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The Simultaneous Electrophoretic Analysis of Esterase D and Phosphoglucomutase Subtyping in Fresh Blood and in Dried Bloodstains

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ABSTRACT: Phosphoglucomutase$_1$ (PGM) subtyping and esterase D phenotyping were simultaneously performed by electrophoresis of bloodstained fibers using agarose and a Tris-maleic acid buffer system, pH 5.4. This method reduces anodal gel shrinkage and shortens development time when compared to the conventional electrophoretic technique for PGM subtyping which is performed at pH 7.4 using an agarose-starch substrate.

KEYWORDS: criminalistics, genetic typing, electrophoresis, phosphoglucomutase, esterase D

The identification of the three common phosphoglucomutase (PGM_1) phenotypes by the electrophoretic analysis of bloodstained threads was first performed in 1967 by Culliford [*1*]. Since that time it has been demonstrated that four PGM loci exist, each of which determines the production of a characteristic set of isozymes [*2-4*]. Conventional starch gel electrophoretic analysis and pedigree analysis showed that the three common PGM phenotypes (PGM_1 1, PGM_1 2, and PGM_1 2-1) were the result of two common locus 1 alleles [*5-6*]. Thus isozymes a+c are present in Type 1, b+d are present in Type 2, and a, b, c, and d are present in Type 2-1.

The use of isoelectric focusing (IEF) in the analysis of PGM_1 isoenzymes results in more complex band patterns. Sutton and Burgess, using IEF on a polyacrylamide gel to analyze red cell lysates demonstrated the existence of four common alleles at locus 1 resulting in ten phenotypes [*7*], thus confirming earlier reports by Bark et al [*8*] and Kuhnl et al [*9*]. The ten phenotypes are classified as 1+1+, 1+1−, 1−1−, 2+2+, 2+2−, 2−2−, 1+2−, 1−2−, 1+2+, and 1−2+ [*8*]. Subtyping increases the discriminating power from 0.55 with three phenotypes to 0.75 with ten phenotypes [*7,9*]. When electrophoresis of red cell lysates was performed on starch gels using a pH 7.4 buffer system Sutton and Burgess were only able to demonstrate the three common phenotypes. Subsequently PGM_1 subtyping was demonstrated by conventional electrophoresis using a starch gel, and a pH 5.9 buffer system

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[*10*]. The benefits of using starch gel over polyacrylamide is that the latter often fails to polymerize properly, it is neurotoxic prior to polymerization, and ammonium persulfate, which is generally used in the preparation of polyacrylamide gels, is known to inactivate certain enzymes [*11*].

Burdett and Whitehead using isoelectric focusing on polyacrylamide were able to separate the variants of PGM, erythrocyte acid phosphatase (EAP), and hemoglobin [*12*]. Using the same technique, esterase D (EsD) variants have been separated [*13*] and PGM and EAP have been simultaneously separated [*14*]. Burdett successfully subtyped PGM_1 using isoelectric focusing with a charge balanced agarose [*15*]. Agarose is an excellent medium because it is relatively transparent, rapidly and easily polymerized, relatively strong, and can also be purchased with various electroendosmosis (EEO) values to suit a particular electrophoretic analysis. Shaler and Dhawan [*16*] and Wraxall and Provost[3] have developed electrophoretic methods of PGM subtyping using low EEO agarose as the support medium. Shaler and Dhawan have developed an assay in which the serum marker Gc (group specific component) can be separated simultaneously with the PGM_1 subvariants [*17*]. These methods however suffer from gel shrinkage adjacent to the anodal wick as the run proceeds. The increased resistance and increased heating of the gel results in further dehydration at the anodal surface. Decreased current flow and increased heating tend to inhibit migration of the proteins under study and tend to denature them. This paper describes a method that reduces anodal gel shrinkage and thus shortens development time and allows for the simultaneous analysis of PGM_1 subtypes and esterase D isozymes.

Materials and Methods

Collection of Bloodstains

Bloodstains were prepared by placing blood, which had been obtained by finger prick, on cotton cloth. These samples were air dried and stored frozen before further analysis. Fibers from the bloodstain were moistened with gel buffer containing dithiothreitol, 3.5 mg/mL. Following a 15-min incubation at room temperature the moistened threads were placed in the appropriate slots in the gel. Hemolysates were prepared from fresh blood and tested immediately.

Buffers

Catholdal Tank Buffer—The cathodal tank buffer consisted of 0.1*M* Trizma base, 0.1*M* maleic acid, 10m*M* ethylenediaminetetraacetic acid (EDTA) (free acid), and 10m*M* $MgCl_2 \cdot 6H_2O$, adjusted to pH 5.4 with sodium hydroxide.
Anodal Tank Buffer—The anodal tank buffer was prepared as a 1:5 dilution of the cathodal tank buffer.
Gel Buffer—The gel buffer was made up as a 1:15 dilution of the cathodal tank buffer.
PGM Reaction Buffer—The PGM reaction buffer consisted of 0.3*M* Trizma base and 0.1*M* magnesium chloride and was adjusted to pH 8.0 with 6*N* hydrochloric acid.
EsD Reaction Buffer—The EsD reaction buffer consisted of 0.05*M* sodium acetate adjusted to pH 6.5 with 2% acetic acid.

Gel Preparation

The 1% gel was prepared using low EEO agarose (relative mobility $[-M_r] = 0.1$) and gel buffer. The gel measured 20 cm by 15 cm by 1.0 mm.

[3]B. Wraxall and J. Provost, "PGM Subtyping—A Conventional Electrophoretic Method," Serological Research Institute, Emeryville, CA, 1980, unpublished communication.

Origin

The origin was cut 8 cm from the cathode.

Reaction Mixtures

The PGM reaction mixture consisted of the following: 35 mg of glucose-1-phosphate containing approximately 1% glucose-1-6-diphosphate, 2.0 mg of NADP (sodium salt), 1.7 I.U. glucose-6-phosphate dehydrogenase, 2.5 mg of 3-(4,5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide (MTT), 2.5 mg of phenazine methosulfate (PMS), 10 mL of PGM reaction buffer, and 1% agarose. The esterase D reaction mixture consisted of 8 mg of 4-methylumbelliferyl acetate dissolved in 10 mL of EsD reaction buffer. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Agarose was purchased from Serological Research Institute, Emeryville, CA.

Voltage and Time of Run

Twenty volts/centimeters were applied to the gel for a period of 4 to 4.5 h. Electrophoresis was conducted on a cooling platform maintained at 4°C. The conventional electrophoretic analysis of PGM and EsD was performed as described by Wraxall [*18*].

Results

Electrophoresis of bloodstains in Tris-maleic acid buffer, pH 5.4, resulted in anodal gel shrinkage and premature termination of the run when the anodal tank buffer was either undiluted or diluted 1:3 with distilled water. However, dilutions of 1:5 or 1:7 minimized these problems and provided good typing results. Figure 1 illustrates the electrophoretic separation of PGM isozymes using the conventional pH 7.4 gel buffer system. The three phenotypes, PGM_1 1, PGM_1 2, and PGM_1 2-1 can be seen. Figure 2 illustrates separation of EsD isoenzymes using the same buffer system as that used for PGM above. The results of a 4-h electrophoretic run using the Tris-maleic acid buffer, pH 5.4, with dilution of the anodal tank buffer are illustrated in Fig. 3. Four of the ten phenotypes known to be found in blood are present. The PGM_1 isozymes migrate cathodally in this buffer system but anodally in the more conventional system. Figures 4 and 5 illustrate the simultaneous subtyping of PGM_1 and phenotyping of EsD, respectively, following a 4.5-h run. No gel shrinkage or heating of the anodal gel surface was evident. Figure 5 shows the three common phenotypes of EsD. Although the banding pattern of this enzyme is more closely spaced when using a buffer system at pH 5.4 when compared to the results obtained using the buffer system at pH 7.4, nevertheless the three variant phenotypes can easily be distinguished from each other.

Discussion

The results of this study demonstrate that PGM locus 1 subphenotyping can be performed on an agarose medium with good discrimination between the four isozymes 1+, 1−, 2+, and 2−, and that the 4.5-h electrophoretic run using the pH 5.4 buffer system provides reproducible results. Furthermore, EsD can be phenotyped simultaneously with PGM_1. The use of conventional electrophoretic methods to accomplish this has several advantages over isoelectric focusing including economy of analysis and resolving ability when typing two or more enzymes systems having isoelectric points that differ greatly from each other. An additional drawback of IEF is that enzymes can be over-focused when voltage is maintained for an excessive period of time. Enzymes have decreased stability at their isoelectric points. Thus enzymic degradation is promoted with a concomitant loss of activity.

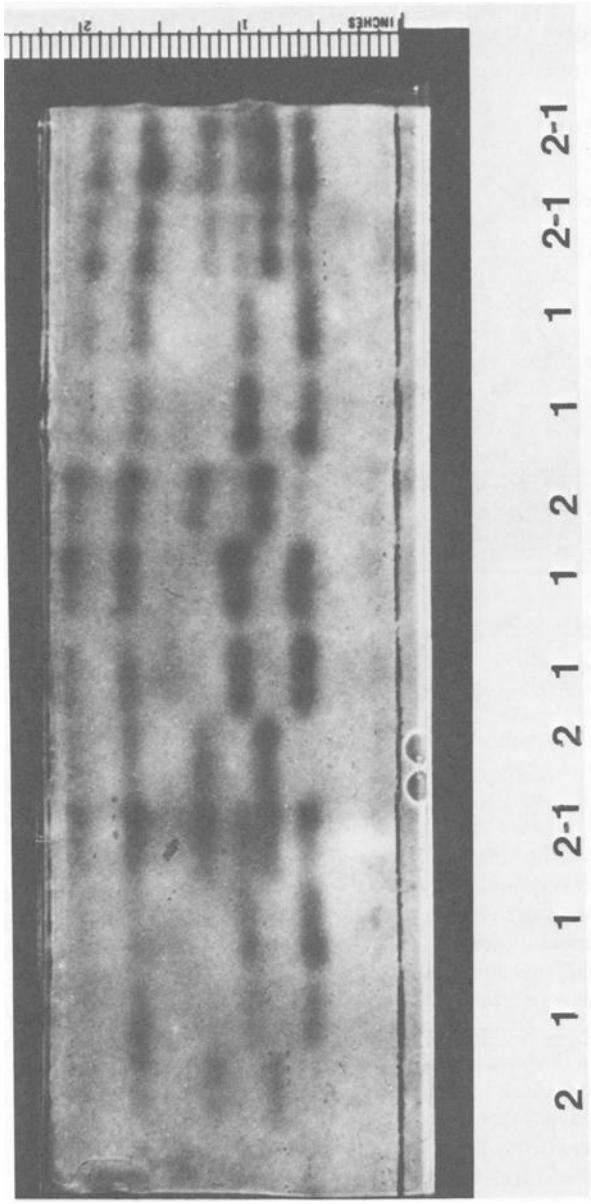

FIG. 1—*Conventional PGM typing using a pH 7.4 buffer system. The three variant forms of this enzyme are represented. From left to right they are: Type 2, Type 1, Type 1, Type 2-1, Type 2, Type 1, Type 2, Type 1, Type 1, Type 2-1, and Type 2-1. 1 in.* = *25.4 mm.*

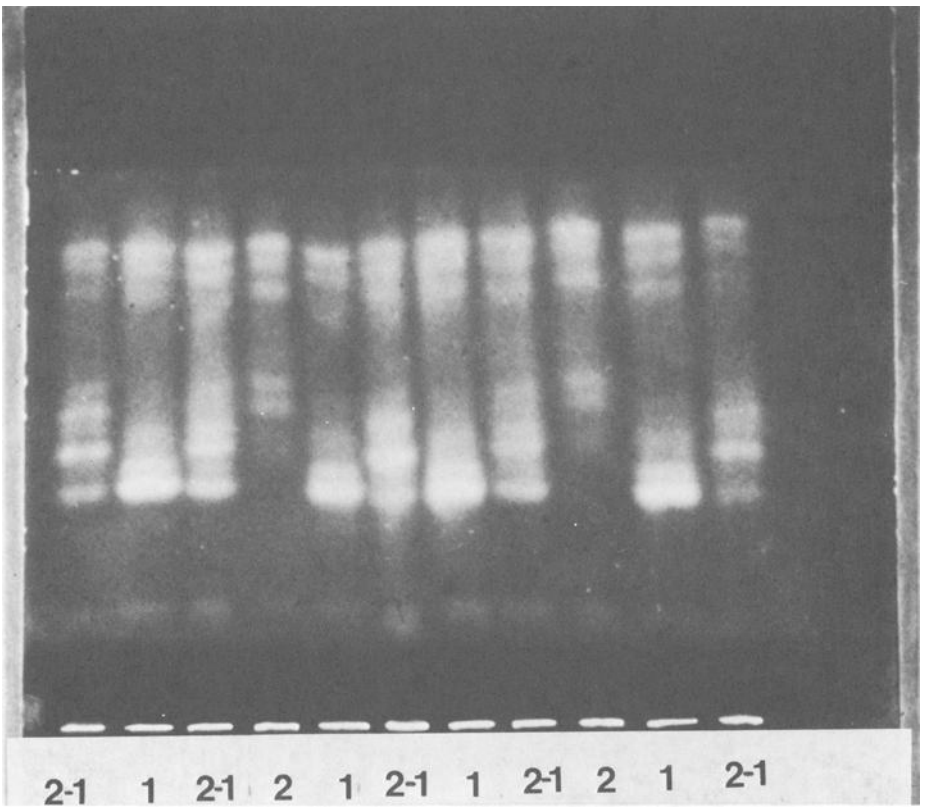

FIG. 2—*Conventional EsD typing using a pH 7.4 buffer system. The three variant forms are represented. From left to right they are: Type 2-1, Type 1, Type 2-1, Type 2, Type 1, Type 2-1, Type 1, Type 2-1, Type 2, Type 1, and Type 2-1.*

The use of agarose rather than starch or polyacrylamide results in a sturdy, transparent polymerized gel which is economical and safe to handle before or after polymerization. Selection of a low EEO agarose reduces the tendency for dissociable cations and water molecules to migrate cathodally thereby interfering with the protein separation by convection within the gel. We find that dilution of the anodal tank buffer significantly improves the phenotyping results primarily because the gel does not become heated and thinned out anodally. Thinning of the gel causes an increase in its resistance and somewhat of a reduction in current flow and thus a decrease in the migration rate of the electrophoresed proteins. At the same time the enzyme could lose its activity as a result of heat denaturation. The ability to phenotype simultaneously other polymorphic protein systems such as EsD in addition to PGM is clearly very beneficial. We are presently working on the development of a system in which glyoxylase I can be included in this group analysis.

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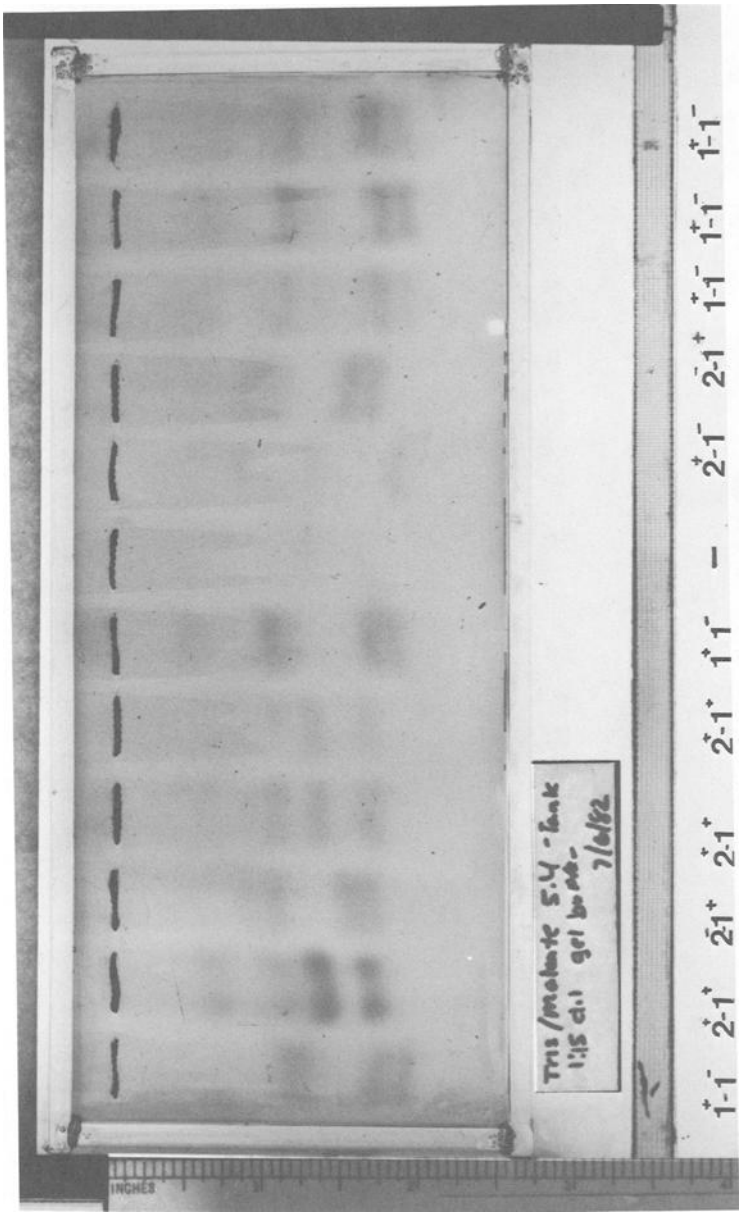

FIG. 3—*PGM subtyping at pH 5.4. Subtypes displayed from left to right are: (1+1−), (2+1+), (2−1+), (2+1+), (2+1+), (1+1−), (not identified), (2+1−), (2−1+), (1+1−), (1+1−), and (1+1−). The run lasted 4 h.1 in. = 25.4 mm.*

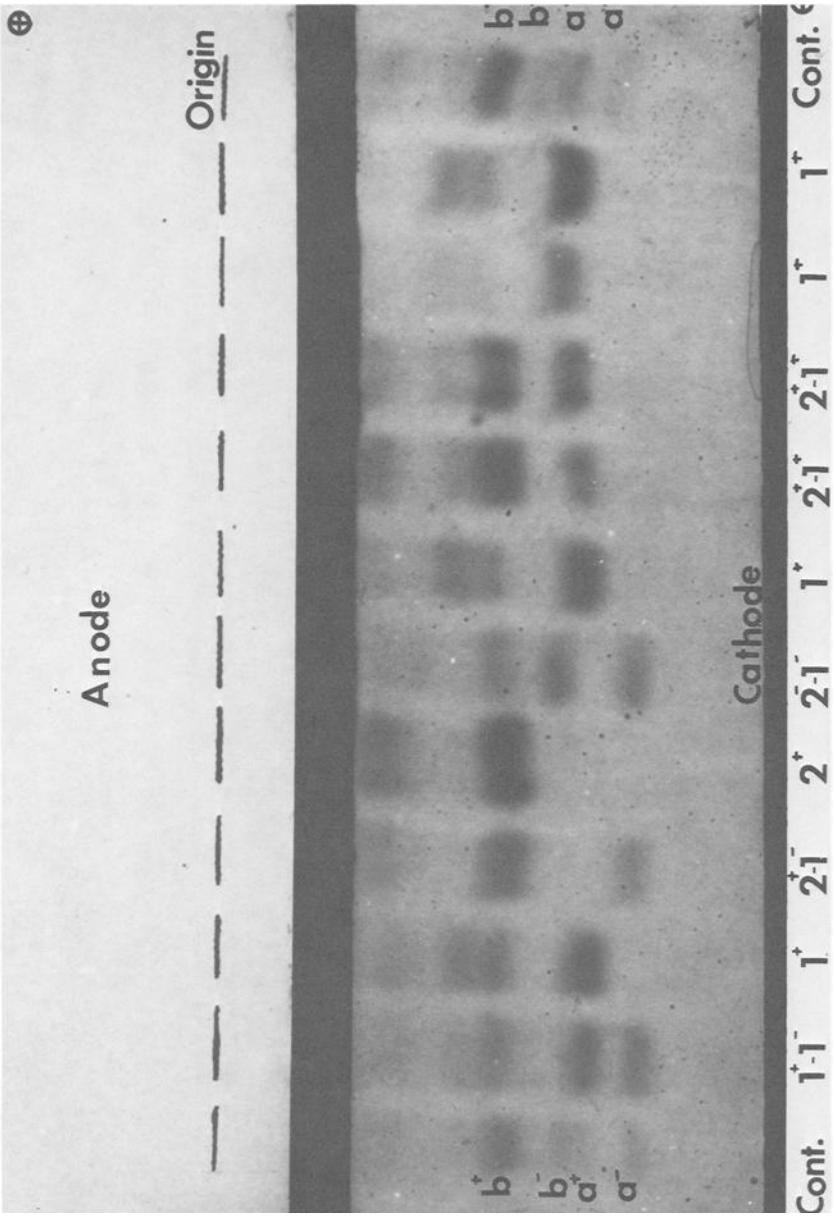

FIG. 4—*PGM subtyping at pH 5.4. The run lasted 4.5 h. The subtypes displayed are from left to right: an artificial control (2+2−1+1−), (1+1−), (1+), (2+1−), (2+), (2−1−), (1+), (2+1+), (2+1+), (1+), and a control (2+2−1+1−).*

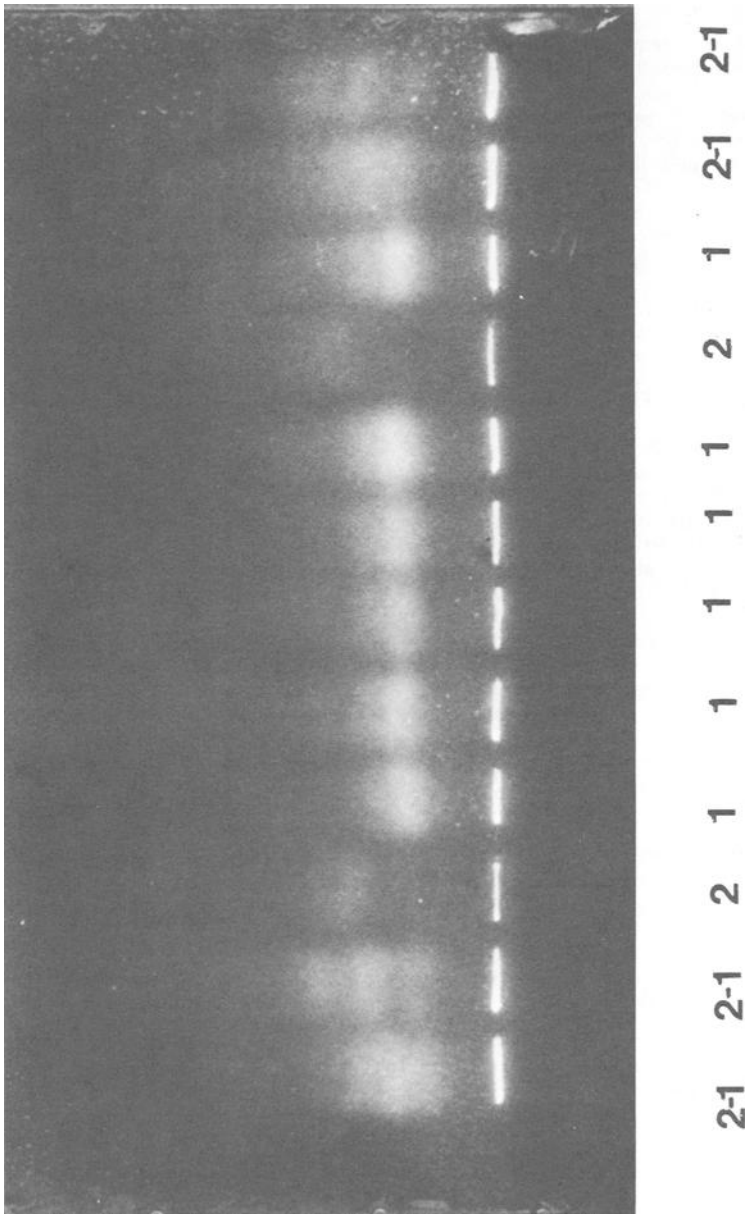

FIG. 5—*EsD typing at pH 5.4, run simultaneously with PGM as seen in Fig. 4. The phenotypes illustrated from left to right are: Type 2-1, Type 2, Type 1, Type 1, Type 1, Type 1, Type 2, Type 1, Type 2-1, and Type 2-1.*

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