J Forensic Sci, Oct. 1975, Vol. 20, No. 4

M. S. Saenger,[1] *B. Sc. and R. G. Yates,*[1] *B. A.*

A Cellulose Acetate Membrane Technique for the Determination of Adenylate Kinase Types in Bloodstains

The genetically determined isoenzyme blood group system of adenylate kinase (AK) has been demonstrated in lysates of human erythrocytes [*1,2*] and in bloodstains [*3*]. The technique employed was horizontal starch gel electrophoresis using either a discontinuous histidine-citrate [*1*], a phosphate [*2*], or a succinate [*4*] buffer system. Since then, electrophoresis on cellulose acetate membrane (CAM) has been introduced as a rapid technique for the determination of AK types in fresh lysates [*5,6*]. We decided to investigate the use of CAM for determining AK types in bloodstains. In preliminary tests with CAM, we found the discontinuous histidine-citrate buffer system [*1,5*] gave clear results with lysates, but unsatisfactory results with even fresh bloodstain material. The phosphate buffer [*6,7*] seemed more promising and this paper describes our evaluation and adaptation of the phosphate system for bloodstain samples.

Materials and Methods

Materials

The tank buffer used was a Biotest [*7*] phosphate buffer (0.014*M*) at pH 6.25 and consisted of 15 g of KH_2PO_4, 5.2 g of $Na_2HPO_4 \cdot 2H_2O$, and 0.1 g of NaN_3 in 1000 ml of distilled water. Before use, one volume of this stock solution was diluted with nine volumes of water. Five by twenty-centimetre CAM strips were employed. The enzyme developing buffer (Biotest [*7*]) was a tris-HCl buffer at pH 7.9. It consisted of 18 g of tris, 10 g of $MgCl_2 \cdot 6H_2O$, and 1 g of NaN_3 made up to 1000 ml and adjusted to pH 7.9 with 0.1*N* HCl. The enzyme developing mixture (Biotest [*7*]) contained 150 g of glucose, 30 mg of adenosine diphosphate (Boehringer), 10 mg of nicotinamide adenine dinucleotide phosphate (Boehringer), 5 mg of phenazine methosulfate (PMS) (Serva), 5 mg of tetrazolium salt (MTT) (Serva), 20 μl of glucose-6-phosphate dehydrogenase (Boehringer), and 20 μl of hexokinase (Boehringer). A 2% aqueous stock agar was used.

Slide Preparation

Five by five-centimetre glass slides were painted with a coat of 0.2% aqueous agar, and allowed to dry. These slides were numbered on the back.

Received for publication 4 March 1975; accepted for publication 17 March 1975.
[1] Forensic serologists, Serology Section, U.S. Army Criminal Investigation Laboratory, Frankfurt am Main, West Germany.

Sample Preparation

Lysates were prepared from fresh blood samples by freezing once washed, packed erythrocytes. Then 2 by 2-mm bloodstain fragments were eluted in a minimum amount of phosphate buffer; a few minutes' elution sufficed for fresh stains, but overnight elution at 4°C was necessary for older stains. We found that the eluates had to be at least pale brown to give readable results.

Method

The electrophoresis tank was filled with buffer, and the CAM strips were soaked in this buffer. The strips were carefully blotted to remove only excess moisture and placed across the dry tank bridges. The anodic and cathodic ends of the CAM should be evenly submerged in buffer. Samples were drawn by capillarity into finely pulled-out pipets, which were then used to draw 1-cm-long sample lines onto the anodic side of the CAM. The sample volume was approximately 0.5 μl. Samples must be applied quickly to minimize diffusion; the method requires practice, but gives much clearer banding and uses less sample than the commercially provided multi-applicators. We ran 12 samples on three CAM strips per tank. Two tanks can be run together, conveniently testing 24 samples simultaneously. The current was switched on as soon as sample application was completed. Electrophoresis was carried out at 300 V, at room temperature, for 70 to 90 minutes, that is, until the hemoglobin band had migrated 4 to 5 cm.

Meanwhile, the enzyme developing mixture was prepared as follows. The enzyme mixture was dissolved in 1 ml of tris-HCl buffer and 4 ml of distilled water. Fifteen grams of stock agar were melted in 5 ml of tris-HCl buffer and 10 ml of distilled water. The agar was cooled to 50°C, the dissolved enzyme developing mixture added in the dark, and this agar mixture poured or pipetted onto six of the numbered glass slides, which were allowed to gel in the dark.

After electrophoresis, each CAM strip was cut at sample origin and along the hemoglobin line. Each CAM segment was numbered sequentially in the top right corner and placed face down on the correspondingly numbered agar developer slide. These slides were incubated in a moisture box in the dark, at 37°C for 30 to 40 minutes, until the purple bands of AK activity developed. The AK types were then read (see Fig. 1).

The CAM segments can be dried and stored, but we found greater clarity was achieved for permanent record by drying the agar slides under filter paper in an oven; the agar developer remains as a film on the slide, and the banding is clear (see Figs. 1 and 2).

Results

Using the above technique, we have tested both lysates and stains from 98 random blood samples and have obtained clear results. Stains were tested one week and four weeks after preparation, and no appreciable loss of activity was seen. We found AK 1 and AK 2.1 types in these samples; the AK 2 type was not encountered. However, the clear differentiation of these three basic AK types on CAM is shown in Fig. 2 (control samples obtained from Biotest GmbH).

Six-month-old AK 1 and AK 2.1 stains from laboratory donors have been successfully typed on CAM; stains older than this failed to give a colored eluate, even after 24-hour elution at 4°C, and failed to give clear AK results.

Conclusion

This CAM method for AK typing has now replaced thin-layer starch [3] as the routine method in our laboratory. Its sensitivity, rapidity, and the possibility of retaining the results as a permanent record render it particularly suitable for forensic purposes.

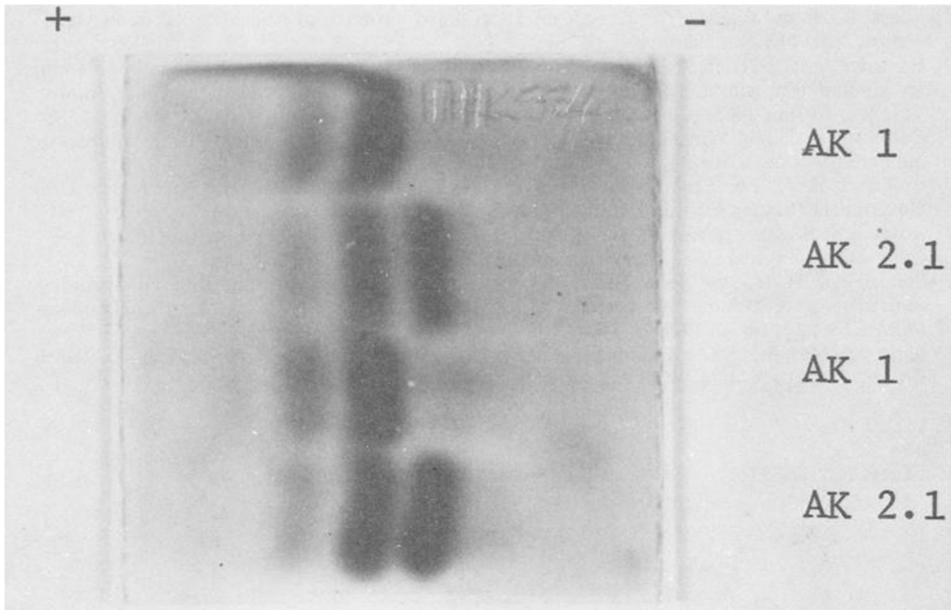

FIG. 1—*Dried slide with results from four bloodstain samples.*

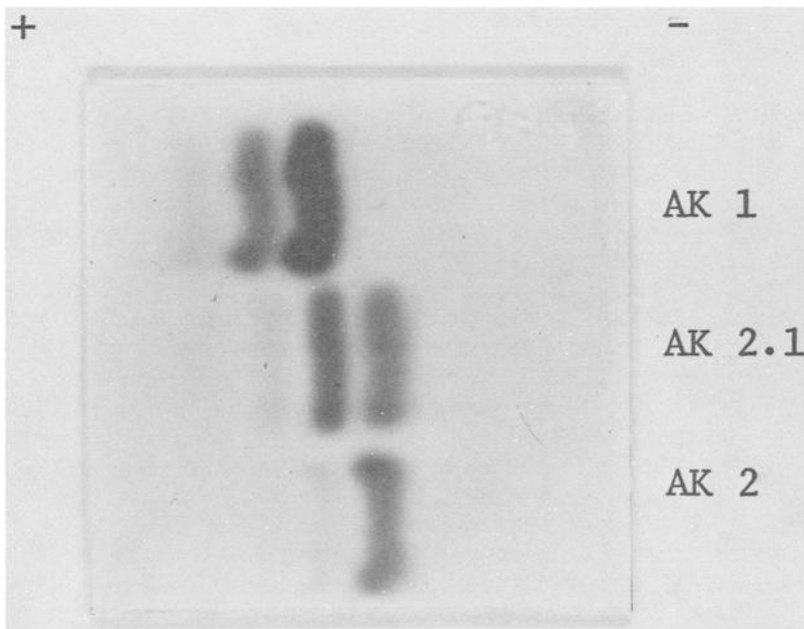

FIG. 2—*Dried slide showing the three basic adenylate kinase types.*

References

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

[*2*] 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, 10 June 1967, p. 1156.

[*3*] Culliford, B. J. and Wraxall, B. G. D., "Adenylate Kinase Types in Bloodstains," *Journal of the Forensic Science Society,* Vol. 8, No. 2-3, 1968, p. 79.

[*4*] Culliford, B. J., *The Examination and Typing of Bloodstains in the Crime Laboratory,* U.S. Government Printing Office, Washington, D.C., 1971, p. 139.

[*5*] Rosalki, S. B., "Separation of Adenylate Kinase Isoenzymes on Cellulose Acetate Membrane," *Clinica Chimica Acta,* Vol. 27, 1970, p. 497.

[*6*] Sonneborn, H.-H. and Renninger, W., "Adenylatkinase-Untersuchung. Eine populationsgenetische Untersuchung mit Celluloseacetatfolien-Elektrophorese," *Das Aerztliche Laboratorium,* Vol. 17, No. 2, 1971, p. 54.

[*7*] Sonneborn, H.-H., "Die Bestimmung der erythrocytaeren Enzympolymorphismen," *Biotest GmbH,* Frankfurt/Main, West Germany, 1972.

USACIL-Europe
APO
New York, N.Y. 09757