Fig. 1. A $^1/_2$ in. $\times$ $^3/_8$ in. piece of foam rubber (B) affixed to each side of the bed plate holds the chromatoplates (G) firmly against the securing edges (C) when the leveling bag (H) is inflated. The addition of guide bars* (D) equipped with ball bushings (E), also shown in Fig. 1, allows the spreader (F) to be moved evenly and smoothly across the plate, producing a very uniform layer of adsorbent on all plate sizes ranging from 2 $\times$ 20 cm up to 20 $\times$ 20 cm. Micrometer heads** can also be added on the applicator's adjustable plate to allow quick, accurate, and reproducible selection of layer thicknesses.

This applicator is routinely used in this laboratory for the preparation of all TLC plates. Many of the chromatoplates are used for the quantitative analysis of lipids by the densitometric technique of BLANK *et al.*[3], where uniformity of the adsorbent layer improves the quantitativeness of the measurement.

Medical Division, Oak Ridge Institute of Nuclear Studies,
*Oak Ridge, Tenn. (U.S.A.)****

RANDALL WOOD§
FRED SNYDER

1 O. S. PRIVETT, M. L. BLANK, D. W. CODDING AND E. C. NICKELL, *J. Am. Oil Chemists' Soc.*, 42 (1965) 381.
2 F. SNYDER AND H. KIMBLE, *Anal. Biochem.*, 11 (1965) 510.
3 M. L. BLANK, J. A. SCHMIT AND O. S. PRIVETT, *J. Am. Oil Chemists' Soc.*, 41 (1964) 371.

Received August 23th, 1965

* One-half-in. stainless steel ball bushings and $^1/_2$-in., 60-core hardened and ground stainless steel rods obtained from Thompson Industries, Inc., Manhasset, N.Y.

** Calibration (0.0–0.500-in. graduations of 0.001 in.) obtained from Brown and Sharp, Precision Park, Northkingston, R.I.

*** Under contract with the United States Atomic Energy commission.

§ USAEC Postdoctoral Fellow under appointment from ORINS.

Thin-layer chromatography of phosphate esters of biologic interest*

The methods available for the separation and quantitation of phosphate esters from tissue extracts are based on either paper or ion-exchange chromatography or electrophoresis. In general, the procedures are either time consuming, not easily adapted to microquantities, or not capable of affording adequate separation. Thin-layer chromatography (TLC) offers the possibility of combining these features. WARING AND ZIPORIN[1] have described a two-dimensional procedure for the separation of a limited number of sugar phosphates with acidic solvent systems, but the ability of the method to separate these compounds from cell extracts was not noted. In our experience the use of acid solvent systems in the first phase produced excessive admixture and uneven solvent fronts, and did not provide adequate resolution. The following two-dimensional procedure has been used satisfactorily for the separation and quantitation of phosphate esters of human red cells. The method has proven

* Supported in part by a research grant from the North Carolina Heart Association.

particularly convenient also, in conjunction with a radiochromatogram scanner, for investigations of cell metabolism with labeled compounds.

Procedure

Preparation of plates. Glass plates (20 cm × 20 cm) were coated 0.25 mm thick with a slurry of unmodified cellulose (MN 300, Machery, Nagel & Co.) (15 g/85 ml H_2O) and air dried overnight. As the cellulose layer contained phosphate impurities sufficient to give a light blue background when sprayed with molybdate reagent[2], the plates were washed by allowing the first solvent to ascend to a distance of 18 cm and redried thoroughly with a warm air stream. To ensure straight solvent fronts during development, an edge of 0.5 cm on each side of the plates was removed.

Solvent systems. The acidic and alkaline solvents of BANDURSKI AND AXELROD[3], as developed for paper chromatography, were found to be the most satisfactory of a large number tested, when their composition was modified and the phase sequence reversed. The basic solvent was methanol–aqueous ammonia (sp. gr. 0.896)–water (7:1:2 v/v). The acidic solvent employed routinely was methanol–glacial acetic acid–water (8:1.5:0.5 v/v). A formic acid system, methanol–formic acid (88% w/v)–water (8:1.5:0.5 v/v), was found useful also. This solvent provided higher R_F values, but distortion and excessive background staining occurred at the solvent front.

Chromatographic procedure. Neutralized tissue extracts (up to 10 μl) were applied to the plates in a spot less than 0.5 cm in diam., at an origin located 3 cm from the bottom edge. Two plates were spotted identically and developed together in the same tank; one plate was used for location of the esters and the other for their quantitation. After an equilibration period of 30–45 min, the plates were lowered into the basic solvent to a depth of 1.5 cm of the cellulose layer and developed at room temp. until the front reached 10 cm above the origin (*ca.* 35 min). They were then removed, thoroughly dried with a warm air stream, turned 90°, re-equilibrated, and developed with the acidic solvent to a distance of 10 cm (*ca.* 30 min). It was essential, before spraying with color developers, to remove all traces of solvent by thoroughly drying at 60° for 1 h.

Detection and quantitation of phosphate esters. Following the first development, the plates were scanned with U.V. radiation (2537 Å) to locate the adenine and pyridine nucleotides which were outlined with a soft lead pencil. After the second development, one plate only was sprayed with 10–12 ml of HANES AND ISHERWOOD's molybdate reagent[2] for the visualization of the phosphate compounds. Inorganic phosphate appeared immediately as a yellow spot. The plate was dried at 80° for 10 min whereupon glucose-1-phosphate appeared as a greenish yellow spot. It was then allowed to cool to 50–60°, held over a steam bath for 2–3 min to hydrolyse the more resistant phosphate esters, and while still moisture saturated, exposed to U.V. radiation in a dark room. The phosphate esters appeared within 10–15 min as blue spots on an essentially white background and became more prominent and distinct with standing for 2–3 h. Distinct spots were obtained from less than 1 μg of compounds as difficult to detect as the fructose esters. If the plate was over-developed with U.V. radiation, a brief exposure to ammonia vapors sufficed to remove excessive background color. Occasionally, for greater contrast, it was resprayed with molybdate reagent and dried at 85° for 5 min.

The stained plate served as a template for outlining the compounds on the second

and unsprayed plate. The absorbent contained in the outlined areas, each a separate compound, was removed with a microhematocrit tube, plugged at one end with washed glass wool, to which a slight negative pressure was applied. All the absorbent of a spot, collected to form a microchromatography column, was eluted with 0.1 *N* HCl. More than 90% of the phosphorus of the compound phosphorus was obtained in 1.5–2.0 ml eluate, and as such was suitable for phosphorus analysis by the modified method of BARTLETT[4] and also for spectrophotometric identification.

Preparation of erythrocyte extracts. Ten ml volumes of heparinized whole blood were withdrawn from normal human donors. The erythrocytes were separated and extracted with trichloroacetic acid according to the method of BARTLETT[4] and the extract decationized by passage through a Dowex 50 X 8-H column. The eluate was neutralized with dilute NH_4OH and lyophilized to a final volume of 4 ml. This solution was either spotted immediately or frozen and stored at deep freeze temp. with no apparent degradation.

Results and discussion

The degree of separation and resolution of phosphate esters that can be achieved by the two-dimensional chromatographic method outlined is illustrated in Fig. 1. This chromatogram shows the separation of the esters of freshly drawn human erythrocytes extracted immediately. Twelve compounds were detected as distinct spots in the majority of the blood samples analyzed while another three appeared infrequently. The pyridine nucleotide, TPN, must be located and quantitated be-

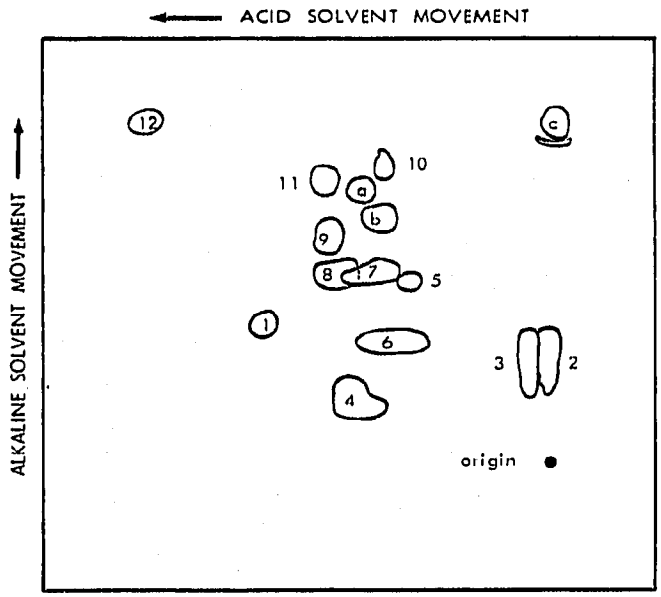

Fig. 1. Two-dimensional TLC chromatogram tracing of the separation of hexose phosphates and triose phosphates in extracts of fresh human erythrocytes. Glycolytic intermediates usually observed: 1 = inorganic phosphate; 2 = adenosine triphosphate; 3 = adenosine diphosphate; 4 = 2,3-diphosphoglyceric acid; 5 = ribose-5-phosphate; 6 = fructose-1,6-diphosphate; 7 = 3-phosphoglyceric acid; 8 = 2-phosphoglyceric acid; 9 = glyceraldehyde-3-phosphate; 10 = glucose-1-phosphate; 11 = glucose-6-phosphate; 12 = pyrophosphate. Spots corresponding to the following compounds were detected occasionally: a = fructose-1-phosphate; b = fructose-6-phosphate; c = triphosphopyridine nucleotide. Reference compounds for a, b, 4, 5, 6, 7, 8 were obtained from C. F. Boehringer and Son; 9, 10, 11 from Sigma Chemical Co.; c, 2, 3 from Pabst Laboratories.

fore developing with the second solvent system as it is lost in this phase. DPN, although present in erythrocytes, is largely removed from the extracts by Dowex treatment and hence is not found on the chromatogram. The color intensity and hue of the spots after reaction with molybdate is dependent not only on the amount of the ester present but also upon the ease by which it can be hydrolysed. Thus the most prominent spots of the chromatogram were the adenine nucleotides, fructose-1,6-diphosphate, 2,3-diphosphoglycerate and the inorganic phosphate, while the hexose monophosphates and the monophosphoglycerates were more difficult to bring out.

The R_F values of the esters found in the blood extract are given in Table I. By careful standardization of the chromatography procedure, reproducible values could be obtained. However minor changes in environmental conditions or absorbent characteristics were capable of producing variations. Also the R_F value varied slightly depending upon whether the ester was run singly, in a mixture, or as an extract

TABLE I

R_F VALUES OF TISSUE PHOSPHATE ESTERS

Compound	R_F	
	Alkaline solvent system	*Acetic acid solvent system*
2,3-Diphosphoglyceric acid	0.14	0.39
Adenosine diphosphate	0.24	0.07
Adenosine triphosphate	0.25	0.02
Fructose-1,6-diphosphate	0.29	0.32
Inorganic phosphate	0.33	0.58
2-Phosphoglyceric acid	0.43	0.44
3-Phosphoglyceric acid	0.45	0.36
Glyceraldehyde-3-phosphate	0.53	0.45
Fructose-6-phosphate	0.58	0.37
Fructose-1-phosphate	0.65	0.39
Glucose-6-phosphate	0.66	0.46
Glucose-1-phosphate	0.70	0.34
Triphosphopyridine nucleotide	0.80	—
Pyrophosphate	0.80	0.79

component. Nevertheless the positions of the compounds relative to an index compound were consistent and proved to be a reliable guide to identification. Inorganic phosphate, normally present in tissue extracts, was selected as a convenient reference. The determination of the relative positions of fructose-1,6-diphosphate and fructose-6-phosphate was aided by the specific cherry red color produced by the acidic naphthoresorcinol reagent of WALKER AND WARREN[5]. The characteristic color produced by the molybdate reagent with glucose-1-phosphate and inorganic phosphate and the U.V. detection of the purine derivatives served for their location. The identification of other compounds, relative to the index compound, was accomplished by chromatographing mixtures of known phosphate esters and also by loading extracts with large amounts of authentic esters.

Although the extract was spotted on two identical plates and developed simultaneously, this procedure is not entirely satisfactory (except in the case of the nucleotides which may be located without staining) because it is not always possible

to be certain of complete recovery of a compound using the template method without the risk of contamination with material from closely neighboring spots. Nevertheless, the chromatograms of 16 normal blood samples yielded values (μmoles P/ml cells) which correspond closely with those determined by ion-exchange chromatography[4]; adenine nucleotides, 2.1–6.0 (3.5 $\pm$ 0.9 S.E.); 2,3-diphosphoglycerate, 6.7–7.9 (7.0 $\pm$ 0.4 S.E.); glucose-6-phosphate, 0.11–0.26 (0.17 $\pm$ 0.06 S.E.) and inorganic phosphate 0.27–0.39 (0.33 $\pm$ 0.06 S.E.). Chromatograms of extracts of 3 week stored acid–citrate–dextrose (ACD) blood, when compared to fresh blood extracts reveal notable differences; the former show decreases in 2,3-diphosphoglycerate with associated increases in monophosphoglycerates, and also a relative absence of adenosine triphosphate (Fig. 2). Also several prominent compounds were found in the stored

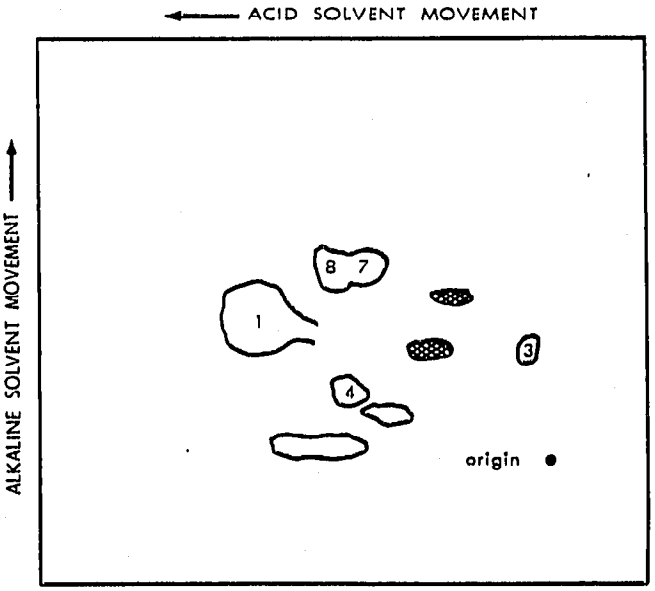

Fig. 2. Two-dimensional TLC chromatogram tracing of the compounds of an extract of 3 week ACD stored erythrocytes. The numbers refer to the compounds as in Fig. 1. Compounds not observed in extracts of fresh cells (hatched spots) absorbed with ultraviolet radiation but did not stain with the molybdate reagent. Unidentified phosphorylated compounds are present also.

erythrocytes which did not appear in fresh cells. These have not been identified, but several of the spots were U.V. absorbing and it is assumed that they are degradations of purine nucleotides.

Department of Pharmacology, The Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N.C. (U.S.A.)

I. W. F. DAVIDSON
W. G. DREW

1 P. P. WARING AND Z. Z. ZIPORIN, *J. Chromatog.*, 15 (1964) 168.
2 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
3 R. S. BANDURSKI AND B. AXELROD, *J. Biol. Chem.*, 193 (1951) 405.
4 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 449, 459, 466.
5 D. G. WALKER AND F. L. WARREN, *Biochem. J.*, 49 (1951) xxi.

First received July 5th, 1965
Modified August 20th, 1965