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Genetic Markers in Semen. III: Alteration of Phosphoglucomutase Isozyme Patterns in Semen Contaminated with Saliva

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ABSTRACT: Contamination of semen by saliva can result in the alteration of seminal phosphoglucomutase (PGM_1) isozyme patterns. The alteration is characterized by the gradual loss of the a and b isozyme bands with the concomitant generation of anodal bands; eventually, all PGM activity is lost. The conversion of PGM isozyme patterns has been shown to be due to a dialyzable heat-labile factor in saliva and a nondialyzable heat-labile factor in semen. The implications of this conversion for PGM typing in sexual assault evidence are discussed.

KEY WORDS: pathology and biology, genetic typing, semen, phosphoglucomutase

Of the enzyme genetic markers known to be present in human semen, the phosphoglucomutase first locus (PGM_1) marker system has thus far proven to be the most useful in the analysis of semen evidence material [*1-4*]. The marker system has good discrimination potential, particularly if PGM_1 subtyping can be done [*5-7*], and the enzyme activity levels in semen are sufficiently high to allow typing of dilute material [*3,4*], for example, semen in vaginal fluids collected from rape victims or weak extracts of semen stains. Accordingly, many laboratories routinely do PGM_1 typing on semen evidence.

The principles and procedures for PGM_1 typing of semen are essentially the same as those used for the typing of blood and other tissues. There is, however, a suggestion that some special problems may be associated with the typing of semen evidence material. Many analysts have experienced PGM_1 isozyme patterns that are weak, unclear, or "not quite right." It may also be confusing that the appearance of seminal PGM_1 isozyme patterns differs somewhat from the typical red cell PGM_1 patterns in the number and relative intensities of the isozyme bands [*1,2*]. These factors may account for the report by Rees and Rothwell [*8*] that several in a series of PGM_1 typings of semen in stains and on vaginal

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swabs were discordant. Price et al [3] also note that problems may be encountered in the PGM_1 typing of seminal material and recommend that seminal PGM_1 typing gels be interpreted only by experienced readers according to strict standards of pattern clarity: no attempt is made to type patterns that are faint or in which the isozyme bands are not well resolved. A significant proportion of stain and swab samples with PGM activity cannot be typed if these standards are honored, and the analyst must thus exercise restraint.

The possibility that the PGM_1 isozymes in seminal material may, under some circumstances, undergo biochemical modification affecting their activity or electrophoretic mobility has not been previously investigated. There are two reasons for considering this possibility. First, ejaculated semen is known to undergo a variety of biochemical changes [9]. For example, ejaculated semen rapidly coagulates and then liquefies; both processes are believed to involve proteolytic and other hydrolytic activities [9]. Other processes, possibly degradative, cause alterations in the electrophoretic behavior of seminal proteins in liquid standing semen (Refs 1 and 9 and unpublished experiments). Any of these or other processes occurring in semen could possibly affect the seminal PGM_1 isozymes. Second, semen proteins may be modified by processes occurring as a result of mixing with other body fluids. The possibility of such interactions must be seriously considered since semen evidence is often contaminated with other physiological fluids such as vaginal fluids, blood, saliva, perspiration, and fecal material.

In this report, we present evidence that seminal PGM_1 isozyme patterns may be altered in semen contaminated with saliva. The first suggestion of this alteration stemmed from observations made by one of us (D. N.) in the course of analyzing the semen evidence in a rape case. In this case, both victim and assailant were PGM_1 Type 1 individuals. Electrophoretic analysis of the stain from the crotch of the victim's underpants yielded the PGM isozyme pattern (Q) illustrated in Fig. 1; Type 1 and 2-1 standards are also present. Although the resolution of isozyme bands was not sufficient to allow typing according to the guidelines recommended by Price et al [3], it is clear that the pattern differs markedly from that expected from a Type 1 sample. In particular, there appears to be a

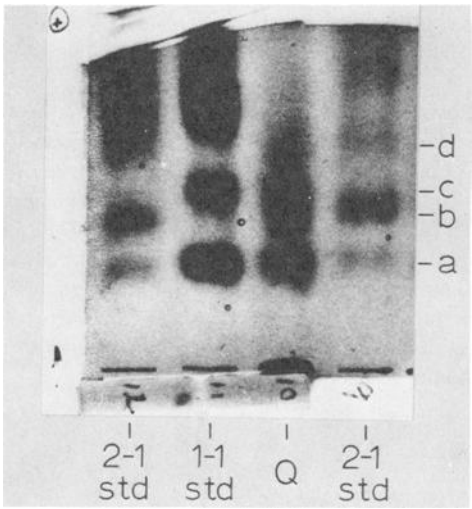

FIG. 1—*PGM isozyme patterns given by semen evidence material* (Q) *and Type 1 and 2-1 standards. The standards were red cell hemolysates. The evidence sample was extracted from the crotch of the victim's panties. The panties were collected at the time of the hospital examination, which was 3 to 5 h after the assault.*

smeary band cathodal to the c band in a position approximating the b band, a smeary band roughly in the d band position, and additional anodal staining material; these additional bands make the pattern more similar to a Type 2-1 pattern than to a Type 1 pattern. The facts of the case precluded contamination by semen from a second male and another explanation for the deviant pattern was sought. The circumstances surrounding the assault suggested the possibility that the stain might contain saliva as well as semen and this was supported by the detection of salivary-type amylase [*10*]. Mixtures of semen and saliva were then prepared and typed for PGM_1 with the result that the PGM isozyme patterns were markedly altered. The characterization of this phenomenon forms the basis of this report.

Materials and Methods

The electrophoretic analysis of PGM was done on starch gels using either the tris(hydroxymethyl)aminomethane (Tris)-maleate pH 7.4 buffer system as described by Harris and Hopkinson [*11*] or a modified Tris-maleate buffer pH 7.4 containing 0.1 *M* Tris base, 0.046 *M* maleic acid, 10 m*M* ethylenediaminetetraacetic acid (EDTA), and 10 m*M* magnesium chloride; the latter buffer has a lower ionic strength, which allows higher voltages to be used during the electrophoresis run. The gels were cast on glass plates at a thickness of 3 mm and samples were applied on thin strips of filter paper inserted into the gel. Typical runs were for 16 to 18 h at 8 to 10 V/cm in the refrigerator. Following the electrophoresis, the gels were sliced into 1.5-mm-thick slabs and stained on the inside face with the conventional agar overlay [*11*].

Semen, blood, and saliva samples were obtained from donors of known PGM type. Semen was collected by masturbation into sterile containers; it was allowed to liquefy before use. In some experiments, whole semen was used; where only seminal plasma was needed, it was separated from the sperm fraction by centrifugation at 20 000 *g* for 20 min at 4°C. Blood was collected by finger prick into isotonic saline; the red cells were washed several times in saline and lysed in an equal volume of distilled water by freezing and thawing. Saliva donors stimulated their salivary secretion by chewing on a wad of parafilm for a few minutes; saliva was collected into sterile glass containers and was clarified by centrifugation (2000 rpm, 10 min, room temperature) before use. Seminal plasmas, red cell hemolysates, and saliva samples were stored frozen between experiments without loss of activity.

Chemicals used in this study were obtained from Sigma Chemicals (St. Louis, Mo.). Dialysis tubing was obtained from VWR Scientific (San Francisco, Calif.) and was prepared prior to use by boiling 15 min in a 10-m*M* EDTA solution to remove trace metals.

A typical experiment consisted of incubating a mixture of semen and saliva in a reaction volume of 40 to 50 μL in a plastic microcentrifuge tube at 37°C in a water bath. At the end of the desired incubation period, the tube containing the mixture was removed from the water bath and a sample was taken for electrophoretic analysis; the remainder could be frozen with no subsequent effect on the PGM isozymes. In tests with inhibitors, the inhibitor solution was added to the incubation mixture; a like quantity of saline or distilled water was added to the control.

Results

Alteration of Patterns

Altered seminal PGM_1 Type 1 and Type 2-1 patterns are shown in Fig. 2 (left and right, respectively). In the illustrated experiment, seminal plasma and saliva were mixed in equal proportions; an aliquot of each mixture was immediately frozen as a control and the

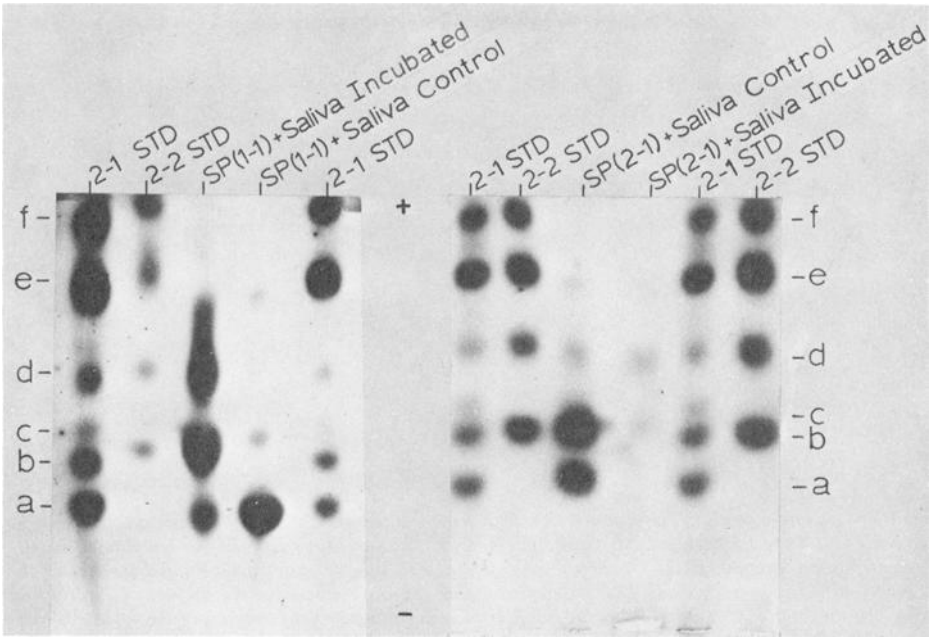

FIG. 2—*Alteration of PGM isozyme patterns in semen/saliva mixtures.* (left) *Seminal plasma* (SP) *and saliva, both from a PGM Type 1 individual, were mixed in equal proportions and incubated at 37°C for 2 h* (center track). *The control mixture was frozen immediately after mixing. Seminal plasma mixed with isotonic saline and incubated under the same conditions showed no pattern alteration. The saliva sample contained no detectable PGM activity.* (right) *Seminal plasma and saliva from a PGM Type 2-1 individual were treated as described above. The standards on both gels were red cell hemolysates.*

remaining solutions were incubated at 37°C for 2 h. The PGM_1 isozyme pattern given by the Type 1 control mixture (left gel) is a typical seminal Type 1 pattern; both a and c bands are present with the a band dominating in intensity. The isozyme pattern given by the incubated mixture is distinctly altered; the a band has diminished in intensity, there is an unresolved smear in the pattern encompassing the b and c band positions, and there is an additional smear extending anodally from the d band position. This altered pattern is similar in its features to the pattern given by the case material illustrated in Fig. 1. The altered pattern is also not dissimilar to a somewhat smeared Type 2-1 pattern; this can be seen by comparing the altered Type 1 pattern with that of the Type 2-1 standard on the left-hand gel.

The alteration of the seminal Type 2-1 PGM_1 pattern (Fig. 2, right) illustrates the relative effects of the alteration on the PGM_1 1 and PGM_1 2 isozymes. The control mixture shows four bands, the PGM_1 1 a and c bands and the PGM_1 2 b and d bands; the a and b bands are dominant. In the incubated sample, the a band is gone and the c band is nearly gone; the b and c bands both remain in diminished intensity and the b band is now somewhat weaker than the d band. The loss of the a band in the altered Type 2-1 pattern makes the altered pattern virtually indistinguishable from a Type 2 pattern.

The progressive alteration of a PGM_1 1 isozyme pattern with time, illustrated in Fig. 3, shows that band a activity gradually disappears with the concomitant generation of anodal bands. This pattern change is consistent with the notion that the a band isozyme is chemically modified to yield the more anodal bands; this, however, remains to be formally demonstrated. Enzyme activity also gradually diminishes during the later stages of the process; this usually becomes apparent only after the a band activity is gone. The alteration

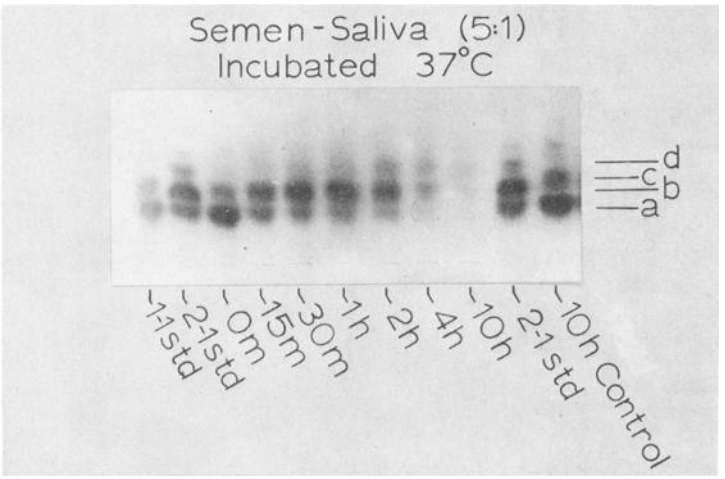

FIG. 3—*Time course of PGM conversion in a semen/saliva mixture. Both semen and saliva were from a PGM Type 1 individual. Mixtures of semen (50 μL) and saliva (10 μL) were incubated for the indicated time intervals at 37°C, then frozen. The 0-min sample was frozen immediately after the semen and saliva were mixed. The 10-h control was the test semen sample incubated for 10 h at 37°C without saliva. The Type 1 and 2-1 standards were fresh seminal plasma samples.*

of PGM_1 2 isozyme patterns follows the same general scheme, that is, the b band gradually disappears with the concomitant generation of anodal bands. However, the rate of alteration of the PGM_1 2 isozyme patterns appears to be slower than for the PGM_1 1 isozymes. As a consequence, conversion of Type 2-1 samples usually passes through a stage in which the a band is missing, resulting in an isozyme pattern that greatly resembles a Type 2 pattern; this stage is captured in Fig. 2.

The rate of PGM_1 isozyme band alteration varies directly with the proportion of saliva present in the semen/saliva mixture. Equal-volume mixtures (1:1) exhibit rapid conversion to the altered pattern (see, for example, Fig. 2); the rate of change slows as the proportion of saliva diminishes, and when the proportion drops below about 40:1 semen/saliva, conversion goes very slowly if at all. The dependence of the conversion rate on the saliva proportion may account for some of the variation in conversion rates seen from experiment to experiment; different saliva samples may contain different amounts of the saliva "conversion" factor or factors. Variation in conversion rates is also seen when semen samples from different individuals or from the same individual collected at different times are used with a single saliva sample. This may be explained by variation in the level of some semen component or by differences in the susceptibility of the PGM_1 subtype isozymes [5–7] to whatever chemical modification is occurring. This latter possibility is currently under investigation.

Since semen evidence is often found or preserved in the dried state, it was important to determine whether PGM_1 conversion occurred in dried mixtures of semen and saliva. Dried stains on filter paper were made from aliquots taken from semen/saliva mixtures that had been incubated for various periods of time at 37°C. The stains were stored for up to one month at room temperature (19 to 22°C) and then characterized for PGM conversion. It was found that the extent of conversion in each stain matched the conversion that had occurred in the liquid mixture prior to drying; there was no evidence of continued conversion in the dried state. If dried stains made from a freshly prepared semen/saliva mixture were subsequently rehydrated and incubated wet, no conversion appeared to occur; thus, drying apparently inactivates the conversion factors.

Preliminary Biochemical Characterization of the PGM_1 Conversion Reaction

A more complete understanding of the conversion reaction requires (a) identification of the conversion factor or factors present in saliva and semen and (b) characterization of the conditions that promote or inhibit the conversion. Some preliminary experiments addressing these questions are described below.

Boiling or dialyzing saliva prior to incubation with seminal plasma resulted in no conversion (Table 1). The loss of conversion activity in the saliva remaining after dialysis suggests a low molecular weight saliva factor. However, incubation of seminal plasma with the salivary low-molecular-weight dialysate fraction (concentrated by lyophilization) did not result in conversion. This observation and the results of other experiments in progress suggest that more than one factor in saliva is involved in the conversion process.

If saliva alone contained all the factors required for conversion, then conversion should be seen in mixtures of saliva with other tissue materials, for example, blood. However, no conversion was seen when saliva/red cell hemolysate mixtures were incubated; this suggests that semen may also contain a factor involved in PGM_1 conversion. To determine whether this was the case, mixtures containing red cell hemolysate from a PGM_1 1 individual and saliva or seminal plasma, or both, from a PGM_1 2 individual were prepared and incubated; the disappearance of the a band isozyme (from the hemolysate) provided indication of PGM conversion regardless of what happened to the seminal PGM isozymes. Conversion occurred only when both untreated semen and saliva were present (Table 2); there was no conversion when either semen or saliva was absent or had been boiled. This indicates that a heat-labile semen factor is involved in the conversion of seminal PGM_1 in the semen/saliva mixtures.

To gain some insight into the biochemistry of the conversion reaction, various chemical agents were added to semen/saliva mixtures before or after incubation; the results of these experiments are summarized in Table 3. The addition of reducing agents before incubation blocks conversion. However, "converted" PGM does not revert if reducing agents are added after incubation. These findings indicate that the conversion does not involve formation of a mixed disulfide complex similar to that formed between red cell acid phosphatase and glutathione [*12*], nor does it involve any other easily reducible oxidation product of PGM_1; the possibility of an irreversible oxidation reaction [*13*] is not excluded although the fact that conversion occurs in a nitrogen atmosphere tends to discount this possibility. Neither of two serine protease inhibitors blocks the conversion reaction,

TABLE 1—*Characterization of PGM_1 conversion factors in saliva.*

Reaction Mixture[a]	Conversion[b]
SP + Sal	++
SP + dialyzed Sal	±
SP + boiled Sal	−
Dialyzed SP + Sal	++
Dialyzed SP + dialyzed Sal	−
Dialyzed SP + concentrated Sal dialysate	−

[a]Seminal plasma (SP) from a PGM_1 1 individual (30 μL) and saliva (Sal) (10 μL) were mixed and incubated for 5 h at 37°C prior to electrophoresis.

[b]Conversion (++) was indicated by the total loss of a band activity, the appearance of anodal bands, and a weakening of total PGM activity. Partial conversion (± and +) was judged by the relative intensity of the a and anodal bands; the ± score indicated a and anodal bands of roughly equal intensity and the + score indicated predominance of the anodal bands. The negative (−) score indicated a PGM pattern indistinguishable from uncontaminated semen.

TABLE 2—*Evidence for a PGM_1 conversion factor in seminal plasma.*

Reaction Mixture[a]	Conversion[b]
Hemo + Sal	−
Hemo + SP	−
Hemo + SP + Sal	++
Hemo + boiled SP + Sal	−
Hemo + SP + boiled Sal	−
Hemo + boiled SP + boiled Sal	−

[a] Hemolysate (Hemo), seminal plasma (SP), and saliva (Sal) were mixed in equal proportions and incubated for 2 h at 37°C.
[b] Scored as described in Table 1.

TABLE 3—*Effect of chemical agents on PGM_1 conversion.*

	Conversion[b]	
Treatment[a]	SP-Sal Mix	PGM Control
Reducing agents added prior to incubation		
Reduced glutathione (25 m*M*)	−	−
Dithioerythritol (10 m*M*)	−	−
Mercaptoethanol (25 m*M*)	−	−
Reducing agents added after incubation		
Reduced glutathione (25 m*M*)	++	−
Dithioerythritol (10 m*M*)	++	−
Protease inhibitors added before incubation		
Bovine pancreatic trypsin inhibitor (2.5 mg/mL)	++	−
Phenylmethylsulfonyl fluoride (5 m*M*)	...[c]	...[c]
Aprotinin (22 trypsin inhibitor units/mL)	++	−
Sulfhydryl reagents added before incubation		
p-Hydroxymercuribenzoate (5 m*M*)	++	−
Oxidized glutathione (25 m*M*)	±	±
Miscellaneous		
Incubation in nitrogen atmosphere	++	−
EDTA (25 m*M*) added before incubation	++	−

[a] Saliva (20 μL), agent (10 μL), and seminal plasma (20 μL) were mixed in that order. Mixtures with Tris (10 m*M*) buffered isotonic solution, pH 7.5, substituted for saliva were used as controls (PGM control) to determine whether the agent acted directly on PGM. Incubation was for 2 h at 37°C.
[b] Scored as in Table 1.
[c] Phenylmethylsulfonyl fluoride irreversibly inhibits PGM activity.

indicating that proteases of this type, known to be present in semen and saliva [*14,15*], are probably not involved in the conversion reaction; the possible involvement of other types of proteases cannot be excluded. Finally, the conversion reaction appears to involve neither active sulfhydryl groups nor divalent cations. A number of plausible biochemical mechanisms of PGM_1 conversion remain to be tested and studies on this problem are continuing.

Discussion

The experiments described in this paper demonstrate that seminal PGM_1 isozyme patterns are substantially altered in semen contaminated with saliva; the rate and extent of the alteration depend on the amount of saliva present in the semen/saliva mixture.

The isozyme pattern conversion follows a characteristic progression: initially the principal isozyme bands (the a and b bands) disappear, anodal bands concomitantly appear, and ultimately enzyme activity is lost. The rates of conversion of the PGM_1 1 and PGM_1 2 isozymes appear to differ, which in combination with the altered appearance of the isozyme patterns may lead to confusion or errors in typing.

The extent of the practical problem posed by PGM_1 pattern conversion depends (a) on the frequency with which the phenomenon occurs and (b) the risk of mistyping when conversion does occur. With respect to the first, PGM_1 pattern conversion may potentially occur in any semen evidence that is contaminated by saliva. It is estimated that about 30% of sexual assaults involve some form of oral sex act [*16*]; thus, saliva contamination is probably not infrequent. In addition, saliva is sometimes used as a lubricant to facilitate vaginal penetration and subsequent copulation; in such situations, levels of contaminating saliva might be substantial. Beyond these considerations, little can be said about the frequency with which semen evidence is contaminated by saliva; hard numbers would help considerably in assessing how often conversion might pose problems. In any case, in particular situations, indication of possible saliva contamination cannot be reliably provided by the victim's statement since many victims are understandably reticent about describing distasteful acts and because the use of saliva as a lubricant may not have been observed. As a consequence, it would be desirable to test all samples for saliva contamination independently of the victim's account.

Although PGM_1 conversion has been demonstrated so far only in semen/saliva mixtures, the possibility remains that conversion may, under some circumstances, occur in uncontaminated semen or in semen contaminated with other body fluids. (For that matter, PGM_1 conversion may also occur in contaminated or uncontaminated blood but the fact that it has not previously been noted suggests that it probably does not.) Promotion of conversion by vaginal fluids under any circumstances would be of obvious concern and this is currently under investigation. Ultimately, the assessment of whether or not PGM_1 conversion is restricted to semen/saliva mixtures will depend on a more complete understanding of the biochemistry of the reaction; if the factors present in semen and saliva are present elsewhere, the possibility of conversion may be more widespread than currently appreciated.

The alteration of PGM_1 isozyme patterns makes more complex the task of the analyst interpreting PGM typing plates. Obviously great caution is called for and the guidelines suggested by Price et al [*3*] appear to provide the best hedge against error. It may be emphasized that relative band intensities as well as band position are important in interpretation; the relative intensification of anodal staining material and the blurring of band resolution provide indications of conversion. The analyst should also be aware that the potential risk of error resulting from conversion differs from type to type and is predictable: PGM_1 Type 1 patterns are altered to produce fuzzy 2-1-like or possibly 2-like patterns, Type 2-1 patterns convert to apparent Type 2 patterns, and Type 2 patterns do not convert to another type but simply fade. In making a typing judgment, the caveat of possible conversion effects should be held in mind.

It would be desirable if some warning signal could be developed to indicate the risk of PGM_1 conversion. At present, detection of saliva contamination and "not quite right" isozyme patterns are the best signals available. Development of a better, more systematic, and reliable warning system depends on a better knowledge of the biochemistry of the conversion reaction.

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