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Genetic Markers in Human Semen. II: Quantitation of Polymorphic Proteins

Semen evidence is almost always present in cases involving sexual assault. The typing of genetic markers in this evidence material can be very valuable in the investigation and prosecution of these crimes. In many cases the victim of the assault cannot identify the assailant and in these cases genetic information gained from the semen can be used to include or exclude potential suspects. Even when the victim can identify the assailant, genetic typing information can provide important independent corroborating evidence. It is therefore clear that more extensive genetic characterization of semen evidence would be beneficial.

In an earlier report [*1*] we reviewed current knowledge concerning the extent and expression of genetic variation in human semen. It was pointed out that semen contains several genetically variable protein, enzyme, and blood group markers that offer great potential for genetic typing analysis of semen evidence. However, it was also pointed out that there are a number of problems that must be understood and overcome before extensive genetic typing of semen can become a practical reality.

Fundamental to the understanding of these problems is a knowledge of the quantitative expression of the genetic markers in semen. Information about the quantity of each marker present in semen provides guidelines for choosing which markers might yield useful results in particular case situations. Genetic markers present at high levels should be relatively easy to type even in dilute or partially degraded semen samples. On the other hand, markers present at low levels relative to their limits of detection may be useful only under special circumstances. Because the amount of semen evidence available for analysis is often limited, it is important to know what analyses are likely to be informative.

In this report we address the question of the quantitative expression of a number of genetically polymorphic proteins and enzymes in human sperm and seminal plasma. For those markers found in both sperm and seminal plasma the relative contribution of sperm and seminal plasma to the total activity in semen has been determined. Because most crime laboratories are familiar with the typing of these markers in blood, we have compared the activity levels in blood and semen. Finally, to put this quantitative data into a

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meaningful context, we have also determined the minimum amount of each marker required for a typing analysis.

The results of this study show that a few markers are present in semen at quite high levels relative to the limits of detection; therefore, there is no quantitative barrier to the routine utilization of these markers. A large number of markers is present in semen at moderate to marginal levels relative to current detection limits. Some of these are potentially useful in particular contexts such as the analysis of fresh stain material; the remaining markers have, for one reason or another, limited practical potential in semen typing.

Materials and Methods

Preparation of Blood and Semen for Analysis

Blood samples were obtained either by finger puncture into acid-citrate-dextrose (ACD) anticoagulant or by venipuncture utilizing a Vacutainer® coated with ethylenediaminetetraacetic acid (EDTA). Red cells and plasma were separated by centrifugation in a desktop centrifuge; the clarified plasma fraction was removed and saved for analysis without further preparation. The buffy coat was removed from the surface of the packed red cells and discarded. The red cells were then washed twice in isotonic saline (0.9% sodium chloride) and were lysed by the addition of two volumes of cold distilled water to the packed cells or by subjecting the packed cells to freezing and thawing. The red cell membrane debris was removed by high speed centrifugation (25 000 *g*) at 4°C. Plasma and hemolysate samples were usually assayed within a few hours after their preparation; all samples were maintained at 0 to 5°C until analyzed.

Semen samples were obtained from volunteers by masturbation; semen collection proceeded in accordance with approved guidelines for the protection of human subjects. After collection, the semen samples were allowed to liquify at room temperature for 30 min; the volume of the sample was then measured and the sperm count was determined by a turbidometric procedure (see below). Seminal plasma was separated from sperm following high speed centrifugation (15 000 *g*) at 4°C for 10 min; the seminal plasma fraction thus obtained was essentially free of all cellular material. The sperm pellet was washed twice by resuspension in isotonic saline followed by centrifugation at low speed (300 *g*, 10 min). The washed pellet was then lysed by a freeze-thaw cycle in an equal volume of water and the cellular debris was separated from the lysate by high speed centrifugation. The sperm lysates were usually analyzed immediately and were kept at 0°C until the time of analysis; these precautions minimized the degradation of sperm lysate material by released sperm hydrolases.

It should be noted that the sperm pellet fraction also contains a significant proportion of nonsperm cells [*2*]. The contribution of the nonsperm cells to the total activity of the sperm pellet fraction was assessed by examining the pellet fraction prepared from ejaculates of aspermic individuals. In every case this nonsperm cellular material was found to possess substantially less activity than would be expected from the sperm in a normal ejaculate. The seminal plasma fractions from the aspermic individuals were also examined; enzyme levels between these and normal seminal plasmas were compared to determine what proportion of the activity in the normal plasma was contributed by leakage from sperm or testicular tissue. The aspermic semen was obtained from three individuals; two had been vasectomized and the third was aspermic as a result of treatment for testicular cancer.

Blood plasma, seminal plasma, and sperm lysate were assayed for total protein by the

method of Lowry et al [3] with commercially prepared Folin-Ciocalteu reagent (Harleco); bovine serum albumin was the protein standard.

The concentration of hemoglobin (Hb) in hemolysates was determined by using the procedure of van Kampen and Zijlstra [4]; the equivalent concentration of red cells was calculated by assuming that 33×10^6 red cells contain 1 mg of hemoglobin [5].

Sperm counts were determined by a turbidometric procedure. The optical density of appropriately diluted sperm suspensions was read at 450 nm; sperm counts were determined from a standard curve that was calibrated with sperm suspensions counted with a hemacytometer. The calibration curve is linear to an absorbance at 450 nm (A_{450}) of 0.600 and has an approximate slope of 0.15 optical density units per million sperm per millilitre.

Assay of Marker Enzymes and Proteins

Unless otherwise indicated, enzyme assays were conducted at 37°C with a constant temperature water bath. Substrates and enzymes were purchased from Sigma and coenzyme [nicotinamide-adenine dinucleotide phosphate (NADP)] was purchased from P. L. Biochemicals. Antisera were purchased from Cappel; their monospecificity was verified by immunoelectrophoretic analysis. All other reagents were analytical grade.

Glucose-6-phosphate dehydrogenase (G6PD) activity was determined in a spectrophotometric assay at 25°C in which the reduction of NADP was monitored at 340 nm [6]. The final assay solution contained enzyme, 0.6 m*M* glucose-6-phosphate, 4 m*M* magnesium chloride, 0.2 m*M* NADP, and 0.1*M* tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl) buffer, pH 8.0 in a volume of 3 ml; the reaction was initiated by the addition of substrate and was read at 5- to 10-min intervals until the A_{340} had changed 0.05 to 0.2 absorbance units. Activity of 6-phosphogluconate dehydrogenase does not significantly interfere with this assay because the levels of this enzyme are not appreciably greater than the G6PD levels.

The assay for 6-phosphogluconate dehydrogenase (6PGD) activity was similar to the assay for G6PD [7]. The reaction mixture was the same except that 0.6 m*M* 6-phosphogluconic acid was substituted for the glucose-6-phosphate; this assay was also conducted at 25°C.

Phosphoglucose isomerase (PGI) activity was determined in a linked assay system in which the PGI reaction product, glucose-6-phosphate, was measured by its subsequent oxidation by G6PD. The reaction mixture (3 ml) contained enzyme, 0.6 m*M* fructose-6-phosphate, 0.2 m*M* NADP, and one unit of G6PD in 0.1*M* Tris-HCl, pH 8.1.

The determination of phosphoglucomutase (PGM) activity also used an assay system linked to G6PD [8]. The reaction mixture contained, in addition to enzyme, 1.67 m*M* glucose-1-phosphate containing glucose-1,6-diphosphate as contaminant, 4 m*M* magnesium chloride, 2 m*M* EDTA, 0.2 m*M* NADP, and one unit of G6PD in a 3-ml volume buffered with 0.05*M* imidazole-HCl, pH 7.5.

Adenylate kinase (AK) activity was determined by linkage through hexokinase to G6PD. The assay mixture (3 ml) contained enzyme, 2 m*M* adenosine-5′-diphosphate, 1.5 m*M* magnesium chloride, 5.3 m*M* glucose, 0.2 m*M* NADP, one unit of G6PD, and two units of hexokinase in the same pH 7.5 imidazole-HCl buffer. For each of these enzyme assays, one unit of enzyme activity is defined as the reduction of one micromole of coenzyme per minute.

Sperm diaphorase was assayed as previously described [9].

Peptidase A activity was assayed in a linked reaction with L-amino acid oxidase and peroxidase with L-valyl-L-leucine as substrate according to the procedure of Sinha et al [10]. One unit of enzyme activity is defined as the hydrolysis of 1 μmole substrate

per minute at 37°C. A mixture containing 1 μmole each of L-valine and L-leucine produces an A_{415} of about 0.330 with the standard assay conditions.

Amylase activity was assayed by a starch precipitation technique that will be described in detail elsewhere.[2] The conventional dinitrosalicylic acid procedure of Bernfield [*11*] is not appropriate for seminal plasma since this fluid contains large quantities of reducing substances.

Proteins with no enzyme activity were assayed by quantitative radial immunodiffusion according to the procedure of Fahey and McKelvey [*12*]. Agarose gels containing antiserum were prepared by mixing one volume of antiserum (appropriately diluted with isotonic saline buffered with 0.01*M* Tris-HCl, pH 7.4) with one volume of melted agarose (2% in the Tris-buffered saline); both antiserum and melted agarose were at about 50°C at the time of mixing. The melted agarose-antibody mixture was gelled in petri dishes at a depth of about 2 mm. For the measurement of antigen, a measured amount of antigen solution (5 to 10 μl) was added to wells (2 mm diameter) punched in the gel. The antigen was allowed to diffuse for 24 to 72 h. The gels were then washed in Tris-buffered saline for 24 h to remove unprecipitated protein, after which they were washed for 1 h in water to remove salt. The gels were subsequently air-dried on glass plates and stained for precipitated protein with amido black (0.2% amido black in methanol, acetic acid, and water in the ratio of 5:1:5). Immunoprecipitates were observed as rings around the antigen wells; the area of the precipitin ring correlated directly to the amount of antigen added. Ring diameters were measured from the enlarged images of the dried and stained diffusion plates projected on a standard photographic enlarger. Standard calibration curves were established by using serial dilutions of normal human serum as the antigen solutions and the concentrations of antigen in unknowns were determined by comparison to the standard curve.

Determination of Minimum Quantities for Analysis

The minimum amount of a protein or enzyme required for an electrophoretic typing analysis was estimated by performing electrophoresis on serial dilutions of a sample containing a known level of protein or enzyme activity; a standard quantity of each dilution was applied to the electrophoresis gel. The minimum level of activity required for a typing determination was then indicated by the most dilute solution yielding a clear typing result. In estimating the minimum required amounts for those enzymes visualized by the tetrazolium staining procedure, the sensitivity titration was done by using AK as reference enzyme. The justification for this approach is that for these enzymes the assays directly and the staining reactions indirectly measure the same reaction process, the production of NAD(P)H. This method indicated that the minimum enzyme activity required to produce a tetrazolium band on an electrophoretic gel was about 0.5 milliunit. For the purpose of these determinations we have not taken into consideration the fact that heterozygous samples generally produce multiple banded patterns; however, in systems, such as PGM, where multiple loci might be expressed, appropriate compensation has been made. The minimum required activity units for the remaining enzymes were determined independently since each is visualized by a distinct staining reaction. The minimum required amounts for those plasma protein markers conventionally typed by electrophoresis were determined in essentially the same fashion. For the immunoglobulin markers Gm and Inv the estimates of the minimum required amounts were based on a standard micro typing procedure [*13*].

[2]E. T. Blake and G. F. Sensabaugh, "Differential Expression of Amylase Loci (Amy_1 and Amy_2) in Human Body Fluids and Secretions," in preparation.

Results

Determination of Enzyme Levels in Sperm and Seminal Plasma

Semen consists of a cellular fraction and a plasma fraction. The cellular fraction of semen consists primarily of spermatozoa but, in addition, contains exfoliated tissue cells and cellular debris; this nonsperm cellular material may make up 30% of the total cellular fraction of semen [2]. The cellular fraction of semen makes up a relatively small proportion of the total semen volume; on a volume basis, semen is 95% or more plasma. To assess the relative contribution of sperm and plasma to the total semen enzyme levels, the cellular and plasma fractions were separated and assayed independently. This approach also allowed the concentration of the marker enzymes in sperm and red cells to be directly compared.

Table 1 shows the activities of seven intracellular enzymes in sperm and in red cells; these activities have been compared on a cell-to-cell basis. It is apparent that some markers such as AK are found at much higher levels in red cells. Peptidase A and PGI, on the other hand, are present in sperm cells at considerably higher levels than in red cells. Sperm diaphorase is not found at all in red cells; red cells do contain, however, other diaphorases. The remaining three enzymes are present at about the same level in the two cell types.

These enzymes are primarily thought of as intracellular enzymes; in semen, however, some of them are found in the plasma fraction as well. Table 2 presents data comparing the specific activities of the seven marker enzymes in seminal plasma and sperm extracts. It is apparent that for each enzyme the specific activity in sperm is higher than in seminal plasma. This might suggest that the activity present in seminal plasma is the result of leakage from sperm. However, analysis of seminal plasma from vasectomized individuals shows clearly that this is not the case; the level of activity in the seminal plasma of vasectomized individuals is comparable to the level of activity found in the seminal plasma of unvasectomized men. One important implication of this finding is that the presence of sperm in semen is not a necessary requirement for the typing of these markers provided that there is sufficient activity in the seminal plasma to begin with.

It is apparent from Table 2 that some of the marker enzymes are present in seminal plasma at relatively high levels. Since the seminal plasma makes up about 95% of the total volume of whole semen, it would be expected that the contribution of the seminal plasma activity to the total activity in whole semen would be considerable. That this is the case is shown in Table 3. Comparing the figures for sperm and seminal plasma shows that for the markers peptidase A, PGM, and PGI about 90% of the total semen activity

TABLE 1—*Comparison of enzyme activity in sperm and red cells.*

Enzyme	Activity, milliunits/10^6 Cells[a]		Sperm/Red Blood Cell Ratio
	Sperm	Red Cells	
Adenylate kinase	0.44 ± 0.2 (12)	7.91 ± 0.72 (10)	0.056
Glucose-6-phosphate dehydrogenase	0.105 ± 0.08 (7)	0.16 ± 0.022 (10)	0.66
Peptidase A	2.09 ± 1.88 (6)	0.048 ± 0.035 (10)	43.5
6-Phosphogluconate dehydrogenase	0.182 ± 0.12 (10)	0.11 ± 0.009 (10)	1.65
Phosphoglucose isomerase	11.1 ± 10.1 (6)	1.36 ± 0.14 (10)	8.16
Phosphoglucomutase	0.688 ± 0.27 (13)	0.218 ± 0.046 (10)	3.16
Sperm diaphorase	0.156 ± 0.08 (8)	absent	...

[a] Number in parentheses indicates the number of determinations.

TABLE 2—*Specific activity of enzymes in sperm and seminal plasma.*

Enzyme	Specific Activity, milliunits/mg Protein[a]		
	Sperm	Seminal Plasma	Seminal Plasma (Vasectomized)
Adenylate kinase	330.8 ± 177.3 (12)	1.58 ± 1.04 (11)	1.96 (1)
Glucose-6-phosphate dehydrogenase	107.7 ± 10.5 (7)	0.114 ± 0.066 (8)	0.087 (1)
Peptidase A	997.2 ± 589 (6)	155.9 ± 129 (13)	248.1 ± 256 (3)
6-Phosphogluconate dehydrogenase	163.3 ± 114.5 (10)	0.207 ± 0.1 (16)	0.135 (1)
Phosphoglucose isomerase	2592 ± 1987 (6)	104.8 ± 47.6 (9)	62.8 (1)
Phosphoglucomutase	525.9 ± 263 (13)	14.5 ± 8.7 (17)	18.7 ± 1.1 (2)
Sperm diaphorase	117.3 ± 61.2 (8)	absent	absent

[a]Number in parentheses indicates the number of determinations from different individuals.

is contributed by seminal plasma. In contrast, for the remaining four enzymes, AK, G6PD, 6PGD, and sperm diaphorase, the contribution of seminal plasma is not as significant. Also included in Table 3 is amylase, a secreted enzyme, which is not found in sperm at all.

For purposes of comparison, the quantitative values for whole blood are also included in Table 3. The whole blood values for the seven intracellular enzymes are predicated on their levels in red cells; the whole blood value of amylase, on the other hand, is based on its blood plasma level because this enzyme is not found in red cells. Only in the case of peptidase A is there more activity in whole semen than in whole blood. The levels of amylase, PGM, and PGI in blood and semen are roughly equal, and the remaining markers are present at very much lower levels in semen than in blood. The practical implication of these findings is that the markers present in semen at reasonable levels will be as easy to type from semen as they are from blood.

Determination of Seminal Plasma Levels of Serum Protein Genetic Markers

Several of the genetically polymorphic nonenzymatic proteins found in blood plasma are also found in seminal plasma; the levels of these proteins in both fluids were estimated by a quantitative radial immunodiffusion assay. The radial immunodiffusion assay for transferrin is shown in Fig. 1 to illustrate the technique. With the radial diffusion assay the levels in semen of eight marker proteins were determined relative to their levels in blood plasma; these results are shown in Table 4.

As indicated in our previous report, four genetically variable blood plasma proteins could not be detected in seminal plasma; these were haptoglobin, group specific component, complement factor 3, and ceruloplasmin. These findings are in agreement with other studies [*14,15*]. The values in Table 4 indicate the sensitivity limit of the radial diffusion assay; these four marker proteins may in fact be present in semen at levels lower than the indicated values but, if so, the markers would be useless for typing purposes.

The remaining four marker proteins are present in seminal plasma at levels corresponding to 1 to 5% of their levels in blood plasma; these values are comparable to those obtained by Tauber et al [*14,15*]. Of particular note are the standard deviations in the levels of these four proteins, which range as high as 90% of the mean value. Comparison of the intra- and inter-individual variances shows that, except for transferrin, the major

TABLE 3—*Comparison of genetic marker enzymes in semen and blood.*

Enzyme	Sperm, milliunits/8×10^7 Cells	Activity, milliunits/ml			Semen Activity from Seminal Plasma, %	Semen/Blood Ratio
		Seminal Plasma	Total Semen[a]	Whole Blood		
Adenylate kinase	35.2	47.8	83.0	37 300	58	0.0022
Amylase	absent	910	910	1 160	100	0.78
Glucose-6-phosphate dehydrogenase	8.4	5.74	14.1	753.6	41	0.019
Peptidase A	167.2	5901	6068	225.4	97	26.9
Phosphoglucomutase	55.04	449.1	504.1	1 037	89	0.49
6-Phosphogluconate dehydrogenase	14.56	8.74	23.3	517.7	36	0.045
Phosphoglucose isomerase	888	4381	5269	6 402	83	0.82
Sperm diaphorase	12.48	absent	12.48	absent	0	...

[a] The contribution of sperm to the total activity in semen is calculated by assuming that the average ejaculate contains 80 million sperm per millilitre.

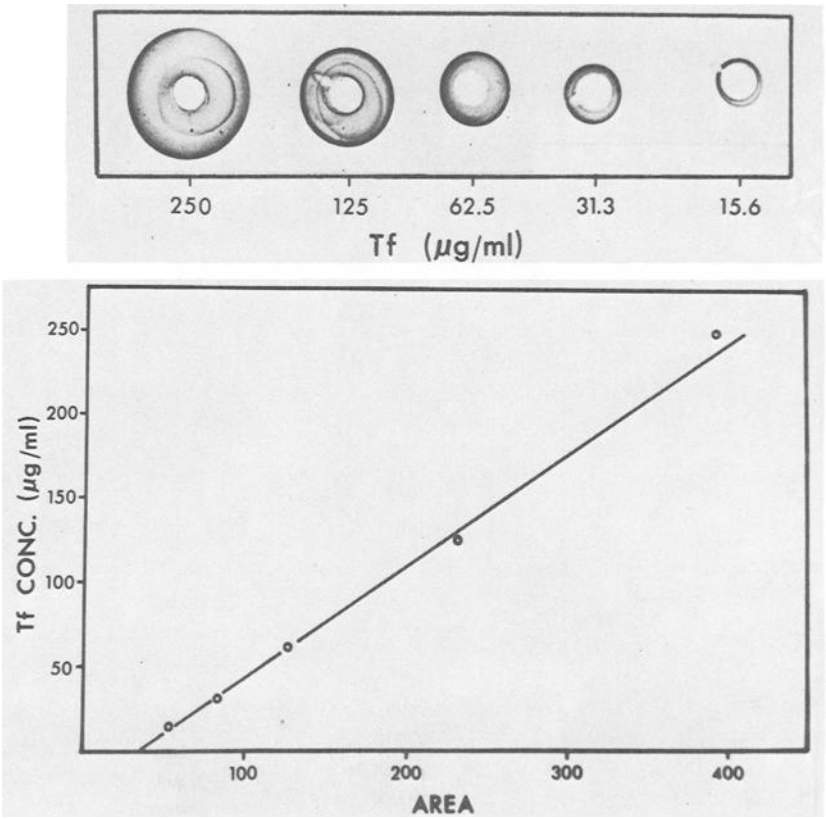

FIG. 1—*Quantitative radial immunodiffusion assay for transferrin* (Tf). *The gel contained 1% monospecific antitransferrin antiserum. The monospecificity of the antiserum was verified by immunoelectrophoresis against purified transferrin, whole blood serum, and seminal plasma. The area enclosed by the precipitin ring* (top) *is directly proportional to the amount of antigen in the sample* (bottom). *This procedure was used to assay the other nonenzymatic proteins surveyed in this study.*

TABLE 4—*Comparison of nonenzymatic proteins in seminal plasma and blood.*

Protein	Amount, μg/ml		Semen/Blood Ratio
	Seminal Plasma[a]	Whole Blood[b]	
α_1-Antitrypsin	68.2 ± 29.2 (18)	1000	0.068
Transferrin	110 ± 98 (20)	1100	0.1
Immunoglobulins (Gm and Inv Markers)			
IgG (Gm and Inv)	98 ± 50 (23)	5500	0.018
IgA (Inv)	25 ± 6 (4)	1100	0.023
Complement C3	not detected at 24	600	...
Gc protein	not detected at 20	375	...
Ceruloplasmin	not detected at 10	225	...
Haptoglobin (Type 1-1)	not detected at 5.6	900	...

[a] For those proteins that could not be detected in seminal plasma the number indicates the sensitivity of the assay.

[b] Whole blood values were calculated by assuming that whole blood is 50% serum. Serum values were taken from the literature.

component of variation is intra-individual. This means that there is considerable variation in the quantitative expression of these markers from ejaculate to ejaculate.

Determination of Minimum Levels Required for Routine Genetic Typing

To put the preceding quantitative data into context, the activity level of each marker was compared to the minimum amount required for a routine genetic typing analysis. The minimum activity required for electrophoretic typing of the marker enzymes was indicated by the most dilute sample yielding a clear typing result. A typical determination, for esterase D, is illustrated in Fig. 2.

The results of these determinations are shown in Table 5, Column 1. From these minimum amount estimates, the equivalent minimal volumes of blood, whole semen, and seminal plasma were calculated (Columns 2 to 4) by dividing the minimum required amount by the concentration of the marker in the indicated fluid. The contribution of sperm (assuming a sperm count of 80×10^6 sperm/ml) to the total semen activity is indicated by the difference between whole semen and seminal plasma.

It will be noted that this table contains data pertaining to glyoxalase. The quantitative expression of this polymorphic enzyme in semen has not been previously described, and this is the subject of an accompanying report;[3] pertinent observations need only be summarized here. Comparison of the glyoxalase activity in serial dilutions of red cell

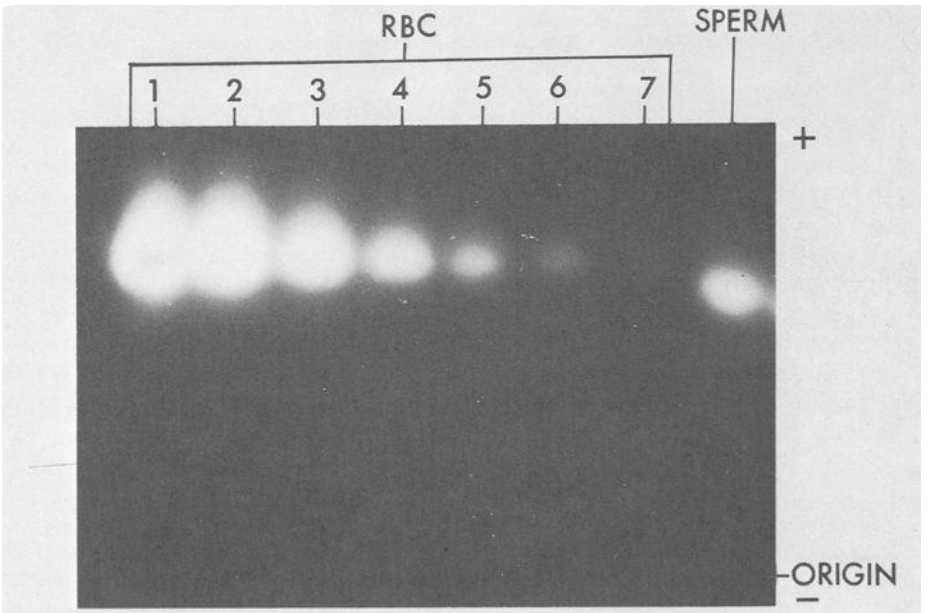

FIG. 2—*Electrophoretic sensitivity titration for esterase D in blood and sperm. Standard aliquots (10 μl) of sample were applied to the electrophoresis wicks. Red cell hemolysate samples* (RBC) *were serially diluted; RBC-1 contains the equivalent of 28 million red cells and RBC-7 contains the equivalent of 0.4 million red cells. After electrophoresis* [16] *the gels were sliced and stained with 4-methylumbelliferone acetate for 30 min. The limit for typing is reached at RBC-6 (0.8 million red cells), which is equivalent to 0.2 μl of whole blood. The sperm sample contains the equivalent of 3.6 million sperm and has the same approximate intensity as RBC-4, which contains 3.5 million red cells. Thus the concentration of esterase D in sperm and RBC is about the same.*

[3] E. T. Blake and G. F. Sensabaugh, "Genetic Markers in Human Semen. III. Expression of Glyoxalase I in Human Sperm and Seminal Plasma," in preparation.

TABLE 5—*Estimates of minimum quantity of semen for typing determinations.*

Genetic Marker	Minimum Amount Detectable[a]	Whole Blood, μl	Whole Semen, μl	Seminal Plasma Alone, μl
Peptidase A	0.25	1	0.04	0.04
Phosphoglucose isomerase	0.5	0.1	0.1	0.1
Phosphoglucomutase$_1$[b]	0.5	1	1	1
Glyoxalase[c]	...	1	5	5
Amylase	5	5	5	5
Adenylate kinase	0.5	0.01	5	10
Esterase D	...	0.2	10	...
6-Phosphogluconate dehydrogenase	0.5	1	25	60
Glucose-6-phosphate dehydrogenase	0.5	1	40	90
Sperm diaphorase	0.5	absent	40	absent
Transferrin	5	5	50	50
Gm (IgG)[d]	10	2	100	100
Inv (IgG and IgA)[d]	10	2	100	100
α_1-Antitrypsin	5	5	100	100
Phosphoglucomutase$_3$[b]	0.5	absent	400	absent

[a] Milliunits for enzymes and micrograms for nonenzymatic proteins.

[b] The activity of PGM_1 in red cells is half the total PGM activity. The activity of PGM_3 in sperm is estimated to be about 2% of the total sperm PGM activity.

[c] Although a value is given for seminal plasma, for reasons described in the text glyoxalase typing from this fluid is very difficult.

[d] Quantities indicate material needed to detect each antigenic determinant.

and sperm lysates indicates that there is about four times more glyoxalase activity in sperm cells than in red cells on a per cell basis. The phenotypic expression of glyoxalase in sperm and red cells is identical; that is, the electrophoretic patterns are the same for a single individual and reflect the genotype. The electrophoretic pattern of glyoxalase activity in seminal plasma, on the other hand, differs from the sperm and red cell patterns; the isozyme pattern from seminal plasma is smeared and has an altered electrophoretic mobility. The smearing and mobility shift do not appear to have a trivial explanation and efforts to reverse the effect have not been successful. Because the seminal plasma glyoxalase activity smears over the sperm glyoxalase isozyme bands, the typing of glyoxalase from whole semen samples poses significant interpretive problems.

Discussion

The genetic analysis of semen evidence is of interest in two contexts: in the analysis of semen stains and in the analysis of semen traces in vaginal swabs or aspirates. In either case, the capacity of any particular genetic marker to be typed depends on quantitative considerations: the amount of the marker initially present, its stability in the evidence material, its dilution in the stain or swab extract, and the detection limit of the typing procedure. The basic objective of this study was to determine the levels of a number of genetic markers in semen and to compare these levels to practical typing detection limits. Semen levels were also compared with blood levels to provide a standard of reference. In making the comparison between semen or blood levels and typing detection limits, one can see how stability and dilution factors might affect the ultimate capacity of a marker to be typed. For example, a marker present in semen at 100 times its minimum typing detection level may be typeable despite substantial loss of activity or dilution whereas a marker present at less than ten times its typing limit level would not be typeable under

the same conditions. Based primarily on quantitative considerations such as these, it has been possible to divide the genetic markers present in semen into three general groups.

The first group contains those markers present in semen at levels substantially higher than their minimum quantitative typing limit levels; there is no major quantitative barrier to their analysis. The three markers in this group are PGM, peptidase A, and PGI. Each is present at a high level in both seminal plasma and sperm. Because of the high levels in seminal plasma the presence of sperm is not required for analysis and the typing of semen from aspermic males is possible. Of these three markers, PGM has the most general value in terms of its discrimination potential; it is not difficult to type in dried and liquid semen as long as one is aware that its phenotypic expression in semen is somewhat different from that in blood [*1,17*]. The potential value of PGM has been increased by the recent demonstration that the three common PGM phenotypes can be subdivided into ten phenotypes with isoelectric focusing [*18,19*]. The value of peptidase A is, at the present time, limited to situations involving semen from black males. There are, however, two additional common phenotypes of peptidase A in European and American white populations which, given current electrophoretic typing methods, cannot readily be distinguished from the common 1-1 type [*10,20*]. Thus, the potential of this marker system is yet to be realized. Finally, the value of PGI as a marker is limited by the fact that variant types are relatively rare, occurring in only about 1% of the population.

The second group of markers contains those proteins that have moderate activity in semen and that can be typed in semen evidence under certain favorable circumstances. Included in this group are AK, amylase, esterase D, glyoxalase, G6PD, 6PGD, and transferrin. Two of the markers, amylase and transferrin, are seminal plasma proteins and can be typed in semen provided that the semen sample has not been appreciably diluted. Even then, the levels of these two proteins in semen are close to the limits of their detectability on typing gels and some concentration step may be required prior to analysis. The remaining five enzymes are basically sperm enzymes; their levels in seminal plasma are sufficiently low to preclude routine typing from this fluid alone. Accordingly, the typing of these five enzyme markers would be possible only in those special situations where sufficient sperm can be isolated from the sample prior to analysis or where a large semen sample is available. Within this group of seven markers, glyoxalase is the most valuable in terms of discrimination potential; however, as noted in Results, there may be a significant problem in the interpretation of the isozyme patterns given by mixtures of sperm and seminal plasma. Esterase D is the next best marker in this group; its discrimination potential is better for Asian Indians and East Asians than for European and American whites and blacks [*21*]. Transferrin and G6PD have discrimination value only in black populations; G6PD is, in addition, a somewhat unstable enzyme and may not be detectable in any but the best sperm preparations. The remaining three markers, AK, amylase, and 6PGD, have relatively low discrimination potentials in all racial groups; the value of AK is further restricted by the apparent preferential degradation of the "2" isozyme in lysed sperm [*1*].

The third group contains markers present at moderate to low levels in semen which for one reason or another are of limited usefulness in the forensic context. This group contains α_1-antitrypsin, the immunoglobulin markers (Gm, Inv), PGM_3, and sperm diaphorase. A critical factor in every case is that relatively large amounts of semen are required for typing. The analysis of α_1-antitrypsin, the immunoglobulin markers, and PGM_3, for example, requires the equivalent of about 0.1 ml or more of whole semen. The fourth member of this group, sperm diaphorase, does not require as much material but is of restricted value because of its limited expression [*9,22*]; there is little point in attempting to type this marker unless appropriate reference material (that is, sperm) can be obtained from a suspect. Of these four, the immunoglobulin markers are by far the most valuable in terms of their discrimination potential; however, Gm and Inv typing requires expertise and reagents not commonly available at present in the forensic science laboratory.

The assignment of the genetic markers in semen to these three groups is of course somewhat relative. The assignments are dependent on the values taken for the minimum amounts required for typing. We have attempted to be conservative in estimating minimum typing limits, and procedures currently in use in some laboratories may reduce the minimum levels by a factor of two or four. The comparative data on the levels of the markers in blood should allow these laboratories to make appropriate adjustments. For the most part, however, the present assignments would not be significantly affected by such a relatively small increase in the sensitivity of typing procedures. Improvement in typing sensitivity of the sort that would promote markers from the second and third groups into the first group would require in most cases gains in sensitivity of 10- to 20-fold or more. In this regard the quantitative data presented here have value in another context: they establish for each marker a standard of sensitivity that improved techniques must exceed in order to diminish quantitative barriers to analysis.

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