrophoretic Methods

The chromatographic and electrophoretic methods which have been proposed as means of identifying seminal stains are based on the separation and identification of one or more lower molecular weight substances found in semen in particularly high concentrations. These are primarily choline, spermine and spermidine. Spermidine has the structure $H_2N\text{-}CH_2(CH_2)_2NH\text{-}(CH_2)_3CH_2\text{-}NH_2$, and is closely related to spermine (Fig. 10.5).

In 1957, Fiori noted that spermine and spermidine could be separated from seminal stains by descending chromatography on Whatman No. 1 filter paper in a solvent system consisting of n-butanol:acetic acid:water:40:10:50. The method was sensitive to 1 $\mu\ell$ of semen, and spermine had an R_f value of 0.03. Thoma *et al.* (1959) proposed a procedure in which spermine was extracted into chloroform



(a) Naphthol Yellow S



(b) Flavianic Acid

Figure 10.7 Naphthol Yellow S and Flavianic Acid

from seminal stain material in solutions of K_2CO_3. The chlorophorm phase was then subjected to paper chromatography. Gültingen (1961) studied this technique and concluded that it was unreliable. He also said that Thoma had told him that they had become aware of many of the problems Gültingen had encountered after the publication of their paper, and had not pursued the matter. Levonen (1960) employed ascending paper chromatography in isopropanol:acetic acid:water::50:10:40 to seminal stain extracts. The stains were extracted with 20% trichloroacetic acid, this material extracted with ether, and the aqueous phase evaporated to dryness and redissolved in dilute HCl. 10 $\mu\ell$ of semen in a stain was required for a positive result. Using Dragendorff's reagent, spermine appeared as a pink spot at R_f 0.32, while choline appeared as a deep purple spot at R_f 0.74. Satch *et al.* (1967) reported a virtually identical method, except that choline was said to have an R_f value of 0.79. They tested vaginal secretions and menstrual blood as well, with negative results, and these contaminants did not interfere with the chromatography. Stains up to 10 days old could be diagnosed. Gültingen (1961) said that he did not think the paper chromatography methods were very reliable. Djalalov (1974) reported a paper chromatographic procedure for the simultaneous separation and detection of spermine, choline, acid phosphatase and seminal amino acids.

Hessel *et al.* (1967) reported the detection of spermine and choline from as little as 1 $\mu\ell$ semen by thin layer chromatography. 1N HCl was used as an extraction medium and as developing solvent on 250 Silica Gel F plates. Choline was detected with Dragendorff's reagent at R_f 0.5, and spermine was detected at R_f 0.85 by overspraying with potassium iodoplatinate reagent. It was found that purified spermine and choline ran slightly faster than when contained in seminal plasma.

Yano (1970) reported a method for the detection of spermine and choline by thin layer chromatography. 10 $\mu\ell$ seminal plasma was required for the test. Stains up to 5 years old gave positive results, and boiling the stain in water for 1½ hrs did not destroy the spermine or the choline. A number of other body fluids, including vaginal secretions, and the juices of a number of fruits were found to contain no detectable choline or spermine. Hallcock (1974) reported a method similar to that of Hessel *et al.* (1967). Saline was used to extract the stain and 30 $\mu\ell$ of the extract was applied to the plate. She developed the spermine spot with potassium iodoplatinate (R_f = 0.85) and the choline spot by over-spraying with concentrated H_2SO_4 (R_f = 0.58).

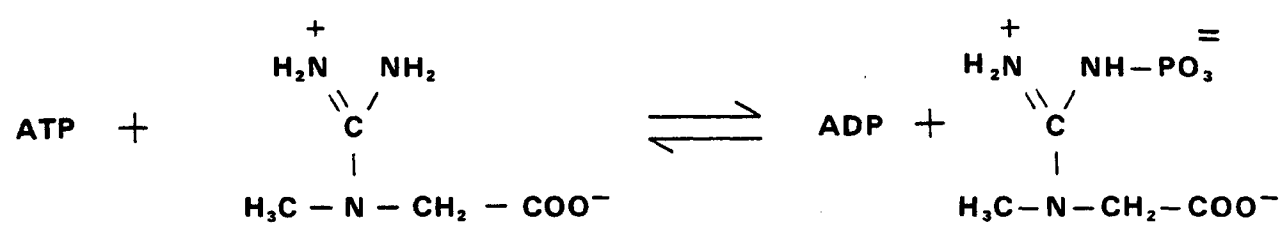
Bureš (1968) used paper electrophoresis in pH 4.6 citrate buffers for the separation of spermine and choline. Samples were extracted with 0.5N HCl, and the extracts spotted anodically. After electrophoresis for 15 min at 15V/cm, the paper was dried and sprayed with Dragendorff's reagent. Vaginal secretions, saliva, perspiration, urine and blood stains gave negative results, and did not interfere.

Kosatík *et al.* (1966) used ascending paper chromatography in butanol:formic acid:water::10:1:5 to detect citric acid for the identification of semen. Semen, it was said, contains from 80 to 410 mg % citric acid, while urine may contain 20 to 120, human milk 127 and other body fluids tested, including vaginal secretions, less than 10. Zipkin and McClure (1949) reported that human saliva contains only 0.2 to 2.0 mg % citric acid. Stains from 3–5 weeks old gave positive results, and the R_f of citric acid in the system was 0.42-0.47. The presence of citric acid in human semen was first noted by Schersten (1930). The physiological role of citric acid in semen was discussed by Humphrey and Mann (1948). Seminal citric acid originates in the seminal vesicles. Kirk had suggested the possible use of citric acid as a seminal marker in 1953.

Nickolls (1956) noted that the detection of fructose might prove valuable as a basis for seminal stain identification, but at that time no one had yet explored the possibility. Fructose is present in human semen, and is the principal reducing sugar (Mann, 1946a). Spermatozoa metabolize the fructose along the principal fermentative carbohydrate pathway, a process called fructolysis. Most cells metabolize glucose principally along this pathway (glycolysis). Once fructose-6-phosphate is formed, of course, the pathways are identical. Seminal fructose originates in the seminal vesicles, and its levels are responsive to testosterone concentration. The metabolism and physiological role of fructose in semen have been extensively studied (Cf Mann, 1945, 1946a, 1946b; Mann and Parsons, 1947). Erben (1971) showed that seminal fructose could be separated from seminal stains by paper or thin layer chromatography, and readily distinguished from glucose or sucrose. Kirk (1953) noted that a kind of paper chromatographic method very similar to the one which had been described for separating blood stains from debris (Cf section 7.2) was equally applicable to the separation of seminal stain materials from debris.

10.7 Creatine Phosphokinase

In 1964, Griffiths and Lehman suggested using the high levels of creatine phosphokinase in semen as a basis for the medico-legal identification of seminal stains. Creatine phosphokinase (creatine kinase; CPK; EC 2.7.3.2) is systematically known as ATP:creatine phosphotransferase, and catalyzes the reaction:



According to these investigators, semen contains from 385 to 14,000 units CPK per mℓ, a concentration higher than in any other body fluid tested, including normal and pathological serum, feces, gastric juice, urine and vaginal secretions. In stains the activity declined as a function of stain age. At 24 hours of age, stains showed a mean activity of 142 mols creatine formed/mℓ extract/hr. The reaction was measured using creatine phosphate and ADP as substrates. By 1 month of age, the mean activity had dropped to 132, and by 6 months of age, to 78. They said the test should be regarded as presumptive evidence for the presence of semen.

In 1968, Suyama *et al.* showed that seminal acid phosphatase hydrolyzes creatine phosphate in the absence of ADP. If a specific SAP inhibitor were added, the CPK activity of semen was found to be very low. They believed that Griffiths and Lehman had actually been measuring the AP activity of semen with creatine phosphate as substrate, and that the method was, therefore, not only not new, but in no way superior to any other acid phosphatase test.

10.8 The Lactic Dehydrogenase-X-Isoenzyme

A brief discussion of lactic dehydrogenase (LDH) was given in section 8.1.5. LDH activity in human semen was first reported by MacLeod and Wroblewski in 1958. They measured total activity in a number of samples, and found that LDH activity was lower in the seminal plasma than in whole (sperm-containing) semen. In 1963, Blanco and Zinkham reported that they had observed an LDH isoenzyme unique to human sperm. It had an electrophoretic mobility on starch in pH 8.6 buffers in between that of LDH-3 and LDH-4. In addition, its heat stability, kinetic properties with pyridine nucleotide analogs and chromatographic behavior on DEAE-Cellulose were likewise intermediate between those of LDH-3 and LDH-4. The enzyme did not occur in seminal plasma, nor in prepubertal testicular tissues. Goldberg, in the same year, independently confirmed the observation using polyacrylamide disc gel electrophoresis. He found that the enzyme could be solubilized by exposure of the sperm cells to sonic oscillations. In 1967, Farriaux *et al.* suggested the application of LDH-X, as the new isoenzyme had been named by its discoverers, to the diagnosis of seminal stains. The enzyme could be found in 35 day old stains, and showed a greater resistance to inhibition by 2M urea than some of the other LDH isoenzymes, and incorporation of the inhibitor into the procedure simplified the detection of LDH-X. The isoenzyme can be detected in men with low sperm counts (10^6 cells/mℓ), as well as in those with necrospermia and altered sperm morphology (Szeinberg *et al.*, 1967). The LDH-X activity as a fraction of total seminal LDH activity varies from one individual to another, and the isoenzyme was not detected in one person with a sperm count of 22 x 10^6/mℓ (Eliasson, 1967). Studies with split ejaculates indicated that the prostate was the primary source for the LDH isoenzymes other than X. Zinkham reviewed the subject in 1968, and again in 1972, noting that the enzyme is unique to sperm cells. The enzymatic and biochemical properties, in comparison with some of the other seminal LDH isoenzymes, were given as well. It was clear, at least in birds, that another structural gene, controlling the synthesis of an additional polypeptide chain, is involved in LDH-X production (Zinkham, 1968). Ressler *et al.* (1967) obtained data in their immunological experiments with anti-LDH-1, anti-LDH-5 and anti-LDH-X which suggested that LDH-X synthesis in humans was controlled by a third allelic locus. Evrev *et al.* (1970) noted that the isoenzyme could be found in tissue cultures of mature human testes and of seminiferous tubule cells, as well as in sperm. Dixon and Gonsowski (1974) mentioned LDH-X in their studies on the use of the LDH isoenzyme determination for menstrual blood identification (see section 8.1.5). Schmechta (1975) reported that the LDH-X activity averaged about 29% of total LDH activity in 12 samples, and that the enzyme could be detected on cellulose acetate membranes from stains up to 36 days old. Hule and Hrdlička (1975) said that LDH-X is sometimes absent in normospermic specimens. Mokachi and Madiwale (1976) recommended a disc gel electrophoretic procedure for examining seminal stains for LDH-X, and said that this method might actually be better than searching for spermatozoa in older stains. The procedure would, of course, be of no value in diagnosing azoospermic samples.

10.9 Sperm and Seminal Esterases

Nonspecific esterases in sperm were described by Beckman and Kjessler (1968). They found up to six electrophoretically separable zones of esterase activity in washed spermatozoa which were not of seminal plasma origin. Two of the zones may have been artifacts of the preparation method. Some individuals showed one zone, others showed two, and still others, three. Not all the sperm specimens examined had activity. Prasad *et al.* (1976) found that the fastest moving α-esterase was absent in vasectomized and oligospermic patients.

Of greater interest for seminal stain identification are the seminal plasma esterases. Tran Van Ky and Muller (1968) carried out a fairly extensive study of some of the enzymes in human seminal plasma. Immunoelectrophoresis was employed to assess the total antigenic protein composition; in addition, some of the precipitin arcs were identified by specific enzyme staining reagents. One lipase, two alkaline phosphatase, one chymotrypsin, two leucine aminopeptidase (see Section 8.2.3) and four esterase antigen-antibody complexes were identified in the immunoelectrophoretogram. All four esterases hydrolyzed α- or β-naphthyl acetate, but only certain ones were active with indoxylacetate, butyrylthiocholine and carbonaphthoxycholine. Only one of the esterases was common to serum. It was also found in the course of the studies that the antibodies to the various enzymes do not all appear in the immunized rabbits at the same time. Weekly injections were given, and the time of appearance of the various anti-enzyme antibodies determined. It required

70 days to obtain an anti-seminal plasma antiserum containing all the anti-enzyme antibodies reported. The authors noted the potential medico-legal application of the technique, and said that it should be superior in many ways to other methods, combining, as it does, the resolving power of immunoelectrophoresis with the specificity afforded by the specific antibodies and the specific enzyme stains. In 1970, Darwiche *et al.*, using a kind of crossed over electrophoretic method, tested the esterase identification method with seminal stains. The enzymes were found to be relatively thermostable. Several extracting solutions were tried with stains of varying age. The best results were obtained with a pH 7.4 phosphate buffer containing 10% Triton-X-100 and 10% "sputolysine" (the latter apparently a commercially available nonionic detergent). Stains from 6 to 12 months old gave positive results with this extraction medium, but stains 14 years old did not. They believed this method to be specific and relatively simple, and recommended it for seminal stain identification. Evrev (1971) tried the technique and recommended it as well. Evrev (1970) had described two esterases which hydrolyzed α-naphthylacetate, and which were detectable in immunoelectrophoretic precipitin bands. These could be detected in stains up to 30 days old, and were absent from serum, vaginal and cervical mucus, urine, saliva, nasal mucus and from the semen of bulls and rams. Roberts *et al.* (1972) characterized these seminal esterases, with β- and $\beta\gamma$- electrophoretic mobilities. The α-mobility enzyme was not always present and formed a complex with lactoferrin. The $\beta\gamma$-mobility enzyme was heterogeneous on starch gel electrophoresis, but gave a single peak on Sephadex G200 which indicated a MW of about 69,000–77,000. Hermann (1972a) described a cholinesterase and a nonspecific aliesterase in seminal plasma, both of which were detectable in antigen-antibody complexes following immunoelectrophoresis. The latter was said to be of prostatic origin.

10.10 γ-Seminoprotein

In 1969, Hara *et al.* carried out immunoelectrophoretic studies on seminal plasma with antisera to human seminal plasma. A number of precipitin lines were observed. One of these could be shown to be a protein common to semen, colostrum and saliva, but not serum. They called this protein β-seminoglobulin (β-Sm). Lactoferrin was also observed, and was called seminoferrin. One protein was found to be present in all seminal samples, and could be detected with anti-human semen serum which had been absorbed with serum, saliva and colostrum. This component was believed to be semen specific, and was named γ-seminoprotein (γ-Sm). Yamasaki (1971) in a similar set of experiments identified an α_1-glycoprotein which was common to semen, saliva and colostrum, and this protein was called α-seminoglycoprotein.

Hara *et al.* (1971) partially purified γ-Sm, and found it to be trichloroacetic acid precipitable and thermolabile. Its antigenic activity was abolished by exposure to papain or trypsin. The material gave a single precipitin line upon immunoelectrophoresis, but could be separated into several components, all with activity, upon polyacrylamide disc gel electrophoresis. Three γ-Sm components with isoelectric points between pH 5.8 and 7.1 could be observed in a purified preparation by isoelectric focusing (Koyanagi *et al.*, 1972). The purified preparation contained 74.7% protein, 0.8% sialic acid, 1.6% fucose, 3.6% mannose, 1.5% glucose, 2% galactose, 2.4% glucosamine and 1.3% galactosamine (Hara *et al.*, 1972a). The MW was estimated on Sephadex thin layers to be 28,000–30,000 (Hara *et al.*, 1972b). By sedimentation equilibrium methods, the protein was found to have a sedimentation coefficient of 2.6S, corresponding to a MW of about 19,900 (Koyanagi *et al.*, 1973a). Examination of 18 samples of seminal plasma from healthy donors indicated that the γ-Sm concentration was 1 to 6.25 mg/mℓ if a ring test was used, and 2–10 mg/mℓ if a radial immunodiffusion test was used (Hara *et al.*, 1973a). It could be shown (Hara *et al.*, 1973b) that γ-Sm was not identical with IgG or any of its components, IgA, IgM, IgD, IgE, GOT, GPT, LAP, LDH, a hyalouronidase or its inhibitor, hyaluronic acid, lysozyme, plasminogen, plasmin, thrombin, fibrinogen, carboxylesterase, PGM, 6PGD, prostaglandins or the 3.72S component of Herrmann and Hermann (1969a, 1969b) (see section 10.13.2). In 1974, Koyanagi showed that γ-Sm had four -SH groups which reacted with p-CMB, and that a very pure preparation had a sedimentation coefficient of 2.55S, corresponding to a MW of 22,500–24,000. Estimation of the MW of this pure preparation on Sephadex and Sepharose yielded a value of 23,000 (Koyanagi *et al.*, 1975a). The molecular heterogeneity of the purified preparation on polyacrylamide disc gel electrophoresis was believed to be the result of variability in the number of sialic acid residues attached to different molecules (Koyanagi *et al.*, 1975b). Hara and Inoue (1975) recommended the use of the immunological test for γ-seminoprotein as a sensitive and specific method for identification of semen in stains.

10.11 Other Methods

Two additional enzyme markers have been suggested as bases for seminal stain identification tests. In 1948, Berg found that semen and retroplacental blood contain much higher levels of diamineoxidase than do other body fluids. The diamineoxidase is a histaminase, and acts upon other substrates containing amino groups as well. He showed that the enzyme was present in azoospermic samples, and thought that this would be a good method for diagnosing seminal stains, provided blood shed at birth or abortion could be excluded. Laves (1948) assayed seminal hyaluronidase, which is a component of the sperm cell, but may occur in seminal plasma to some extent as well. He thought that this enzyme could be used as a marker for semen in stains in medico-legal cases. Neither of these techniques have been widely used. Berg (1954) mentioned them, and noted that the assays are particularly difficult and involved, and that acid phosphatase determination is probably preferred by most workers for that reason.

In 1926, Marcusson-Begun described a non-specific agglutinin in potato juice. Human and a number of animal red cells were readily agglutinated by this lectin. The agglutinin has been purified and found to be a glycoprotein containing arabinose (Marinkovitch, 1964). In 1965, Barsegyants observed that human semen inhibits red cell agglutination by the potato phytagglutinin, and on this basis, a hemagglutinin inhibition test for semen and seminal stains was devised. In 1970, he said that even seminal stains which had been washed could be detected in this way (Barsegyants, 1970a). Jakliński *et al.* (1968) carried out studies on the technique and found it to be satisfactory for seminal stain identification. It is not specific, however, since human serum and human milk both inhibit the agglutination reaction as well.

In 1954, Fiori conducted studies of a color test for spermine in terms of its applicability to the medicolegal identification of seminal stains. This test, he referred to as the Fuchs-Tokuoka reaction. Fuchs (1939) had observed that spermine reacts with copper carbonate powder to give a deep blue product upon heating. Tokuoka (1950) said that he had found spermine in neoplastic tissue and in the serum of cancer patients, and was interested in assaying the spermine as a means of diagnosing the disease. He used the copper-carbonate powder reagent to test for spermine in serum, and said that the reaction was very sensitive. Fiori (1954) found that the test compared favorably to Puranen's test (see Section 10.4.3) Although the Fuchs-Tokuoka reagent did not detect semen in stains at the lowest concentration tested in the sensitivity studies, and Puranen's reagent did detect it, both reagents reacted with the other seminal samples tested, and gave negative reactions with a variety of other body fluids and secretions which could potentially contaminate seminal stains. Gültingen (1961) could obtain positive Fuchs-Tokuoka reactions only with fluid samples. Stains, even fresh ones (5 days old), did not give the indicated color change.

Suzuki *et al.* (1980) described an enzyme-coupled technique for the determination of spermine in stains, which employed a bovine amine oxidase. The procedure was quick, they said, and could be used as a preliminary screening test.

Cortivo *et al.* (1979) examined the amino acid composition of several body fluids and secretions including semen. They noted that the amino acids present, and their relative levels, were characteristic, and suggested that the profile might provide an identifying marker pattern for semen, urine, vaginal secretions and sweat. Stains prepared from these fluids retained the characteristic patterns.

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.

10.13 Composition of Semen

A number of constituents of semen, which have been employed as markers for the identification of seminal stains, have been discussed individually in Sections 10.1–10.9. In this section, a more general, over-all discussion of the composition of semen is given. Apart from the spermatozoa, seminal plasma may be thought of as containing three major categories of components: low molecular weight compounds; enzymes; and non-enzymatic proteins. All the proteins, whether they possess an enzymatic activity or not, are potentially antigenic, and preparations of antisera to seminal plasma may contain antibodies to any of them. In addition, there are a number of antigenic proteins on the sperm cell, which may be intrinsic cellular constituents, or seminal plasma proteins which have been absorbed onto the cells (so-called sperm-coating antigens). The soluble antigens of the ABH and Lewis blood group systems will not be considered in this section, but in a later one.

10.13.1 Sperm cell antigens

The spermatozoan has various antigenic components (Scacciati & Mancini, 1975). The corresponding antibodies may be sperm agglutinins, may arise spontaneously, i.e., autoantibodies (Samuel *et al.*, 1975), and are important in considerations of fertility and sterility. Sperm agglutinating antibodies sometimes arise in women, presumably due to an antigenic stimulus, although it is not altogether clear how this process takes place (Tyler and Bishop, 1963). Various sperm agglutinins are known which bring about head-to-head agglutination, while others cause tail-to-tail agglutination (Friberg & Tilly-Friberg, 1977). The literature on this material is quite extensive, and since the subject is not of major importance to the medicolegal identification of semen, it is not reviewed in detail. The reviews of Tyler and Bishop (1963) and of Shulman (1971 and 1974) may be consulted for details and documentation. Voisin *et al.* (1975) noted that sperm antigens may be characterized as autoantigens, in which antibodies may be induced in the same organism which produced the antigen, and alloantigens, in which antibodies may be produced in other members of the same species. They used the term isoantigens to refer to spermatozoal antigens which cause the production of antibodies in women, and which may be found in serum, cervical mucus or both.

10.13.2 Seminal plasma proteins

Most of the non-enzymatic proteins of seminal plasma have been detected and studied by immunological methods. Any of the proteins may be an antigen. Some of the components are serum proteins, while others are specific to semen (Searcy *et al.*, 1964; Leithoff and Leithoff, 1965a). All the immunological tests for semen are based on the use of antisera prepared against seminal proteins. Most often, the antisera are complex mixtures of antibodies; in a few cases, such as with anti-SAP or anti-γ-Sm, they are prepared against specific components. The antigens of seminal plasma are of great importance in forensic immunology, for the immunological tests for semen in stains are not of great value if they cannot be employed in the detection of azoospermic samples.

Any number of workers have used immunoelectrophoretic techniques to study seminal plasma antigens, and up to a dozen precipitin arcs can usually be observed. The major arc areas have sometimes been designated by letters, "A" being most anodal. Individual components within the areas are then called A_1, A_2, C_3, and so forth. But the nomenclature is by no means universal. Similar designations may be used by different investigators to denote different components, and the relationships between the different components reported by different laboratories are seldom clear. There is some evidence that the antigenic composition of prostatic fluid may be different from the prostatic antigenic composition of ejaculated semen (Barnes *et al.*, 1963 and 1965). Shulman and Li (1974) and Li and Beling (1973) purified their E_1 protein component, and found that it has a MW of about 31,000 Li *et al.* (1976) purified the B_1 component, finding it to be a sperm-coating lactoferrin of seminal vesicle origin with a MW of about 33,000. Hekman and Rümke (1969) had established that lactoferrin as a prominent sperm coating antigen in seminal plasma. Herrmann (1966) purified his B_2 component, finding it to be a ceruloplasmin derivative. Herrmann and Hermann (1969a and 1969b) have purified a component having a sedimentation coefficient of 3.72S, corresponding to a MW of about 50,000. These proteins were among the eight immunoelectrophoretically distinguishable components previously described (Herrmann and Schirren, 1963). The 3.72S protein does not change its electrophoretic mobility upon treatment and neuraminidase, indicating that N-acetyl-neuraminic acid residues have not been removed (Herrmann, 1972b). The existence of sperm-coating antigens was discovered by Weil *et al.* (1956) who observed that antibodies to seminal plasma could be obtained by immunization with washed spermatozoa.

Acharya *et al.* (1966) studied seminal antigens that migrate with γ-globulin mobility on agar gel electrophoresis. These were found to be trichloroacetic acid soluble, and could be separated into three components, called TSC-1, TSC-2 and TSC-3. TSC-2 was identified as a sialomucoprotein (Kaleker *et al.*, 1967). Amano & Behrman (1968) isolated two antigens from pronase-treated seminal plasma. Both were mucoproteins, and had MW of about 40,000 and 20,000. Scacciati de Cerezo (1974) isolated two glycopeptide antigens from the trichloroacetic acid soluble fraction of seminal plasma, and determined that both their molecular weights were in the neighborhood of 10,000.

Kojima (1966) observed seven precipitin arcs by immunoelectrophoresis, calling them ρ, A_1, and P_1 through P_5, where P_5 was the most cathodic. Three of these were said to be unique to semen. Yamasaki (1971), using an anti-human semen serum which had been absorbed with serum, colostrum and saliva, as well as an unabsorbed serum, said that γ-Sm was the only component unique to semen. Albumin, an α_1-glycoprotein, α_1-antitrypsin, α_1-lipoprotein, transferrin, ceruloplasmin, Hp, fibrinogen, IgG, IgA and IgM were found to be common to serum and semen. Lactoferrin, β-SM and α-seminoglycoprotein were common to semen, saliva and colostrum. Quinlivan and Sullivan (1972) identified albumin, transferrin, lactoferrin, AP, serum β_1-globulin, IgG, seminal vesicle β_1-globulin, seminal vesicle β_2-globulin and a prostatic β_2-globulin in semen. Tauber *et al.* (1975) studied split ejaculates to try and determine the particular accessory gland origin of some of the seminal components. IgG, IgA, albumin and transferrin seemed to originate from other than the seminal vesicles, while fructose and lactoferrin were probably of seminal vesicle origin. IgM, β_1C/β_1A-globulin (C'3 component of complement), ceruloplasmin and fibrinogen could not be detected. In 1976, Tauber *et al.* continued these studies, and indicated that lysozyme, α-amylase, neutral proteinase and plasminogen inhibitor occurred in the initial fraction of the ejaculate, indicating prostatic or Cowper's gland origin. The high MW proteinase

inhibitors were of approximately the same concentration throughout the fractions, except for the pancreatic trypsin inhibitor activity which appeared to be of seminal vesicle origin. Plasminogen, prothrombin, clotting factor VIII, and several proteinase inhibitors were not detected by immunoelectrophoresis.

Recently, Sayce and Rees (1977a) looked at seminal plasma antigen profiles of a number of men, some attending a fertility clinic, and some post-vasectomy patients. Very little or no Gc-globulin, prealbumin, haptoglobin, plasminogen, cholinesterase, fibrinogen or α_2-macroglobulin was observed in these people, and no differences between the groups could be detected. Orosomucoid (α_1 acid glycoprotein), α_1-antitrypsin, albumin, transferrin, ceruloplasmin and lysozyme were detected in addition to the proteins mentioned above. Subsequently, 400 post-vasectomy patients were studied with respect to the orosomucoid component (Sayce and Rees, 1977b), using anti-human orosomucoid and anti-human α_1 acid glycoprotein sera obtained commercially (see also in Section 45.4). About 14% of the men gave a strong precipitin arc in immunodiffusion tests, there being no difference between post-vasectomy patients and patients attending infertility clinics. The orosomucoid was of serum origin. Those men not showing the strong (S) reaction showed a weak (W) reaction with the antisera, but the precipitin band was not found in the same location. Selective absorption experiments showed that the S reaction band was different from the W reaction band, i.e., that the reactions were being given by non-identical antigens. The possibility of the strong reaction precipitin band as a marker in human semen in medico-legal inquiries was raised, but the necessity of carrying out further studies to verify the specificity was noted. It could be shown that the reactivity was consistent within the same individual over a considerable number of months.

10.13.3 Enzymes and low molecular weight components of seminal plasma

Suominen *et al.* (1971) determined the activities of proteinase, AP, and an esterase in 205 seminal samples. They determined the fructose concentration as well. Semen was found to have a high fibrinolytic activity, a fact which could have serious implications in attempting to interpret results of fibrinolytic assays for menstrual blood identification if semen were present (Cf Section 8.1.2). The proteinase activity was assayed with casein as substrate, and the esterase activity was one which hydrolyzed the synthetic ester p-toluenesulfonyl-L-arginine methyl ester. Molnar *et al.* (1971) studied the ejaculates of individuals suffering from pathological conditions which caused seminal vesicle constituents to be absent. Citric acid and acid phosphatase were clearly of prostatic origin, but they believed the seminal vesicles to be the source of LDH and GOT. Nun *et al.* (1972) reported that SAP levels were significantly higher in vasectomized and azoospermic patients. Atanasov and Gikov (1972) reported that they could localize LDH, MDH, AP, alkaline phosphatase, GPT, GOT, LAP, peroxidase and α-amylase in polyacrylamide disc gels after electrophoresis of prostatic fluid. Blake and Sensabaugh (1975) surveyed human semen for enzymes displaying polymorphism, and found that AK, antitrypsin, amylase, esterase-D and peptidase-A were all present. It had been known previously that semen contains PGM.

10.14 Seminal Protein p30

Sensabaugh (1977 and 1978) has recently reported isolation of an apparently seminal fluid-specific protein, designated p30. The protein is antigenic, and was detected as one of a number of precipitin arcs given by seminal plasma by immunoelectrophoresis against anti-human seminal plasma serum. The protein has been purified to a significant degree, and specific antisera have been raised against it. It was strongly suggested that monospecific antisera to semen-specific proteins, as p30 appears to be, would provide the soundest basis for an immunological test for the identification of semen in stains. Sensabaugh could show that p30 reacted with an antiserum prepared against the E_1 component of Li and Beling (1973) (see above), indicating that E_1 and p30 are immunologically identical if not in fact the same protein. The p30 protein is a glycoprotein with MW about 30,000, probably prostatic in origin. Upon subjection to isoelectric focusing, the protein splits into several bands with isoelectric points between pH 6.5 and 8.0. The concentration of the protein in 17 men ranged from 0.24 to 5.5 mg/mℓ. These biochemical characteristics are quite similar to those which have been reported for γ-seminoprotein (section 10.10), and although direct experimental comparisons would be required to test the two proteins for identity, it may be the case that p30 and γ-seminoprotein are identical. Specific antisera to p30 have been tested with a variety of materials, including stomach contents, bile, cow milk, egg yolk, egg white, coffee, coke syrup, karo syrup, detergents, suntan lotions, Conceptrol birth control foam, several commercially available lotions, and several animal semens, and been found to be unreactive. The antiserum has been employed on casework materials as well with completely satisfactory results. Seminal stains up to a year old reacted with anti-p30. The protein was not always detected in vaginal washings from sexual assault victims, probably because the protein was too dilute.

The concentrations of a number of substances in human seminal plasma are shown in Table 10.3.

Table 10.3 Concentrations of Some Components of Human Seminal Plasma

Substance	Average Content (in mg/100 mℓ semen unless otherwise noted)	Range (same units as average)
Water (%)	91.8	89.1-94.4
Ca^{++}	18	7-30
Cl^{-}	43	28-57
Mg^{++} in meq/ℓ	12	–
Na^{+}	117	100-133
Zn^{++}	14	5-23
Total Phosphorus	112	90-120
Inorganic Phosphorus	11	–
Total protein	4.1	3.4-5.5
Acid Phosphatase★	370,000	50,000-800,000
Spermine	–	20-350
Choline	–	11.2-14.4
Citric Acid	480	0-2,340
Fructose	300	50-600
Urea	72	–
Uric Acid	6	–
Creatine	20	–
Sialic Acid	–	64.5-219

Data from Blood and Other Body Fluids (1961), White and Macleod (1963) and Santoianni (1967)

★ in King-Armstrong units (pH not given)

SECTION 11. IDENTIFICATION OF SALIVA

Saliva is encountered much less frequently than are blood and semen in evidentiary materials submitted for examination. Accordingly, it has received comparatively less attention in the literature over the years. Many authorities have not brought up the subject of saliva stain identification at all (e.g. Vibert, 1980; Hamilton and Godkin, 1894; Tidy, 1884; Lucas, 1935 and 1945; Simpson, 1965; Glaister, 1931; Gradwohl, 1954). The methods for identifying saliva are based primarily on the identification of the inorganic anions thiocyanate and nitrate, or of the enzymes alkaline phosphatase or amylase. Microscopical and immunological methods have been employed as well.

11.1 Identification of Inorganic Ions

11.1.1 Thiocyanate

According to Krüger (1898), the presence of thiocyanate in human saliva was first noted by Treviranus in 1814, but the substance was only identified as being thiocyanate twelve years later by Tiedemann and Gmelin in 1826. The old literature on the subject was reviewed by Krüger (1898) and again by Lickint (1924). In 1948, Fischman and Fischman determined that normal saliva contains from 3.1 to 27.5 mg SCN^-/100 mℓ saliva. The average content for nonsmokers was 11.7, while for smokers it was 17.5. These values are quoted in *Blood and Other Body Fluids* (1961) as well. Ruddell *et al.* (1977) determined that the concentration of thiocyanate in saliva was about 1.6 mM, or about 93 μg/mℓ (or 9.3 mg/100 mℓ). The average content was about 2.5 times higher in smokers than in nonsmokers.

Thiocyanate may be detected by addition of a small amount of dilute $FeCl_3$ to the slightly acidified sample. A pink to red color indicates a positive test. This technique is mentioned in the forensic literature by Mueller(1953), Kirk (1953) and by Dérobert (1974). The most extensive experiments on the method were done by Nelson and Kirk (1963). Fresh saliva was found to contain 150 μg SCN^-/mℓ. Freshly dried stains gave comparable values, and aqueous extracts of 7½ month old stains showed about 67% of the original concentration. After 28 months, stains on linen retained about 20% of the original concentration, while stains on cotton had only about 5%. The test is sensitive to about 3μg SCN^-, and with older stains, more and more stained cloth had to be eluted to achieve a positive result. It is believed that thiocyanate does not occur in these concentrations in other body fluids, and the test, when positive, was considered specific. Thiocyanate may be absent, or in undetectably low concentrations, however, and a negative test does not exclude the presence of saliva in the stain (Mueller, 1975). Berg (1957) said that a positive test should not be regarded as a specific test, and Dérobert (1974) does not appear to regard it as conclusive either.

11.1.2 Nitrite

Detection of nitrite anion for the identification of saliva stains was studied by Nelson and Kirk (1963). NO_2^- was detected by the Griess test (Cf Feigl and Anzer, 1972), in which α-sulfanilic acid is allowed to react with the nitrite to form a diazonium compound, which reacts with α-naphthylamine in the reagent to form the pink to red product, p-benzenesulfonic acid-azo-α-naphthylamine. A reagent consisting of 0.5% α-naphthylamine in 5N acetic acid and 0.8% sulfanilic acid in 5N acetic acid was used in the salivary stain studies. Saliva contained about 10 μg NO_2^-/mℓ, but even in stains which had been dried a few weeks previously, the ion was undetectable. This fact, coupled with the presence of nitrite in decaying nitrogenous matter, severely limits the applicability of this test. It was noted that a positive result would, however, give an indication that the stain had been deposited relatively recently. Ruddell *et al.* (1977) determined that saliva contained an average of about 100.4 μM NO_2^-, which is of the order of 4.6 μg/mℓ, in good agreement with the findings of Nelson and Kirk (1963). Gastric juice contained only about 5% as much nitrite as saliva.

That nitrite occurs in human saliva has been known at least since the work of Savostianov in 1937, who said that the concentration varied from 0.01 to 0.1 mg %. Ville and Nestrezet were said by Varady and Szanto (1940) to have suggested that the presence of nitrite in saliva could be accounted for by oral microbial reduction of dietary nitrates which are secreted in saliva. Savostianov had an identical view in 1937. Studies on the effect of dietary nitrate intake on salivary nitrite have indicated that ingested nitrates are indeed the source of salivary nitrite (Spiegelhalder *et al.*, 1976; Tannenbaum *et al.*, 1976).

11.2 Alkaline phosphatase

Nelson and Kirk (1963) assayed saliva samples for alkaline phosphatase in pH 9 barbital buffers. It was found that 24 hr incubation periods were required, and the activity of whole saliva was about 540 μg phenol liberated from phenyl phosphate/24 hrs at 37° per mℓ. Freshly prepared salivary stains had similar activity, but stains stored for 7½ months retained only about 20% of the original activity. Since alkaline phosphatase is found in many other physiological fluids, this test cannot be considered more than presumptive, but it may have some value in corroborating the presence of saliva in stains. Moon and Bunge (1968a) reported, for example, that 55 samples of seminal plasma had alkaline phophatase

activity ranging from 2.3 to 106.6 Sigma units/mℓ. The presence of alkaline phosphatase in human saliva was first noted by Chauncey *et al.* in 1954. It is not clear whether the enzyme originates in the salivary glands or in the oral epithelium (Levitskii *et al.*, 1973). There may be some contribution from the oral flora as well. With p-nitrophenylphosphate as substrate, saliva had an alkaline phosphatase activity of about 0.04 μmol p-nitrophenol liberated/min/mℓ (Lindqvist *et al.*, 1974). Pini Prato (1970) said that two alkaline phosphatase activity bands could be isolated from human saliva by polyacrylamide disc gel electrophoresis.

11.3 Amylase

11.3.1 Applications of amylase detection to saliva stain identification

Detection of amylase, and in some cases quantitative determination of its activity, is almost unquestionably the most extensively used test for the identification of salivary stains. Amylase is one of the oldest enzymes known. Chittenden (1881) who studied the enzyme quite extensively, said that the activity in human saliva was first noted by Leuchs in 1831. Dixon and Webb (1964) noted that Payen and Perzoz had described an amylase activity in alcoholic precipitates of malt extract in 1833, which they had called "diastase". This term persisted in the literature for many years. Later, salivary amylase came to be known as "ptyalin". Roberts (1881) said that Kühne had proposed the general name "enzyms" for the soluble "ferments", including amylases, and Roberts suggested that the term be Anglicized as "enzymes". Roberts was conducting studies of pancreatic amylase and his paper is one of the oldest references to the now familiar starch-iodine test. Iodine solutions cause starch to turn a deep blue color. In the presence of starch-hydrolyzing enzymes, the disappearance of the blue color can be used as an indicator of the progress of the reaction. Schoch (1961) noted that iodine reacts with the linear molecule amylase rather than with amylopectin (see section 11.3.2) to give the blue color. Amylose in solution forms helices, each turn of the helix containing six glucose units. The blue color is attributed to the binding of one diatomic iodine per turn of the amylose helix. The color intensity and shade will depend on the number of helical turns and, therefore, on the length of the chain. Amylose chains of less than 12 glucose units and two helical turns give no color. As chain length and number of helical turns increase, the color is brown, red, purple, and finally the characteristic blue at chain lengths of more than 45 residues and 9 helical turns. Because amylopectin has branches of about 20–30 residues, Schoch said that it gives a red color with iodine.

Mueller (1928) was the first medico-legal investigator to suggest the identification of salivary amylase as a basis for diagnosing salivary stains. Using 8 hr incubations, he detected substrate disappearance using Lugol's iodine, and product (glucose) formed by means of Fehling's solution. He carried out a number of studies on the assay of the enzyme in saliva, and was able to get positive results from salivary stains. Kirk (1953) noted that the amylase test for saliva stain identification had not yet been standardized. Berg (1957) mentioned the amylase test, and said that he carried it out using iodine to detect the starch. Nickolls (1956) discussed the test in his book, and recommended it as a method for saliva stain identification.

Schaidt (1956) proposed a modification of the amylase procedure, taking advantage of an improved method for detecting the reducing sugar products on a micro scale. He employed the Folin-Wu technique for the colorimetric detection of glucose. Folin and Wu published their basic method in 1919, and a number of refinements in the technique were offered as time went along (Folin and Wu, 1920; Folin, 1929). In effect, the method employs a slightly alkaline solution of Cu^{2+} tartrate for oxidation of the sugar, the Cu^{+} then being detected colorimetrically using a phosphomolybdic acid-sodium tungstate reagent. Schaidt read the color at 440 nm. Good results were obtained, he said, even with a saliva "stain" from a piece of a postage stamp. Incubation times with starch were for 10 min at 40°, much shorter than had been used by Mueller in the original studies in 1928.

In 1960, Yoshida reported that salivary traces on postage stamps and envelope sealing flaps could be detected using a modified amylase test. Stain extract was incubated with starch solution for 30 min at 37.5°, and the reducing sugar detected with triphenyltetrazolium chloride in base. The tetrazolium salt was thereby reduced to a red insoluble formazan. Thoma (1961a) who had apparently arranged for a translation of Yoshida's paper, and discussed some of the experimental details contained in it, conducted some experiments on this method. Reliable results could not always be obtained using Yoshida's procedure, but Thoma presented a modified technique which was said to be reproducible. Pieces of postage stamps or envelope sealing flaps 0.5 cm^2 were said to give positive results, provided the incubation period was long enough. It has been known for a long time that triphenyltetrazolium chloride reacts with ascorbic acid as well as with reducing sugars (Feigl, 1966). Thoma (1961c and 1964) said that this fact should provide a basis for using the tetrazolium salt to determine saliva traces based upon the ascorbic acid content of saliva. Glavind *et al.* (1948) determined that there was 2.4 μg/mℓ (0.24 mg/100 mℓ) levels of Vitamin C in unstimulated saliva, and 1.7 μg/mℓ (0.17 mg/100 mℓ) in paraffin-stimulated saliva. Hafkesbring and Freeman (1952) noted that the level ranged from zero to 0.372 mg/100 mℓ, this figure being quoted in *Blood and Other Body Fluids* (1961) as well. Thoma said that the characteristic red formazan precipitate formed with saliva stain extract in the presence of the tetrazolium salt, while with water controls either no color was obtained or a red color formed but without any precipitate. Radam (1965) critically evaluated this method. He did not think that the test was measuring the ascorbic acid content of saliva at all. Taking the ascorbic acid concentration determined by

Glavind *et al.* (1948) for saliva, 2.4 μg/mℓ, and assuming that the amount of saliva on a postage stamp is 10μℓ, he calculated that the amount of ascorbic acid Thoma was actually testing with the tetrazolium salt, after dilutions and removal of the aliquot for the test, was 1.2 ng. Feigl (1966) said that the limit of detection of ascorbic acid with triphenyltetrazolium chloride was 0.2 μg. Saliva does contain some endogenous glucose, from 11.28 to 28.08 mg/100 mℓ according to *Blood and Other Body Fluids* (1961). This amount alone would not be sufficient to account for the positive tetrazolium test using Radam's assumptions and method of calculation, but he said that saliva contained some dextrins (partially hydrolyzed starch polymer) as well, and that the action of amylase on these compounds would increase the endogenous concentration of reducing sugars. He thought, therefore, that Thoma was in reality measuring reducing sugars. Radam preferred the starch-iodine test in the amylase determination in salivary stains. Mueller (1975) said that von Haas, in his 1968 dissertation at the University of Marburg, had been unable to confirm Radam's explanation for the tetrazolium test being positive in the absence of added starch substrate with saliva extracts.

Thomas (1961b) suggested an alternative method for detecting reducing sugar products of the salivary amylase reaction with starch. It was based on a spray reagent developed by Partridge (1948) for the detection of reducing sugars following paper chromatography. The reagent consisted of 0.1N $AgNO_3$ in excess (5N) NH_4OH, and the reducing sugars brought about deposition of elemental silver on the paper. Thoma applied the amylase reaction products to filter papers which had been previously impregnated with $AgNO_3$, and then exposed them to ammonia. The method, he said, was very sensitive.

In 1974, Willott applied dyed starch substrates for the amylase assay to the medico-legal identification of saliva for the first time. A commercially available blue starch polymer was used as amylase substrate (Phadebas test, Pharmacia). The blue dye is covalently attached to the starch, and upon hydrolysis, a product is obtained which is readily determinable colorimetrically. Saliva, blood, semen, fecal material on anal swabs, vaginal swabs without semen, urine, sweat, human milk and a number of vegetable and plant materials were tested for activity. The test can be carried out quantitatively by determination of the OD at 620 nm following incubation, or qualitatively by incorporation of the substrate into agar gel, and noting the color change upon hydrolysis visually. Saliva stains showed considerable variation in activity (0.013 to 0.183 I.U./ℓ; these values represent the actual activity measured in $3mm^2$ segments of stained cloth in a final volume of 5 mℓ, converted to I.U./ℓ from the OD-activity chart supplied by the manufacturer). Fecal stains showed wide variation as well, two samples having high amylase values (0.4–0.5 I.U./ℓ). One of the vaginal swabs showed 0.021 I.U./ℓ. Since some of the materials tested did show amylase activity levels which fell into the range of activities shown by saliva stains, albeit rarely, the amylase test, even when carried out quantitatively, could not be regarded as being entirely specific for saliva. Blood and semen levels were found to be lower than those of saliva, and identification of saliva in the presence of either of these should not present a problem.

The use of dyed starch substrates for assaying amylase was introduced in 1967 by Rinderknecht *et al.*, who could covalently link a blue dye, Remazol Blue R (Farbwerke Hoechst AG) (see Table 5.3) to starch. Upon hydrolysis, soluble blue product was released, and could be determined by its absorbancy at 590 nm. Ceska *et al.* (1969b) found that a blue dye called Cibachron blau F3G-A (see Table 5.3) could be covalently linked to starch polymer. This material in turn could be cross-linked to form a three dimensional polymer network which swelled in water. The formation of soluble blue product was proportional to substrate concentration over a wide range, and the reaction was linear with time for about 10 min. This substrate was shown to be completely suitable for amylase determinations in serum and urine (Ceska *et al.*, 1969a). In 1971, Sax *et al.* employed a different substrate called Procion Brilliant Red M-2BS Amylopectin (available at the time from Reliable Reagents, Maryland Heights, MO, USA) (see in Table 5.3). This substrate was shown to be suitable for amylase determination in serum and urine, the OD of liberated product being read at 517 nm. In 1973 Rosalki and Tarlow evaluated several commercially available dyed starch substrates in amylase assays. The activity data correlated well with the Remazol Brilliant Blue R Amylopectin (Amylopectin-RBB, Calbiochem), the Cibachron Blue Starch (CB Starch, Pharmacia) and the Procion Brilliant Red Amylopectin (Amylopectin PBR, Dade), but the results varied from those obtained with a quantitative starch-iodine assay. These authors recommended the dyed substrates. Lehane *et al.* (1977) compared nephelometric, dyed starch and starch-iodine techniques for the determination of amylase in serum and urine. The nephelometric and dyed-starch results correlated with one another, but not with the starch-iodine result. Furthermore, the nephelometric and dyed-starch techniques gave above-normal activities in every case of clinical pancreatitis, but the starch-iodine method gave normal values in two of eight cases. Nephelometry was considered more convenient than dyed-starch, and starch-iodine technique was said to be less reliable, based on the results. Rosalki (1970) reported that the Remazol Brilliant Blue Starch (Amylose Azure) could be used to localize amylase on cellulose acetate membranes following electrophoresis. The cellulose acetate foil was placed onto an agar gel containing the substrate, and incubated at 37°. Salivary and pancreatic amylases which had been separated could be localized as blue spots. Hall *et al.* (1970) noted that salivary and pancreatic α-amylases showed differential substrate specificity with soluble starch and Amylose Azure.

Baxter and Rees (1975) carefully compared the starch-iodine assay with a method employing Remazol Brilliant Blue R Amylopectin (Amylose Azure) for the identification of salivary stains. The starch-iodine assay was carried out on

a series of doubling dilutions of buffer extracts of approximately 0.5 in^2 stain, after incubation with soluble starch for an hr at 37°. A boiled extract was included as a control, as were identically treated cloth control extracts. The iodine test for starch was negative to about 1:128 with saliva stain extract, whereas boiled extract and cloth controls were negative to about 1:4 to 1:8. The Amylose Azure assay was found to be somewhat more sensitive, and to give good results after 40 min incubations at 56°, even with extract dilutions of 1:1000. The test was usually done with extracts from ⅛ in^2 of stained cloth. Saliva stains were tested, along with semen, blood, serum, urine, milk, tea, coffee, mixtures of saliva with urine, blood and semen, extracts of malt, potato and yeast, and stains made from dilutions of saliva up to 1:32. The test was specific for saliva, but did not detect salivary stains made from saliva more dilute than 1:4. It was successful in diagnosing saliva on cigarette ends, postage stamps and envelope sealing flaps.

Kipps and Whitehead have done extensive studies on the use of Procion Red MX2B Amylopectin as an amylase substrate and its applicability to the diagnosis of amylase in body fluids. A quantitative assay method for amylase was devised using agar gel plates. The substrate, dissolved in phosphate buffer at pH 6.9 and containing 10 g NaCl/ℓ, was incorporated into 1% gels. Small wells, into which test solution could be placed, were punched into the gel. After 24 hrs incubation at 37°, a red concentric ring indicating amylase activity formed around the well, its diameter being proportional to enzyme activity (Kipps and Whitehead, 1974). Substrate concentration was inversely proportional to diameter. The diameter of the ring could be related to absolute units of enzyme activity by using purified amylase of known specific activity in one of the wells. This technique was employed to assess the amylase concentration in saliva and a number of other body fluids (Kipps and Whitehead, 1974 and 1975). These results, along with the results obtained by several other workers, are given in Table 11.1.

In examining Table 11.1, note that Somogyi units were originally defined for serum amylase, the activity being detected by the use of copper reagents to determine reducing sugar product colorimetrically. The number of Somogyi units/100 mℓ serum is equivalent to that amount of enzyme which yields copper reducing power from starch equal to that number of mg of glucose. For example, 120 U/100mℓ would mean that 100 mℓ serum had the same copper reducing power as if it had contained 120 mg glucose (Somogyi, 1938, 1940, 1941, and 1960). Street-Close Units were defined for an assay using amylose as substrate, and iodine

Table 11.1 Amylase Concentrations in Various Body Fluids

Body Fluid	Mean or Average	Range	Units	Reference
Saliva	0.42	—		
Submaxillary	0.25	—		
Sublingual	0.26	—	mg amylase/mℓ	Schneyer, 1956
Parotid	1.03	—		
Saliva				
Caucasians	616	—	Somogyi/mℓ (auto)	
	37.15	—	I.U./mℓ (manual)	Boettcher &
Aborigines	1071	—	Somogyi/mℓ (auto)	De La Lande, 1972
	64.57	—	I.U./mℓ (manual)	
Saliva	350,000	72,000-1,300,000		
Serum	160	84-300		
Urine	850	130-3500		
Sweat	575	45-895		Kipps &
Tears	—	870-2150	I.U./ℓ	Whitehead, 1975
Lip Mucous	903	170-2900		
Semen	95	28-200		
Azoospermic	22	3-150		
	—	680-13,000		
Semen				
Normal	9	3-75		
Abnormal	8	2-22	Street-Close/100 mℓ	Moon & Bunge, 1968b

to detect residual reactant. A non-enzyme-containing "standard" was run next to the "test" solution, and activity was defined as:

$$\text{Units} = \frac{100}{S}(S - T), \text{ per 100 m}\ell \text{ serum, where}$$

S and T were OD readings at 620 nm. If all the starch were hydrolyzed, the OD would go to zero in "T", and Units would equal 100 per 100 mℓ serum, i.e.

$$\text{Units} = \frac{100}{S}(S - O) = 100 \text{ per 100 m}\ell \text{ serum}$$

(Street and Close, 1956). Amylase was first reported in semen by Goldblatt (1935) and Povda (1962) located amylase in paper electropherograms of seminal plasma. Brantzaeg (1971) examined amylase secretion rates in saliva in terms of μg/min/gland, and noted that the amylase values rose 16-fold upon extended gustatory stimulation.

Kipps and Whitehead (1974) noted that Schneyer's value of about 0.4 mg amylase/mℓ corresponded to about 100,000 I.U./ℓ, and that Moon and Bunge's values could be converted to a range of 17 to 1434 I.U./ℓ (see Table 11.1).

The data of Kipps and Whitehead (1975) indicated that the lower limits of amylase concentration in saliva could be close to the upper limits shown by some of the other body fluids. In one experiment, the amylase level in the saliva of the same person varied from 14,500 to 832,000 I.U./ℓ at 11 different times over the course of about 3 months time. Care must therefore be exercised in the interpretation of amylase assay results from salivary stains. Moreover, depending upon the lower limit of sensitivity of the assay being used, a negative test might not necessarily indicate the absence of saliva.

Whitehead and Kipps (1975) devised a test paper for saliva stains based on the Procion Red dyed starch. Moistened test paper and cloth were placed in contact for 15 to 30 min. Test paper areas which had been in contact with saliva stained areas showed pale regions on the paper in contrast to the red background. The resolution could be improved by giving the paper a water rinse, since the soluble products were then washed away. Negative or very weak tests were given by other body fluid stains, and this technique is perhaps the best available for localizing salivary stains on garments. It is analogous to the procedure devised by Kind (1964) with acid phosphatase test papers for localizing seminal stains (Section 10.2.2). Kipps *et al.* (1978) carried out studies on the detection of saliva stains located underneath bloodstains on several different substrata, using the amylase test paper method. They were successful in being able to identify such stains. Willott and Griffiths (1980) have recently described amylase test papers, prepared from blue dyed-starch substrate.

Kipps *et al.* (1975) noted that saliva stains could be detected on the fronts, cuffs and pockets of worn clothing sampled randomly and not related to any case. These findings show that the finding of a stain on garment and the assessment of its evidentiary value are different matters, just as has been previously discussed in connection with bloodstains (Owen and Smalldon, 1975; Hunt *et al.*, 1960) (section 6.8) and seminal stains (Owen and Smalldon, 1975) (section 10.2.2).

Dawes (1972) showed that the secretion of a number of substances in saliva exhibited circadian rhythm. The rhythms were not the same in stimulated and unstimulated saliva. Barsegyants (1970b) said that amylase in salivary stains could be detected after washing in detergents. In 1971, he indicated that a number of pathological conditions, treatment with various drugs and alcohol ingestion did not affect the ability to detect amylase in stains from the saliva of individuals so affected.

11.3.2 Some properties of amylase and starch

Amylases are found in a wide variety of plants and animals. They hydrolyze the C_1–O_4' bond of α1→4 linked glucose polymers, with transfer of the glucosyl residue to water. Amylases are found in animals and some plants, and yield hydrolytic products of the α-configuration. They are endoenzymes, acting at most internal bonds, can bypass branch points (α1→6 linkages) in branched polymers, and yield a mixture of oligosaccharide products. β-Amylases appear to be restricted to plants. They are exoenzymes, hydrolyzing every other bond from the non-reducing end of the chain, and yielding maltose as product. They cannot bypass branch points. α-Amylase is systematically called α-1,4-glucan-4-glucanohydrolase (EC 3.2.1.1) and β-amylase is called α-1,4-glucan maltohydrolase (EC 3.2.1.2). Salivary amylase in an α-amylase. Amylases is general were reviewed by Thoma *et al.* (1971).

Salivary amylase, like other α-amylases, has an absolute requirement for Cl^-, and all assay mixtures contain chloride salts, usually NaCl. The amino acid composition of the purified enzyme is known, and Metzbaum and Schulz (1965) reported that the MW of the pure enzyme, calculated from the sedimentation coefficient, is 55,200.

It may be noted that starch, the storage polysaccharide of higher plants, consists of two types of glucose polymers, amylose and amylopectin. Amylose is a linear polymer of glucose units held in the chain by α1→4 glucosidic linkages (Fig. 11.1). Amylopectin is a branched polymer, containing α1→6 linkages at the branch points (Fig. 11.2) and is a much larger molecule than amylose. The amylases do not hydrolyze the α1→6 linkages, and cannot therefore degrade amylopectin completely. Another enzyme, an α1→6 glucosidase, or debranching enzyme, is required to hydrolyze the branch point bonds.

11.4 Immunological Methods

The first medico-legal application of an immunological method to saliva identification appears to be that of Diniz (1925). He was concerned with the determination of whether dead infants had been born dead or alive, sometimes called docimasia. There was a connection, he believed, between inflation of the lungs with the first breath of air taken by a newborn, salivary gland secretion and the swallowing reflex.

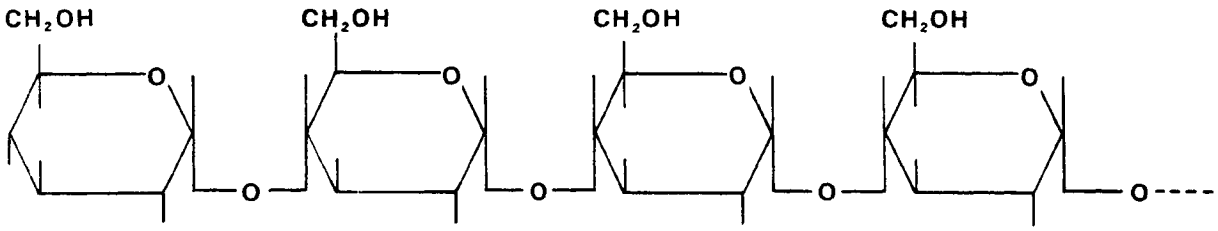


Figure 11.1 Amylose

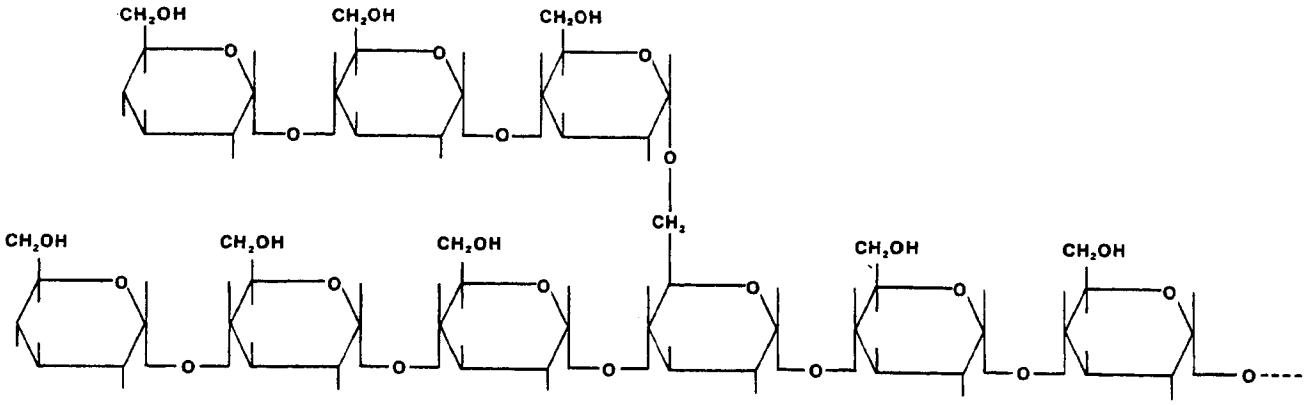


Figure 11.2 Amylopectin

Identification of saliva in the infant stomach indicated that it had been born alive. Few experimental details were given, but he appears to have prepared an anti-saliva serum, and said that the antigen-antibody reaction could be detected, with good results, by precipitation, complement fixation and anaphylaxis. Seitz (1913) could get anaphylactic reactions in guinea pigs using human saliva as both sensitizing and shocking injection material (see section 10.3.2). Mueller (1928) noted that the absence of experimental detail in Diniz's paper made it impossible to repeat the experiments. He said that he was not set up to carry out complement fixation tests, but he did conduct experiments on the precipitin and anaphylaxis methods. The anaphylactic shock studies yielded equivocal results, and Mueller did not think that this approach was particularly promising. Anti-human serum serum gave a precipitin test with fresh saliva and salivary stain extracts, but large areas of stained material were required, and the antiserum naturally cross reacted with a number of other body fluids. Mueller noted that the solution might lie in preparing a specific anti-saliva serum. Witebsky and Henle (1933) prepared rabbit immune precipitin antisera to human saliva. It cross reacted with serum, but reacted more strongly with saliva. Similarly, anti-serum serum cross reacted with saliva, but reacted much more strongly with serum. Rex-Kiss (1942) confirmed these observations, using saliva from group 0 people for immunization to avoid the problem of antibodies being formed to the soluble blood group substances. Some animal anti-saliva sera which he prepared showed salivary specificity, but the human one cross reacted with serum. Saliva and serum share a number of antigens in common, but saliva does contain some specific proteins.

Stoffer *et al.* (1962) found by immunoelectrophoresis that saliva contained the serum proteins albumin, ceruloplasmin, β-lipoprotein, transferrin, β_2-macroglobulin and gamma globulin. There was no fibrinogen. Leach *et al.* (1963) confirmed these findings, and reported serum α_2-glycoprotein, α_1-lipoprotein and β_{2A}-globulin in saliva as well. Ellison and Mashimo (1958) and Ellison *et al.* (1960) found a number of saliva specific proteins using rabbit anti-saliva serum. Simons *et al.* (1964) found nine antigenic components in saliva using an anti-saliva serum, and the antiserum contained an anti-amylase antibody. Furuya (1968) reported that an anti-human saliva serum prepared in rabbits gave 10–13 precipitin arcs upon immunoelectrophoresis, but that after absorption with serum and semen, only one precipitin arc was obtained against saliva, and this could be identified as amylase-anti-amylase complex. Masson *et al.* (1965) identified several salivary globulins by immunoelectrophoresis. Culliford (1964 and 1967) noted the desirability of a specific antiserum to salivary proteins for use in the immunological tests. He also observed that anti-human semen serum reacted more strongly with saliva than did antihuman serum serum. Baxter and Rees (1975) tested an antiserum to

human group A saliva, and found that it cross reacted with seminal plasma. Absorption with pooled semen failed to render the antiserum saliva-specific, and amylase activity could not be detected in antigen-antibody complexes.

11.5 Microscopical Methods

Microscopy may be used to identify the characteristic exfoliated buccal epithelial cells associated with saliva. Most authorities extract the stain, centrifuge the extract, and examine the particulate material thus obtained. A wide variety of histological stains may be employed for examining smears. Radam (1965), for example, used a hematoxylin stain following alcohol fixation. If enough cells are available, cytological sexing methods may be attempted with these cells. Cytological sexing techniques are discussed in a subsequent section. Microscopical methods were discussed briefly by Camps (1968), by Mueller (1975) who did not think the method was as useful as some of the others, and by Dérobert (1974) who indicated that the microscopical confirmation of buccal epithelial cells represented the only certain method of saliva identification.

11.6 Fluorescence Under Ultraviolet Light

Saliva stains exhibit a bluish-white fluorescence under ultraviolet light, which is very useful for carrying out preliminary examinations of garments. The fluorescence helps merely to localize areas for more detailed examination; it does not have any value by itself in the identification of any specific body fluid stain (Mueller, 1953 and 1975; Kirk, 1953).

SECTION 12. IDENTIFICATION OF URINE

Methods for identifying urine have been based on the detection of inorganic anions, especially phosphate and sulfate, and of a number of organic compounds which concentrate in urine, including creatine, creatinine, urea, urinary indican, urochrome, free purine and pyrimidine bases and steroid derivatives. Even though a number of these occur in urine in higher concentrations than in other body fluids, some authors have advised the detection of two or more constituents in an effort to make the identification more probable. Methods have been proposed based on the identification of a single constituent as well.

12.1 Microscopical Methods, Ultraviolet Fluorescence and Odor

Human beings excrete about 9 to 29 mℓ urine/kg body weight/day, containing about 55–70 g solids (*Blood and Other Body Fluids,* 1961). Microscopical methods take advantage of the fact that the "solids" in urine contain various crystalline substances, and epithelial cells characteristic of the urinary tract linings. This approach may provide satisfactory presumptive evidence of the presence of urine in liquid specimens, but has not been very successful in the examination of stains. In the presence of infections, bacteria can be identified as well (Balthazard and Rojas, 1922).

Urine stains fluoresce under ultraviolet light, a fact which helps in localizing stains on garments for subsequent analysis (Kirk, 1953). The color of the fluorescent light varies if abnormal substances are present, e.g., in glycosuria (Hansen, 1945).

One of the more characteristic properties of urine is its odor. Some authors have recommended gentle heating and detection of this odor. Camps (1968) mentioned this method, and Kirk (1953) said that the characteristic odor detection might be among the most specific tests which can be done for urine in stains.

12.2 Inorganic Ions

Balthazard and Rojas (1922) mentioned that urine contained relatively large amounts of Cl^- and PO_4^{3-}, the former being determined with $AgNO_3$, the latter with ammonium molybdate reagent. Kirk (1953) mentioned both phosphate and sulfate as being present in urine in appreciably higher concentrations than in other biological fluids. Sulfate may be detected as its barium salt, and phosphate may be detected as crystals of magnesium ammonium phosphate. According to *Blood and Other Body Fluids* (1961), "inorganic" phosphate is present in serum, saliva, semen and sweat at concentrations of 2.4–3.6, 7.4–21.1, 11 and 0.009–0.043 mg/100mℓ, respectively. Urine contains 10–15 mg phosphate/kg body weight/day. Assuming a 70 kg person, excreting 1ℓ urine in a day, the concentration of phosphate would be 70–105 mg/100 mℓ, assuming constant concentration over time. Similarly, the sulfate concentration is higher than that of serum or perspiration. Chloride in urine is excreted at the rate of 40–180 mg/kg/day. Using the same assumptions as above, the concentration would be 280–1260 mg/100 mℓ, as compared with 295 for serum, 29.8–62.8 for saliva, 99.4–202.4 for semen and 36–468 for sweat. The assumption that the concentration of a substance in urine is constant with time is almost surely not true, but the values give at least some idea of what general ranges of concentrations might be expected. The ions are not unique to urine, although the phosphate and sulfate concentrations are normally considerably higher than in other common body fluids. Swaroop (1973) presented a simple method for detecting sulfate in urine, in which a known amount of barium is complexed with sodium rhodizonate forming a chromophore which absorbs at 520 nm. In the presence of sulfate, the barium is precipitated and the absorbancy is decreased. The sulfate concentration was determined by the absorbancy difference, which was shown to be linear over the range of 0–32 μg sulfate/mℓ.

12.3 Urea

In 1914, Policard suggested the use of xanthydrol, which forms a crystalline compound with urea, for the identification of urine in stains. The test could be carried out on a few threads of stained material, and the characteristic crystals identified microscopically. The reaction had been described by Fosse (1907 and 1912). Maiocchi (1915) carried out studies on the xanthydrol test, noting that some other body fluids including serum, saliva and tears, gave a positive reaction. Balthazard and Rojas (1922) recommended the xanthydrol test, and said that they had gotten negative results with blood, egg white, semen, milk and fecal material. The xanthydrol was dissolved in 95% alcohol, and the test reagent prepared by mixing this solution with glacial acetic acid just prior to use. Ordinarily crystal formation occurred within 30 min, even with older stains. In 1945, Manson took up studies on the procedure. He refined the technique for the actual identification of the crystals by applying a microsublimation procedure described by Kofler and Kofler (1945). Material containing the suspected stain was teased apart into fibers, and treated with alcoholic xanthydrol and acetic acid, and the crystals allowed to form. The crystals were then subjected to the microsublimation procedure and their melting point determined. He said the test was specific for urea and regarded it as virtually specific for urine. The

reaction of xanthydrol with urea is indicated in Fig. 12.1. Ishler *et al.* (1947) said that they had obtained false positive reactions with the xanthydrol test.

In 1940, Gee reported that several of the commonly used tests for urine including color, odor, behavior under ultraviolet light, biuret test, and tests based on ammonia evolution, were not very satisfactory for urine stains on woolen carpets because of interference by substances within the wool. A method was devised in which the urea was extracted into alcohol-acetone, dried down, and the residue again extracted with acetone. The last extract was slowly evaporated, one drop at a time, on a microscope slide, keeping the area occupied by the "spot" as small as possible. Characteristic, needle-shaped crystals soon formed, which could be identified microscopically. The urea crystals could then be converted to urea nitrate crystals by treatment with concentrated HNO_3 as well. Gee said that the extraction was necessary because substances in the wool itself could give false positive results with the test otherwise. Nickolls (1956) recommended Gee's test. Ishler *et al.* (1947) noted that they had encountered problems in trying to convert the urea crystals to urea nitrate.

Ishler *et al.* (1947) utilized a urease enzymatic test for urea in order to identify urine stains on fabrics. Urease (EC 3.5.1.5) is systematically known as urea aminohydrolase, and catalyzes the reaction:

$$\text{urea} + H_2O \rightleftharpoons CO_2 + 2\,NH_3$$

The ammonia may be detected in a number of ways. Ishler *et al.* moistened a small portion of the stained cloth with urease solution, and placed it over a steam bath in contact with a test paper impregnated with Mn and Ag nitrates. A black spot appeared on the test paper within 30 sec if urine was present in the original sample. The test paper reagent was based on the reaction (Feigl and Anger, 1972):

$$Mn^{2+} + 2Ag^{+} + 4\,OH \rightleftharpoons MnO_2 + 2\,Ag^{\circ} + 2\,H_2O$$

Sulfides would give false positive tests if in direct contact with the test paper. A control was recommended in which test paper was placed into contact with fabric material that had been moistened with a non-urease solution. With thick fabrics, a fabric wetting solution could be employed. A positive test could be obtained with a stain made from 0.01% urea solutions.

Cook (1948) suggested a urease test for urea (not specifically for urine) in which the ammonia was detected by its basicity using the acid-base indicator bromthymol blue. The test could be conducted with test paper impregnated with urease and BTB or in 2% agar gels into which the reagents had been incorporated. A bright blue color was a positive test. Thrasher (1970) noted that the agar technique was greatly superior to the impregnated filter paper method in cases where it was difficult to achieve good contact between the filter paper and the contaminated material. He was concerned with the detection of mammalian urine contamination of wheat grains, and said that the agar gel method would detect 2.5 μg urea distributed over the surface of a single grain. Thoma and Kuchinke (1953) reported a urease method for stain examination in which the ammonia was detected using Nessler's reagent. Test papers treated with reagent were employed in the test.

Kirk (1953) did not think that the detection of urea was of very much value in identifying urine in stains.

Rhodes and Thornton (1976) recently described a test for urine in stains based on the reaction of urea with p-dimethylaminocinnamaldehyde (DMAC). It had been observed by other workers that 0.1% solutions of DMAC could be used to detect latent fingerprints. This concentration was too high for a urine test, since all urea-containing physiological fluids (Table 12.1) reacted. At concentrations of DMAC of 0.005%, however, the reagent (prepared in 180 mℓ acetone and acidified with 2 drops concentrated HCl) reacted with the higher urea concentrations found in urine, but not the lower ones found in semen, saliva, perspiration or serum. Stains suspected of containing urine were tested by thoroughly extracting a 1 cm^2 piece of material with 1 mℓ distilled H_2O. A piece of filter paper, dipped into the extract and allowed to dry, is then sprayed with or dipped into a solution of DMAC reagent and heated. Positive results were indicated by a dark pink to red reaction product. In much the same way, the procedure could be used to search for urine stains on fabrics, bedding, etc., by placing the suspected material in between two wet pieces of filter paper, sandwiching them between polyethylene sheets, and allowing the assembly to sit under a weight for an hour. The filter paper could then be dried, and tested. Background color develops in 15 min to 1 hr, so the results had to be read right after the test. DMAC does exhibit reactions with other com-

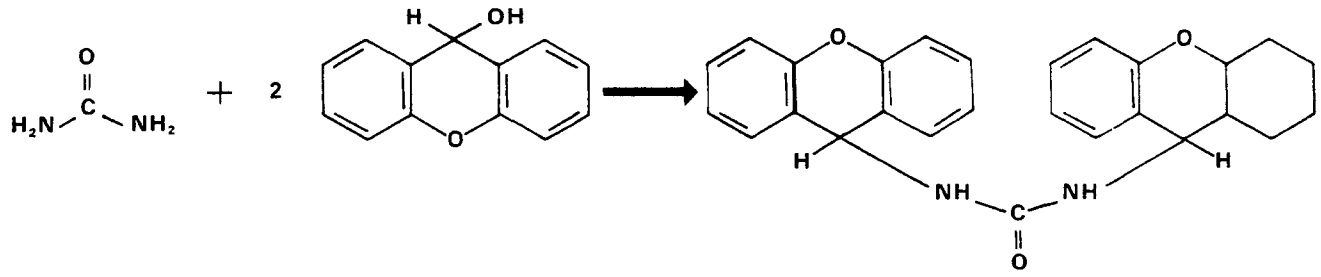


Figure 12.1 Reaction of Xanthydrol (Xanthanol) with Urea

pounds, and it was said that this was not a specific test, but it was recommended as a presumptive test and as a searching aid in locating urine stains.

Human urine contains 200–500 mg urea/kg body weight/day (*Blood and Other Body Fluids,* 1961), corresponding to a concentration of 1400–3500 mg urea/100 mℓ in a 70 kg person who produces 1ℓ urine in a day, assuming no fluctuations in concentration over time. By comparison, serum contains 16–35 mg urea/100 mℓ, semen 72 mg/100 mℓ and sweat 12–57 mg/100 mℓ. Saliva contains 0–18.1 mg/100 mℓ, according to *Blood and Other Body Fluids* (1961). Updegraff and Lewis (1924) determined that saliva contained an average of 4.2 mg/100 mℓ, while Wu and Wu (1951) said that the value ranged from 7.4 to 18.6 mg/100 mℓ.

12.4 Creatinine

The determination of creatinine in urine samples has been of interest to clinical chemists for at least 75 years. One of the oldest tests for the detection of creatinine is that of Jaffe (1886). He found that picric acid reacts with creatinine, in the presence of base, to form a bright red product. In 1904, Folin applied this test to the clinical determination of creatinine in urine. The Jaffe test, as it is usually called, has undergone scores of modifications over the years, primarily by the clinical chemists. The test is applicable to the detection of creatinine in urine stains. Heredia del Portal *et al.* (1971) showed that urine spots dried on filter paper for 15 days could be tested for creatinine using a modified Jaffe test without significant reduction in activity. Many structures have been proposed for the red Jaffe reaction product. These were reviewed in 1974 by Blass *et al.*, who isolated and carried out structural studies on the product. They proposed the structure indicated in Fig. 12.2.

Varma *et al.* (1968) reported a quantitative test for creatinine based on the so-called Salkowski test, in which sodium nitroprusside gives a prussian blue when heated with creatinine. Potassium ferricyanide was employed as oxidizing agent, and a soluble prussian blue complex could be obtained, the absorbancy of which at 680 nm showed a linear relationship with creatinine content over the range 0–10 μg.

Narayan and Appleton (1972) used a countercurrent distribution method to test the specificity of the Jaffe test and the Sakaguchi test for creatinine. The latter, first described by Sakaguchi in 1925 and studied in detail by van Pilsum *et al.* (1956) is based on the reaction of creatinine with o-nitrobenzaldehyde. Narayan and Appleton found that the Jaffee test was more specific for creatinine.

Creatinine occurs in urine to the extent of 15–30 mg/kg body weight/day. A 70 kg person excreting 1ℓ urine per day would have a urinary creatinine concentration of 105–210 mg/100 mℓ, assuming no concentration variation with time. Values of creatinine concentration in other body fluids in the same units (mg/100 mℓ) are serum 0.6, saliva 0.275–0.455, and sweat 0.1–1.3 (*Blood and Other Body Fluids,* 1961). It is known that creatinine excretion varies as a function of time, however, as well as from one individual to another. Greenblatt *et al.* (1976) found that the mean excretory rate in 8 individuals was 21.4 to 25.4 mg/kg body weight/day. There was considerable variation in the mean mass of excreted creatinine by the same individual on different days.

Ladell (1947) studied creatinine losses in sweat during work in hot, humid environments. The rate of excretion decreased at first, but in some cases increased again to values in excess of the zero time value. The salivary creatinine was unaffected (mean value 0.455 mg/100 mℓ), and ingested creatinine which increased serum levels, did not affect the excretory rate in sweat.

12.5 Indican

Indican is excreted in urine at the rate of 0.5–2.0 mg/kg body weight/day, corresponding to 3.5–14 mg/100 mℓ in a 70 kg person excreting 1ℓ urine per day, with no concentration change over time. Serum contains about 0.095–0.105 mg/100 mℓ of this substance (*Blood and Other Body Fluids,* 1961).

Indican is 3-indoxylsulfuric acid. In 1920, Lattes discussed the determination of indican as a method for the diagnosis of urine in stains. He did not think the test was specific for urine stains.

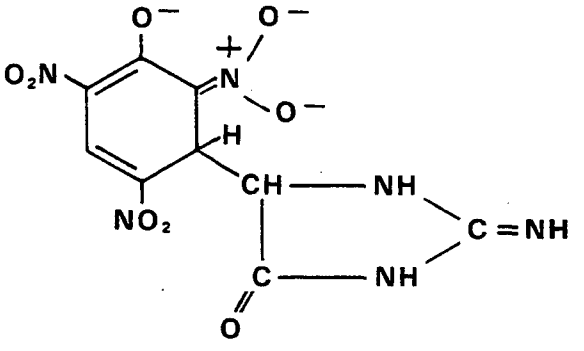


Figure 12.2 Jaffe Reaction Product (after Blass et al., 1974)

12.6 Chromatographic Methods

In 1956, Thoma devised a paper chromatographic method with butanol:acetic acid:water::120:70:10 as the developing solvent (Thoma, 1956a). Urea was detected at R_f 0.4 using p-dimethylaminobenzaldehyde reagent. Rhodes and Thornton (1976), in their paper on the DMAC test (section 12.3), noted that urea reacts more strongly with DMAC than with p-dimethylaminobenzaldehyde. Urine stains could be distinguished in the system from blood, saliva, semen and bile. Thoma (1956b) also noted that allantoin occurs in animal urine but not in human urine. Detection of allantoin using a phenylhyrazine reagent could thus be used to distinguish human from animal urine. In 1966, Weinke *et al.* reported a thin-layer chromatographic method using n-butanol:acetic acid:water::4:1:1 as developing solvent. The solvent front advanced about 10 cm in 70–80 min. Urea was detected as a yellow spot at R_f 0.5 with HCl-p-dimethylaminobenzaldehyde, and creatinine was detected as an orange spot at R_f 0.19 using 5% alcoholic picric acid containing 10% NaOH. Urine stains on various substrates were tested after aging at room temperature. Both compounds could be detected in most cases after 10 days, and in some cases after as much as 40 days.

Giusti and Panari (1972) proposed that several other components of urine be detected by thin layer chromatography, in addition to creatinine and urea, in order to make the identification more specific. Urochrome, indican, purine and pyrimidine bases and steroid derivatives were detected on Silica Gel G plates with isopropanol:concentrated ammonia:water::10:1:1 as developing solvent. They recommended that urea and creatinine be detected first using the method of Weinke *et al.* (1966). Urine stains could be differentiated from perspiration stains, in that the latter showed only urea, creatinine and the purine-pyrimidine base spot at R_f 0.00.

Concentrations of some of the substances in urine and in other body fluids which have been quoted in Section 12 are collected in Table 12.1.

12.7 Immunological Methods

Lee *et al.* (1977) reported that they had prepared an anti-human urinary protein serum, and tested its applicability to the detection of urine in stains. The antiserum was not entirely satisfactory, but the approach was regarded as a promising avenue for future experiments.

Table 12.1 Concentrations of some components of urine and other body fluids

Substance	Urine (mg/kg body weight/day)	Urine (mg/100ml) ★	Serum ●	Saliva ●	Semen ●	Sweat ●
phosphate	10-15	70-105	2.4-3.76	7.4-21.1	11	0.009-0.043
sulfate	3.5-17.5	14.5-122.5	0.45	—	—	0.7-7.4
chloride	40-180	280-1260	295	8.4-17.7 ✓	28-57 ✓	36-468 385 profuse [4] 469 profuse- [5] intermittant [4] 1091 intermittant [4]
creatinine	15-30 21.4-25.4 [1]	105-210	0.6	0.275-0.455	—	0.1-1.3
creatine	0-2	0-14	2.7	—	20	—
urea	200-500	1400-3500	16-35	0-18.1 4.2 [2] 7.4-18.6 [3]	72	12-57 68 profuse [5] 275 intermittant [5]
uric acid	0.8-3.0	5.6-21	1.6-3.9	0.5-8.7	6	0.07-0.25
indican	0.5-2.0	3.5-14	0.095-0.105	—	—	—

Values in Table from Blood and Other Body Fluids (1961) unless otherwise noted

● Values in mg/100 mℓ unless otherwise noted

✓ Values in meq/ℓ

★ Calculated from the mg/kg body weight/day value assuming a 70 kg person excreting 1 ℓ urine per day with no variation in concentration as a function of time

[1] Greenblatt et al., 1974

[2] Updegraff and Lewis, 1924

[3] Wu and Wu, 1951

[4] Lobitz and Osterberg, 1947c

[5] Lobitz and Osterberg, 1947a

SECTION 13. IDENTIFICATION OF FECAL MATERIAL

Microscopy is the oldest method for identifying fecal material and still probably the best technique for achieving a reasonably satisfactory result. The undigested residues of food material ingested 12–24 hrs previously will be found in fecal material. Characteristic fibrous material from animal meats, fish and from vegetables and fruits, seeds, pits and other plant material which is undigestible can all be identified, if present, by an experienced observer. One of the oldest papers devoted to the medico-legal identification of fecal material is that of Moeller (1897) although Robin and Tardieu had identified fecal stains in a case on which they reported in 1860. Vibert (1908) discussed the microscopical method in his text. In 1899, van Ledden Hulsebosch published a complete monograph on fecal examination, with complete descriptions of the various materials that might be found and over 250 plates showing photomicrographs. This book was a standard reference in forensic medicine for several decades. Van Ledden Hulsebosch's son published a paper in 1922, describing an apparatus he had devised for preparing fecal material for microscopical examination. Reuter (1934) reviewed the subject briefly, and Hepner (1952) described in detail his technique for photomicrography in connections with examination of fecal material.

There was always interest in finding methods for comparing fecal samples, which might indicate common origin. Microscopical examination can be of help in establishing that samples were deposited by the same person within a relatively short period of time. Kraft (1929) took advantage of what he thought should be a fairly unique property of fecal material at any one time, its chlorophyll content. Ethereal extracts of fecal material contain chlorophyll if green plant foodstuffs have been ingested, and these solutions show the characteristic chlorophyll fluorescence. The amount of chlorophyll is a function of the amount of green plant food materials which have been eaten. Kraft said that, in one case, he could differentiate two fecal samples on this basis.

Hoen (1929) took an interesting, if somewhat complicated, approach to the problem of individualizing fecal samples. There was evidence that the different strains of coliforms inhabiting the G-I tract, and appearing in feces, showed a certain degree of individual specificity. Hoen thought that, by preparing antisera to the bacteria and differentiating them serologically, different samples should be able to be compared as to common origin. In the particular case at issue, he found the two specimens to be identical by microscopical examination, and felt that the serological identity of the bacteria, which he demonstrated, gave a very strong indication that the specimens had a common origin. The serological profile of the coliforms was relatively constant in the same person over the course of time, and the approach was regarded as very promising. The technique is quite involved, of course, it being necessary to culture and harvest the bacteria, prepare antisera to them, and then carry out the serological tests.

Another method for the identification of fecal material is the detection of bilirubin, a precursor of the normal fecal pigment. The compound may be oxidized to a pink-to-red product with $HgCl_2$, and this test may be used for relatively small fecal stains (Asada and Kominami, 1924). Nickolls (1956) referred to a variation of this test in which 10% $HgCl_2$ in amyl alcohol was used as reagent, and $ZnCl_2$ added to the supernatant fluid following centrifugation. This, he referred to as the Edelman test. Mueller (1975) said that the test was not always reliable.

Vanni (1949) reported on the potential importance of parasitology in examination of fecal material. Any number of harmless, nonpathogenic parasites may inhabit the human gut, and the parasites themselves or their eggs may be found in fecal samples. Vanni elaborated a procedure for carrying out the examination for parasites. It is noteworthy that Robin and Tardieu, in 1860, identified the eggs of *Ascarides lumbricoides* in fecal stains on a garment, and used the finding as a criterion for judging the stains to be fecal in nature. It is necessary to have a sample from the suspected person in order to perform a parasitological comparison for identification purposes. Jarosch and Marek (1959) reviewed briefly the medico-legal examination of fecal material, and included a discussion of bacteriological, parasitological and morphological techniques.

Johnson (1948) reported on a case in which a defendant was convicted of burglary, largely on the strength of expert testimony linking fecal matter at the scene with that found on his overshoes at the time of arrest. The expert compared the samples as to color, odor, and consistency, and carried out a detailed microscopical examination in which a number of animal and plant products could be identified. On this basis, the expert testified that there was only a slim possibility that the samples were from different sources. The New Hampshire Supreme Court agreed that the evidence supported the guilty verdict [State v Burley, 57A (2d) 618, N.H., 1948].

Giersten (1961) gave a very good review of the entire subject. He thought the detection of urobilin was a much more specific test for fecal material than the detection of bilirubin from which it is derived. The latter may occur in vomit as well. Camps (1968) recommended the detection of urobilin in identifying urine stains. The nomenclature of the fecal and urinary prophyrin derivatives is confusing because the early investigators based many of their conclusions on work with impure materials. Both urine and feces contain

two urobilinogens, mesobilane ("urobilinogen"), and tetrahydromesobilane ("stercobilinogen"), and two urobilins, mesobilene ("urobilin IX") and tetrahydromesobilene ("stercobilin") (Lemberg and Legge, 1949). Giersten also noted that identification of enterococci is strong presumptive evidence for the presence of fecal material.

Konzak (1980) suggested coprostanol as a marker for fecal material. The compound could be detected by TLC of an extract of the test substance. Petersen Inman (1980) said that detectable amounts of IgA immunoglobulin, alkaline phosphatase and amylase could be detected in fecal material extracts. These were proposed as potential identification markers for fecal material. It may be noted, too, that the amylase detected in fecal material is pancreatic in origin (AMY_2 locus) and is known to show some genetic variation (see in section 37.3).

SECTION 14. IDENTIFICATION OF OTHER BODY FLUIDS AND SECRETIONS

It may be necessary on occasion to attempt to identify uncommon physiological fluids or secretions. These include amnionic fluid, meconium, colostrum, milk, pus, nasal mucus, sweat and tears. Textbooks in forensic medicine have often discussed one or more of these substances. Most authorities recommend microscopical examination as the best method for differentiating these materials, except for perspiration and tears which are seldom mentioned. In many cases, histologically identifiable cells, globules or other characteristic structures are present which serve to indicate the origin of the material. Proteinaceous fluids may be distinguished form urine, sweat and tears by means of one of the general protein tests, such as the biuret test.

Sweat and tears are similar in composition to urine except that they contain less urea and creatinine. Kirk (1953) noted that urine can best be distinguished from perspiration on the basis of its odor upon heating. Giusti and Penari (1972) noted that their TLC method distinguished urine from perspiration, as discussed in Section 12.6. Lobitz and Osterberg (1945, 1947a, 1947b and 1947c) carried out extensive studies on palmar sweat. The concentrations of various substances varied, depending upon whether sweating was profuse, profuse-intermittent, or intermittent. These data are collected in Table 12.1 There was considerable individual variability in the rates of excretion of various substances.

Josephson and Lockwood (1964) prepared an antiserum to human tears, and examined the antigenic composition of lachrymal fluid by immunoelectrophoresis. Several serum proteins including ceruloplasmin and β_2A-globulin were found, and mild trauma, such as rubbing the eyes, caused transferrin, serum albumin and γ-globulin to appear. The data indicated, however, that tears do contain specific, non-serum proteins. In 1956, Erickson described an albumin in tears which had an electrophoretic mobility faster than that of serum albumin, and was said to constitute about half the total tear albumin. Josephson and Weiner (1968) studied this protein further, saying that it did not occur in serum, saliva, cerebrospinal fluid or urine. It had a MW between that of lysozyme and serum albumin by gel filtration, and was referred to as the anodal tear protein. Electrophoretically, it behaved as a prealbumin. Bonavida *et al.* (1969) named the protein "specific tear prealbumin", and found that it had a MW by gel filtration of about 15,000. It could be further shown that the protein is synthesized in the lacrimal gland. Some animals have this protein in their lacrimal gland secretions as well (Erickson, 1956; Bonavida *et al,* 1969).

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† Spelling may vary: Moench, Mönch

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§ 1 *Japanese Journal of Legal Medicine* (*Jpn. J. Leg. Med.*) has Japanese title: *Nippon Hoigaku Zasshi*

§ 2 *Reports of the Scientific Police Research Institute* has Japanese title: *Kagaku Keisatsu Kenkyujo*

§ 3 *Rendiconti dell'Accademia Scienze Fisiche Mathematiche* (Napoli) has alternate title: *Reale Accademia Scienze Fisiche Mathematiche* (Napoli). *Rendiconti.*

§ 4 *Acta Criminologiae et Medicinae Legalis Japonica* has Japanese title: *Hanzaigaku Zasshi* and see note §10 to References for Unit II

§ 5 *Acta Medica* (Fukuoka) has Japanese title: *Igaku Kenkyu*

§ 6 *German Medical Monthly* is a cover-to-cover translation of *Deutsche Medizinische Wochenschrift*

§ 7 *Journal of the Kumamoto Medical Association* has Japanese title: *Kumamoto Igakkai Zasshi*

¶ Symbol indicates that a translation of the paper appears in Unit IX