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Identification and Demonstration of Three Enzyme Polymorphisms from Bloodstains by Simultaneous Electrophoresis

Adenylate Kinase (AK), Adenosine Deaminase (ADA), 6-Phosphogluconate Dehydrogenase (PGD)

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Summary. The demonstrability of isozyme polymorphisms adenylate kinase, adenosine deaminase and 6-phosphogluconate-dehydrogenase from stored bloodstains was studied. Bloodstains from individuals with known and with unknown phenotypes were investigated. A special method for preparation is given. Samples were separated by a simultaneous electrophoretic method.

Limits for identification were different and found four weeks for PGD, five months for ADA and at least eleven months for AK. AK isozymes and sometimes ADA isozymes were detected in older bloodstains.

Zusammenfassung. Die Nachweisbarkeit der Enzympolymorphismen: Adenylatkinase (AK), Adenosindeaminase (ADA), und 6-Phosphogluconat-Dehydrogenase (PGD), aus gelagerten Blutspuren wird untersucht. Blutspuren von Personen mit bekannten und unbekannten Phänotypen wurden verwendet. Eine spezielle Aufarbeitungsmethode wird angegeben. Die verimpften Proben wurden mittels Kombinationsmethode auf alle drei Enzympolymorphismen untersucht.

Die Grenzen des zeitlichen Nachweises aus gelagerten (20—25° C) Spuren waren unterschiedlich: 4 Wochen für die PGD, 5 Monate für die ADA und mindestens 11 Monate für die AK. In älteren Spuren waren regelmäßig noch AK-Isoenzyme (bis zu 6 Jahren) und gelegentlich noch ADA-Muster erkennbar.

Key words: Bloodstains, Isozyme polymorphismus — Adenylate kinase — Adenosine deaminase — 6-phosphogluconate-dehydrogenase.

Several red cell enzyme polymorphisms are useful genetic markers in human blood [1]. Some of these are routinely typed in forensic serology: Erythrocyte acid phosphatase (EAP), phosphoglucomutase (PGM), adenylate kinase (AK), adenosine deaminase (ADA), 6-phosphogluconate dehydrogenase (PGD).

Methods for the identification of human blood stains that make use of three polymorphisms (EAP, PGM, AK) have been described [2–10].

In order to reduce the seize of the necessary sample of bloodstain some methods have been employed using thin-layer starch gel electrophoresis [5, 6, 8–10]. In this paper we describe a method for starch gel electrophoresis that employs small pieces of filter papers in a thick layer of starch. This method allows the determination of *three* enzymes in *one* run.

Rather discrepant data are available on the time limits of enzyme determination in stored bloodstains [2, 4, 6–10]. All workers, including those from our

laboratory [12], were employing conventional methods of preparation without special regard to the enzyme's biochemical properties. By the use of new general procedures the time limits of enzyme decay could be prolonged considerably. These methods are given too.

Materials and Methods

Bloodstains

1. Venous blood samples from individuals with known phenotypes: AK 1, AK 2, AK 2-1, ADA 1, ADA 2-1, PGD A, PGD AB were applied to stain carriers immediately after they were drawn. Drops, equivalent each to 50 µl, were pipetted on glass, paper and cotton and stored at 20–25° C.

2. Another series of bloodstains was of unknown origin and derived from a collection of the police. These stains had been stored up to six years at 20–25° C and were located on multiple carriers (paper, glass, metal, plastic, wool etc.).

Methods of Preparation of Bloodstains. Prior to electrophoresis the dried stains were redissolved in different ways: stains that could be scraped off were dissolved by grinding them in a solution of 60 mM mercapto-aethanol. Stains that were absorbed by cotton or paper were cut out and minced thoroughly. This material was layered with mercapto-aethanol (30 mM) for 3–4 hours or overnight at 4° C. The extract was separated from the trace carriers by centrifuging the mixture for 10 min at 5000 rpm through a plastic filter (Saran No. 4/67) that retained the fragmented paper or cotton. The solution was dry frozen in small plastic tubes[1] and redissolved by the addition of 45 mM mercapto-aethanol. Fresh stains were redissolved in about 4/5 of the original blood volume, older samples were dissolved in a smaller volume.

Redissolved samples, stains of which had been stored up to six weeks, were applied to filter papers Whatman No. 1, seize 3×9 mm. The older samples were applied to filter papers Schleicher & Schüll No. 598, seize 3×6 mm. 10 µl were needed to soak one filter paper sufficiently.

Electrophoresis. Electrophoresis was carried out on horizontal starch gels by a simultaneous method [11], that allows determination of all *three* enzyme polymorphisms after electrophoresis of *one* sample.

The discrepancy between the height of the filter paper (3 mm) and that of the gel (7 mm) was compensated by the fact that the upper half of the gel (4 mm thick) was rejected.

The staining procedure was extended considerably when old stains had been separated. Thus AK isozymes became visible after 60–90 minutes and ADA isozymes after three hours of incubation time.

Results

PGD. The identification limit of PGD phenotypes A and AB was reached after four weeks of storage. After this limit the specific enzyme stain was no longer visible. The time limit was independent of the phenotype and of the kind of stain (Fig. 1). Stains of unknown origin were older than two months and exhibited no enzyme activity.

ADA. Phenotypes ADA 1 and ADA 2-1 were still identified after 5 months of storage. Since the diagnosis of both phenotypes was based on the different migration of the slow isozymes while the rapid ones sometimes were invisible, one cannot distinguish phenotype ADA 2-1 from the rare ADA 2. When older samples were investigated there was a faded and blurred staining of the enzyme without clearly separated zones (Fig. 2).

In fresh samples ADA 1 and ADA 2-1 migrated with the same rate as in stain extracts. In older stains additional isozyme activity was sometimes visible which was located more towards the anode (Fig. 2b).

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1 Eppendorf-Reaktionsgefäße (Fa. Netheler & Hinz, Hamburg).

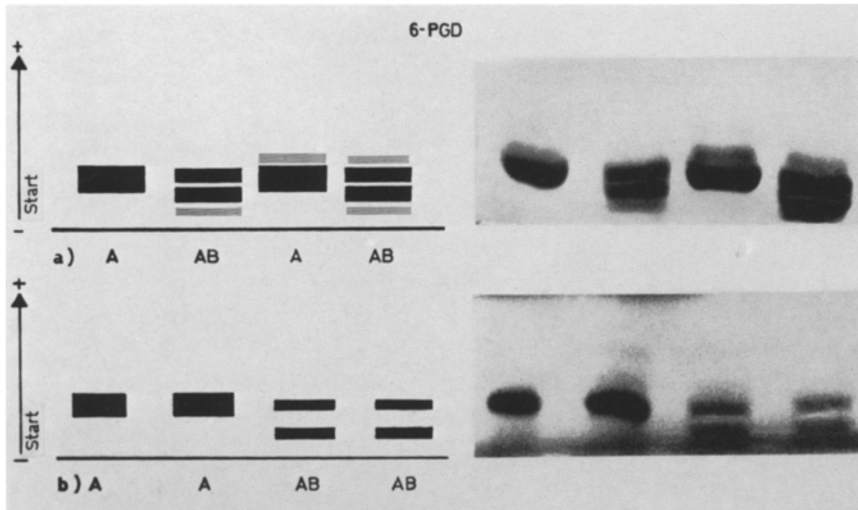

Fig. 1a and b. Scheme and corresponding photo of 6-PGD from bloodstains. a Three days old stains. b 24 days old stains

The stains of unknown origin decayed correspondingly except for some: Although these were up to 28 months old the phenotypes could be identified clearly (Fig. 2c). They consisted of hard blood crusts which were up to 3 mm thick.

AK. Phenotypes AK 1, AK 2-1 and AK 2 could still be diagnosed after 11 months of storage (Fig. 3). In order to find out the absolute time limits for the identification of isozymes 40 blood spots of unknown origin were investigated. These stains, up to 6 years old, exhibited visible isozyme activity corresponding to the major band of type AK 1 (Fig. 3c).

All stains of known origin that were older than 3 months, were extracted from absorbent stain carriers.

Discussion

Although there is a small chance to find one of the uncommon heterozygous phenotypes this chance is enhanced, when *all* variant enzymes with low incidence are typed. There is an additional possibility to estimate the approximate age of the stain when storage conditions are known. However this point needs further clarification.

An astonishing fact arose from the finding that ADA phenotypes could be identified still after five months as they decay much more rapidly in blood samples which are stored at 4° C [13–15].

These changes on storage could be contributed to the bonding of reactive sulphydril groups of the enzyme with oxydized glutathion that increases on storage [16]. The altered enzyme migrates more rapidly towards the anode. Thus the slow enzymes become invisible. Our findings therefore lead to the conclusion that these changes proceed much more slowly in dried samples.

If a blood stain exhibits a slow zone corresponding to the gene product of ADA^2 only the presence of that gene can be stated.

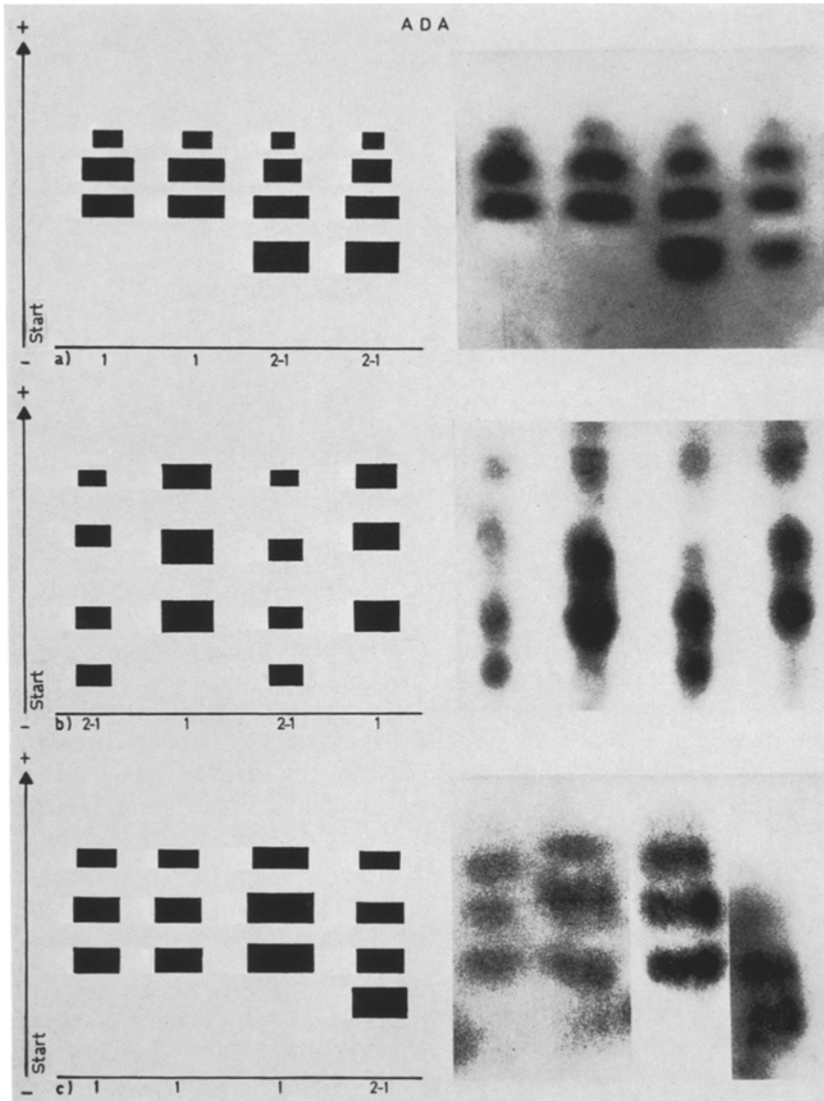

Fig. 2a–c. Scheme and corresponding photo of ADA from bloodstains. a Three days old stains. b $4^1/_2$ months old stains. c 28 months old stains. The 2-1 phenotype is a 6 months old control of stain with known phenotype stored for 6 months

Another interesting point arose from the fact that AK enzyme activity was still detectable after 5–6 years of storage. These findings however have to be interpreted cautiously since there was a statistical chance to find an AK 2-1 type which we failed to observe. Thus we cannot decide at present whether the isozyme, corresponding to AK^2 vanishes earlier after long periods of storage.

The described procedures for preparing the stain and performing electrophoresis were developed stepwise: Thin layer electrophoresis turned out to be

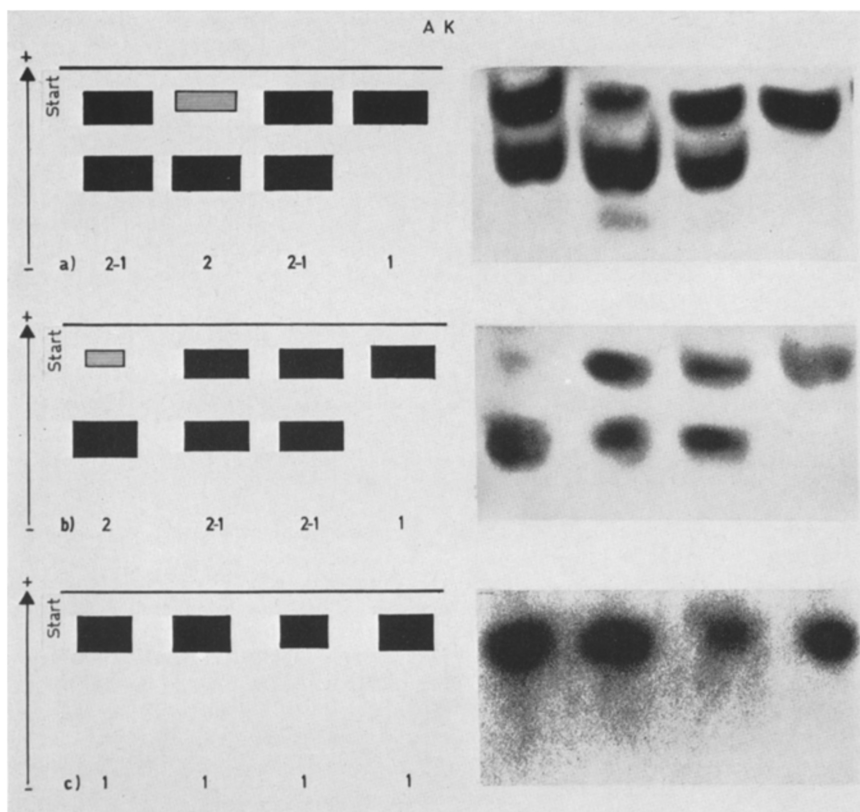

Fig. 3a–c. Scheme and corresponding photo of AK from bloodstains. a Three days old stains. b 11 months old stains. c 4 to 6 years old stains of unknown origin

disadvantageous since the gels often teared during electrophoresis. Furthermore the enzyme staining was more intense when the *cut* surface was stained because of better contact between the separated enzyme and the sandwich ingredients. Since only 3 mm heigh papers were inserted this method was comparable to thin layer methods as to the amount of sample necessary. The technique of blood extraction was apparently quite efficient, since e.g. stain carries consisting of white cotton became nearly white again after this procedure. There was a great enhancement in staining intensity when mercapto aethanol was used instead of distilled water only.

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