J Forensic Sci, Jul. 1977, Vol. 22, No. 3

P. M. Stombaugh, Jr.,[1] *B.S. and J. J. Kearney,*[1] *M.S.*

Determination of Adenylate Kinase Variants in Two Washington, D.C., Population Samples: A Microcellulose Acetate Procedure

In the forensic science laboratory erythrocyte adenylate kinase (AK) polymorphism has been used successfully to further individualize dried bloodstains [*1,2*]. Three distinct phenotypes of adenylate kinase, AK-1, AK2-1, and AK-2, have been identified in the population and are directed by two common autosomal alleles AK^1 and AK^2 [*3*]. Several other less common variants have also been reported [*4*].

The phenotypes of adenylate kinase can be demonstrated in extracts of dried bloodstains by using classical electrophoretic methods. Earlier electrophoretic techniques called for the use of starch gel as the supporting medium [*1,3,4*]. This procedure tends to be somewhat tedious and lengthy. Recently a more efficient method using cellulose acetate as a supporting medium has been described [*2,5*].

The purpose of this report is to introduce a modification of the cellulose acetate procedure proposed by Saenger and Yates [*2*]. This modified system is fast, specific, requires minimal sample volumes for analysis, and allows for extended AK phenotyping of aged dried bloodstains up to 18 months. Sixteen blood samples can be analyzed with a single electrophoretic run.

An AK stability study and the incidence of AK phenotypes in two different Washington, D.C., area population samples are also presented.

Materials and Methods

Preparation of Samples

Blood samples used for the incidence study of AK phenotypes were obtained from a metropolitan Washington, D.C., hospital. Lysates of whole blood samples were prepared by first washing packed red blood cells two times with a 0.8% saline solution and then adding 2 volumes of distilled water to 1 volume of packed cells.

Dried bloodstains used for the stability study were prepared by placing a small amount of whole blood onto a piece of clean cotton sheeting. Each stain was allowed to completely air dry at room temperature. Portions of each stain were then stored at −16, 4, 25, and 37 °C for various lengths of time.

Extracts of dried bloodstains were prepared for electrophoresis by placing a 1-cm^2 portion of stained material into a 12 by 75-mm test tube and adding 2 to 3 drops of cell buffer (pH 6.25, 1.50 g $NaH_2PO_4 \cdot H_2O$, 0.52 g $Na_2HPO_4 \cdot 7H_2O$ made up to 1 litre of distilled water) [*2*]. The stained cloth and buffer were then ground with a glass rod and allowed to soak for approximately 20 min. Longer elution times were required for older

Received for publication 13 Dec. 1976; accepted for publication 3 Jan. 1977.

[1]Chemists, Biological Research Unit, Scientific and Technical Services Division, Federal Bureau of Investigation, Washington, D.C. 20535.

stains. A portion of the extract was then pipeted onto the surface of a small sheet of Para-film® and covered with a small beaker. Samples were transferred onto the cellulose acetate membrane by use of a Beckman single sample applicator.

Electrophoresis

Electrophoresis was carried out on the Beckman R-101 Microzone® Electrophoresis System with accessories.

A cellulose acetate membrane was floated onto a portion of the phosphate cell buffer described previously. The membrane was then immersed in the buffer for 30 s, withdrawn with forceps, blotted between filter papers, and placed onto the spring-loaded membrane holder. The membrane and holder were placed into the electrophoresis cell. After a 1-min equilibration period, the extracted samples were applied to the membrane from the "A" slot position with the single sample applicator.

Electrophoresis was carried out at a constant voltage of 300 V for 70 min. The initial milliamperage was 1.5 mA and slowly rose to 3.0 mA at the completion of the run. The migration of the AK isozymes was toward the cathode.

Assay for Adenylate Kinase Activity

The best assay results were obtained when fresh AK reaction plates were used. These plates were routinely prepared during the 70-min electrophoretic run.

The following ingredients were added to a 25-ml Erlenmeyer flask which had been wrapped in aluminum foil to prevent exposure of the reaction mixture to light: 150 mg dextrose, 30 mg adenosine 5′-diphosphate, 10 mg nicotinamide adenine dinucleotide phosphate (NADP), 5 mg phenazine methosulfate, 5 mg [3-(4,5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide] (MTT), 14 units hexokinase, 6 units glucose 6-phosphate dehydrogenase (all Sigma products), 4 ml of distilled water, and 1 ml of reaction buffer [pH 7.9, 18.0 g tris(hydroxymethyl)aminomethane (Tris), 10.0 g $MgCl_2 \cdot 6H_2O$ made up to 1 litre of distilled water]. The flask was covered and the reaction solution thoroughly mixed.

Agarose (0.4 g), 10 ml distilled water, and 5 ml of the reaction buffer were added to a 50-ml Erlenmeyer flask. This solution was then brought to a boil with constant stirring and subsequently allowed to cool to 60°C.

The reaction mixture prepared earlier was then added to the 60°C agarose solution. The entire mixture was quickly stirred and poured into a 9 by 9-cm petri dish and allowed to solidify in the dark.

Immediately after the 70-min electrophoretic run the holder and membrane were removed from the electrophoresis cell. A 3 to 4-cm piece was cut off each end of the cellulose acetate membrane. The membrane was removed from the bridge assembly and placed facedown onto the reaction plate which had been previously incubated for 10 min at 37°C. Care was taken not to entrap any air bubbles under the membrane, insuring complete contact between membrane and reaction gel. The cover was then placed over the gel and the entire petri dish incubated in an inverted position at 37°C until sufficient activity was observed for typing.

Sixteen-Sample Electrophoretic Run

Sixteen blood samples were run electrophoretically on the same cellulose acetate membrane by applying blood samples 1 to 8 in the "B" slot position located on the Microzone cell cover. The membrane and the bridge assembly were then removed from the cell, rotated 180 deg, and placed back into the cell. Blood samples 9 to 16 were then placed in the "A" slot position. Electrophoresis was as previously described.

Results

Shown in Figs. 1 and 2 are AK zymograms obtained from 8 and 16-sample electrophoretic runs of extracted dried bloodstains. Consistently good migration and a distinct resolution between all major AK isozymes present in each AK phenotype, AK-1, AK2-1, and AK-2, were obtained.

The incidence of AK phenotypes was determined in two different metropolitan Washington, D.C., population samples. The results of this study are summarized in Table 1.

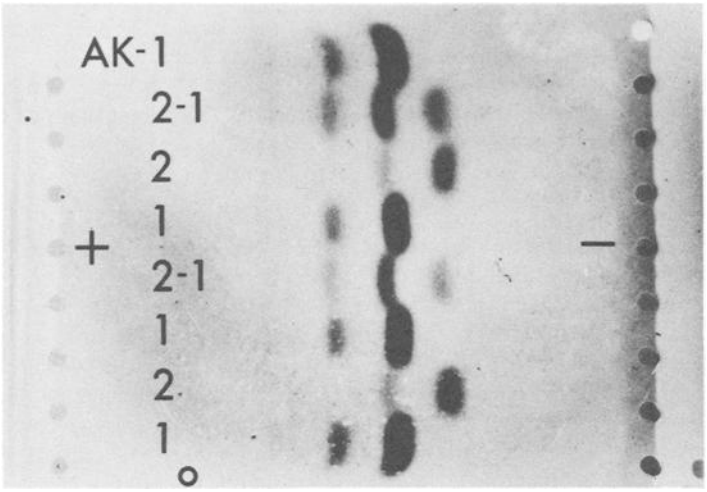

FIG. 1—*An eight-sample cellulose acetate electrophoretic run of extracts taken from dried bloodstains showing phenotypes AK-1, AK2-1, and AK-2.*

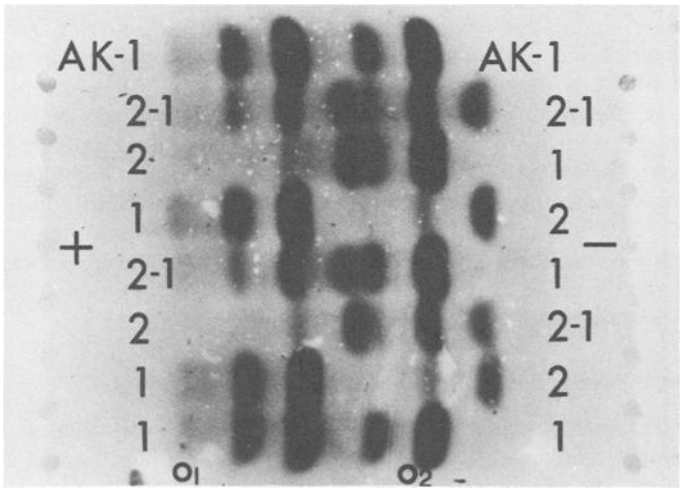

FIG. 2—*A 16-sample cellulose acetate electrophoretic run of extracts taken from dried bloodstains showing phenotypes AK-1, AK2-1, and AK-2.*

TABLE 1—*Incidence of AK phenotypes occurring in two Washington, D.C., area population samples.*

Population Sample	Phenotypes				Gene Frequencies	
	AK-1	AK2-1	AK-2	Totals	AK^1	AK^2
Caucasian						
Number	338	25	1	364	...	...
Incidence observed	0.9286	0.0687	0.0027	...	0.9629	0.0371
Incidence expected	0.9272	0.0714	0.0014	...	...	...
Negro						
Number	75	1	0	76	...	...
Incidence observed	0.9868	0.0132	0.0000	...	0.9934	0.0066
Incidence expected	0.9868	0.0132	<0.0001	...	...	...

Blood from 364 Caucasians was examined, and the frequency of occurrence of the common AK phenotypes was determined to be 0.9286 for AK-1, 0.0687 for AK2-1, and 0.0027 for AK-2. In the 76 Negro bloods examined, the frequency of occurrence of the AK phenotypes was 0.9868 for AK-1 and 0.0132 for AK2-1. No AK-2 phenotypes were observed.

Figure 3 shows the effect time has on AK activity present in dried bloodstains that have been stored at 25 °C. Although AK activity decreased with time, a sufficient

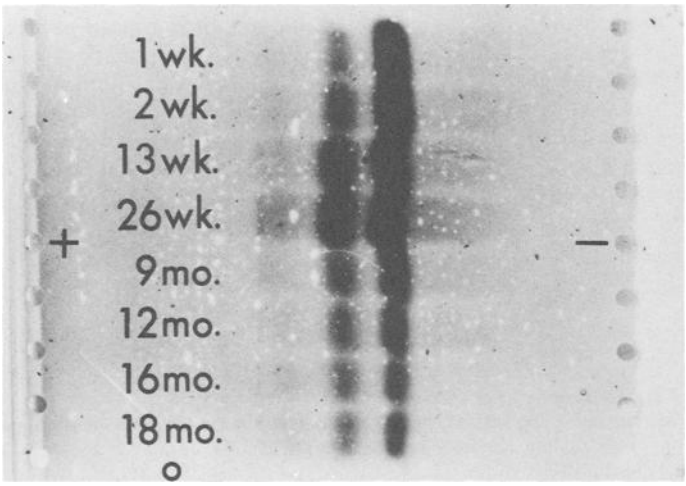

FIG. 3—*A cellulose acetate electrophoretic run showing the effect time has on AK-1 activity in dried bloodstains stored at room temperature (25 °C).*

amount remained after 18 months to permit typing. Bloodstained material stored at −16 and 4 °C also retained sufficient AK activity to allow reliable typing of the three most common AK phenotypes up to 18 months. However, bloodstains stored at 37 °C could only be typed up to 3 months.

In comparing the 17-h starch gel electrophoretic procedure (Tris-maleic acid buffer system [*6*]) for the simultaneous typing of phosphoglucomutase and AK to the cellulose acetate procedure described in this report, it was found that dried bloodstains stored at

25°C for periods up to 18 months could be typed with both systems. However, the clear and distinct isozyme band resolution achieved with the more rapid cellulose acetate procedure readily permitted a more reliable identification of the various AK phenotypes.

Discussion

Erythrocyte adenylate kinase polymorphism was first described by Fildes and Harris in 1966 [3]. From that time, the use of starch gel electrophoresis as a means of identifying the genetically determined variants of this highly stable enzyme has become well known [1,4]. Recently, Saenger and Yates [2] and Rosalki [5], working individually, have described cellulose acetate electrophoretic procedures for the typing of AK. The clinical procedure of Rosalki [5] employs the use of the Microzone electrophoretic apparatus and a histidine-citrate buffer system at pH 7.0. The procedure of Saenger and Yates [2] has been used forensically to type bloodstains and employs a phosphate buffer at pH 6.25. The present report introduces a procedure that utilizes the phosphate buffer used by Saenger and Yates [2] and the Microzone apparatus. This combination, coupled with the good stability of the AK isozymes in dried bloodstains, has produced a forensic AK typing procedure that is rapid, reliable, and of increased sensitivity and utility. Dried bloodstains stored at −16, 4, and 25°C were successfully typed for periods up to 18 months. As many as 16 samples may be typed during a single electrophoretic run, with each sample volume as small as 0.25 μl. In the hands of the authors the convenience, utility, and excellent resolution of the AK phenotypes experienced while using the procedure described in this report precludes advantages found by running AK typings in tandem with other enzyme systems.

While the incidence of the common AK phenotypes in European population samples has been well examined [7], little data exist for United States populations. The results of three AK studies which sample the Negro and Caucasian populations of Seattle, Wash., Chicago, Ill., and New York, N.Y. have been reported by Giblett [8], Bowman et al [4], and Mondovano and Gaensslen [9], respectively. In our laboratory 364 Caucasian bloods were examined and AK gene frequencies of 0.9629 for AK^1 and 0.0371 for AK^2 were obtained. Gene frequencies of 0.9934 for AK^1 and 0.0066 for AK^2 were observed after examining 76 Negro bloods. These results are consistent with the AK gene frequencies presented by Giblett [8], Bowman et al [4], and Mondovano and Gaensslen [9].

References

[1] Culliford, B. J. and Wraxall, B. G. D., "Adenylate Kinase (AK) Types in Bloodstains," *Journal of the Forensic Science Society,* Vol. 8, Oct. 1968, pp. 79–80.

[2] Saenger, M. S. and Yates, R. G., "A Cellulose Acetate Membrane Technique for the Determination of Adenylate Kinase Types in Bloodstains," *Journal of Forensic Sciences,* Vol. 20, No. 4, 1975, pp. 643–646.

[3] Fildes, R. A. and Harris, H., "Genetically Determined Variation of Adenylate Kinase in Man," *Nature,* Vol. 209, No. 5020, 1966, pp. 261–263.

[4] Bowman, J. E., Frischer, H., Ajmar, F., Carson, P. E., and Gower, M. K., "Population, Family, and Biochemical Investigation of Human Adenylate Kinase Polymorphism," *Nature,* Vol. 214, No. 5093, 1967, pp. 1156–1158.

[5] Rosalki, S. B., "Separation of Adenylate Kinase Isoenzymes on Cellulose Acetate Membrane," *Clinica Chimica Acta,* Vol. 27, No. 3, 1970, pp. 497–499.

[6] Spenser, N., Hopkinson, D. A., and Harris, H., "Phosphoglucomutase Polymorphism in Man," *Nature,* Vol. 204, No. 4960, 1964, pp. 742–745.

[7] Stedman, R., "Human Population Frequencies in Twelve Blood Grouping Systems," *Journal of the Forensic Science Society,* Vol. 12, No. 2, 1972, pp. 379–413.

[8] Giblett, E. R., *Genetic Markers in Human Blood,* Blackwell Scientific Publications, London, 1969, p. 516.

[9] Mondovano, J. A. and Gaensslen, R. E., "Distribution of Adenylate Kinase and Phosphoglucomutase Isoenzymes in the Population of the City of New York," *Human Heredity,* Vol. 25, No. 6, 1975, pp. 488–492.

Biological Research Unit
Scientific and Technical Services Division
Federal Bureau of Investigation
Washington, D.C. 20535