A PROCEDURE FOR THE ANALYSIS OF ACID-SOLUBLE PHOSPHORUS COMPOUNDS AND RELATED SUBSTANCES IN MUSCLE AND OTHER TISSUES

T. WOOD

Biochemistry Department, University of Sydney, Sydney, N.S.W. (Australia)

(Received November 14th, 1960)

In 1956, THRELFALL[1] published a procedure enabling him to separate and estimate quantitatively 10 different phosphorus compounds present in an acid extract of rat muscle. The chromatographic methods used to separate these substances on paper were described only briefly. The object of the present work has been to enlarge and extend the methods of THRELFALL[1] and other workers so as to develop a simple but comprehensive procedure for investigating phosphorus compounds in aqueous tissue extracts. Procedures for the water-soluble non-phosphorylated compounds have already been described by the author[2-4].

MATERIALS AND EQUIPMENT

Chromatography was carried out with the "Universal Chromatography Outfit" supplied by Aimer Products Ltd.*. The apparatus was adapted by the author to eliminate all metallic parts. Trays were constructed of 1/4 in. polythene sheet welded together at the edges; the frames consisted of 1/4 in. polythene sheet joined by four 8 mm diameter glass rods, and the spacers were of 1 1/4 in. lengths of polythene tubing, 3/8 in. internal diameter. All chromatograms were of the ascending type on 10 in. square sheets of Whatman No. 541 paper. Norit SX 30 Special was a gift from Swift & Co. Ltd., Sydney; it was washed three times with 10% (v/v) aqueous pyridine to remove ultraviolet-absorbing impurities, then several times with water and dried at 110°. Celite 535 was obtained from Johns Manville Co. Ltd., London. The "Chromatolite" was purchased from Hanovia Ltd., Slough, Bucks.

Reference compounds

Acetyl phosphate (Ac-P), creatine phosphate (Cr-P), carbamyl phosphate (Carb-P), and phospho-enol-pyruvate (PEP), were the synthetic compounds obtained from Dr. G. M. KELLERMAN of this department. Ethanolamine-O-phosphate (Et-P), choline phosphate (Ch-P), and serine phosphate (Se-P), were synthetic compounds, the gift of Mr. H. G. WESTALL, University College Hospital Medical School, London.

* 56, Rochester Place, London, N.W. 1.

Glycerylphosphorylcholine (GPC) was the gift of Dr. I. G. WHITE who obtained it from Dr. R. M. C. DAWSON, Institute of Animal Physiology, Babraham Hall, Cambridge. Glycerol-1-phosphate (Gl-1-P) was prepared from the 2-phosphate by the method of BAILLY[5]. Uridine diphosphate glucose (UDPG) was a gift from Dr. J. F. TURNER, C.S.I.R.O. Plant Physiology Unit, University of Sydney. The flavinadenine dinucleotide (FAD) was from yeast and was supplied by Dr. A. W. LINNANE of this department. Sedoheptulose-7-phosphate (S-7-P) and ribulose-1,5-diphosphate (Ru-1,5-PP) were the gift of Professor B. L. HORECKER, Department of Microbiology, New York University College of Medecine. Phytic acid (PA) was donated by Dr. J. W. LEE, C.S.I.R.O. Wheat Research Unit, Bread Research Institute of Australia, N. Ryde, N.S.W. Adenosine diphosphate ribose (ADP-R) and adenosine triphosphate ribose (ATP-R) were the main products of the action of 3 % perchloric acid on DPN and TPN respectively for 30 min at room temperature[6]. 2,3-Diphosphoglyceric acid (2,3-PPGA) was the principal phosphate present in the barium-insoluble fraction of rat blood[7,8]. Adenylsuccinic acid (AMPS) was prepared by the method of DAVEY[36] and propane-1,2-diol-1-phosphate (PDP) was synthesized as described by RUDNEY[37]. Diphosphopyridine nucleotide (DPN) and the reduced nucleotide (DPNH), triphosphopyridine nucleotide (TPN) and the reduced nucleotide (TPNH), adenosine-3'-, and -5'-phosphates (AMP-3' and AMP-5'), adenosine diphosphate (ADP), adenosine triphosphate (ATP), inosine monophosphate (IMP), diphosphate (IDP) and triphosphate (ITP), uridine-3'- and -5'-phosphates (UMP-3' and UMP-5'), uridine diphosphate (UDP) and triphosphate (UTP), cytidine-3'- and -5'-phosphates (CMP-3' and CMP-5'), cytidine diphosphate (CDP) and triphosphate (CTP), guanosine-3'- and -5'-phosphates (GMP-3' and GMP-5'), guanosine diphosphate (GDP) and triphosphate (GTP), flavin mononucleotide (FMN), thiamine pyrophosphate (TPP), fructose-1-phosphate (F-1-P), fructose-6-phosphate (F-6-P), glucose-1-phosphate (G-1-P), glucose-6-phosphate (G-6-P), fructose-1,6-diphosphate (F-1,6-PP), ribose-5-phosphate (R-5-P), 3-phosphoglycerate (3-PGA), 2-phosphoglycerate (2-PGA), glycerol-2-phosphate (Gl-2-P), galactose-6-phosphate (Ga-6-P), mannose-6-phosphate (M-6-P), 6-phosphogluconate (6-PG), dihydroxyacetone phosphate (DHAP), and glyceraldehyde-3-phosphate (GAP), pyridoxal phosphate (Py-P), adenine (Ad), adenosine (Ao), hypoxanthine (Hy), inosine (I), guanine (Gu), glucose (G), fructose (F), ribose (R), galactose (Ga), and maltose (Ma), were the purest obtainable commercially. Solutions were made in water or 10 % (v/v) isopropanol and stored at 2° or —10° depending on the lability of the compound.

EXPERIMENTAL METHODS AND RESULTS

Preparation of extracts

10 to 20 g of tissue were extracted with 3 % (v/v) perchloric acid, rapidly filtered and the filtrate neutralised with 10 % (w/v) potassium hydroxide. These operations were carried out in the cold-room at 2°. The neutralised filtrate was stood at least 4 h, or overnight, at 2° to allow precipitation of potassium perchlorate to occur.

The solution was filtered and freeze-dried. When the volume of extract was greater than 20 ml, further amounts of potassium perchlorate were removed by redissolving the freeze-dried material in 10 to 15 ml of water, filtering and freeze-drying the filtrate. The freeze-dried extracts were stored over phosphorus pentoxide *in vacuo* at 2° and solutions were kept frozen at —10°.

Removal of calcium and magnesium ions

It was thought desirable to remove calcium and magnesium ions which are known to form complexes with phosphate esters and interfere with their chromatography[9]. THRELFALL[1] used Amberlite IR-120 (H^+) for this purpose. The equivalent resin, Zeo-Karb 225 (H^+) was tested but chromatography of the extracts before, and after, treatment with the resin showed that some of the phosphates had been removed in addition to the cations. A column of Zeo-Karb 226 (NH_4^+) gave better results and development of chromatograms of the effluent with 8-hydroxyquinoline[10] showed the removal of calcium ions without loss of phosphates. When barium precipitation was to be used subsequently to separate the phosphates, the removal of calcium and magnesium was omitted.

Barium fractionation

The procedure described by GERLACH AND WEBER[11] was followed, but omitting the reprecipitation of each fraction. The compounds appearing in fractions A (barium-insoluble), B (barium-soluble, alcohol-insoluble), and C (barium-soluble, alcohol-soluble) agreed fairly well with the distribution reported by these authors with the exception of DHAP which sometimes appeared in fraction A instead of B, F-1, 6-PP which tended to appear in fraction B instead of A, and DPN which sometimes precipitated in fraction B but most often remained in fraction C. The occurrence of any one compound in a particular fraction appeared to depend on the exact conditions and on the other compounds present, so this information was only used as an *indication* of the nature of that compound.

To prepare solutions for chromatography, the barium precipitate was resuspended in water and stirred with either Zeo-Karb 225 (H^+) or Zeo-Karb 226 (NH_4^+). Treatment with Zeo-Karb 226 did not result in the liberation of acid but there was some danger that the barium salts would not be completely decomposed under these conditions and sometimes, on centrifuging, a small amount of undecomposed material could be seen lying on top of the resin. When Zeo-Karb 225 was used, there was little likelihood of the precipitate remaining undissolved but a distinct possibility that any acid-labile phosphates would be decomposed by the acid liberated before this could be neutralised after removal of the resin. Also, as mentioned previously, it was found that some organic phosphates could adsorb onto Zeo-Karb 225, so, in general, treatment with Zeo-Karb 226 was preferred. If, after 5 to 10 min stirring, the suspended barium precipitate had not all been decomposed, more resin was added. After centrifuging, the supernatant was removed with a pipette, the pH adjusted and the solution freeze-dried. The alcohol was removed from fraction C

at 40° under reduced pressure, the barium removed with ion-exchange resin and the solution freeze-dried.

Paper chromatography

The freeze-dried material, dissolved in a minimum amount of water, was applied to the paper by a platinum wire loop, and the spot dried by a blast of cold air from a hair drier. 0.6 g of ethylenediaminetetraacetic acid was added to each 200 ml of solvent placed in the tray.

Solvents

(1) Isobutyric acid–*N* ammonium hydroxide (100:60 by vol.)[12].

(2) *n*-Propanol–0.880 ammonium hydroxide–water (60:30:10 by vol.)[9].

(3) *n*-Butanol–acetic acid–water (60:15:25 by vol.)[13].

The following two solvents were chosen from the five described by GERLACH, WEBER AND DÖRING[14], and are designated GW1 and GW3 respectively. Both were used twice in the same direction.

(GW1) Isopropyl ether–*n*-butanol–90% (w/v) formic acid* (30:30:20 by vol.).

(GW3) *n*-Butanol–*n*-propanol–acetone–80% (w/v) formic acid–30% (w/v) trichloracetic acid (40:20:25:25:15 by vol.).

The above solvents were selected from the many described in the literature and each had its own particular virtues. Cr-P was stable in propanol–ammonia, so this solvent was used for its separation from creatine and detection with diacetyl. The solvent GW1 left nucleotides and most sugar phosphates at the origin and was suitable for the detection of three-carbon phosphates. GW3 had the great advantage of producing a good spread of compounds across the paper and of being able to move large amounts of orthophosphate; only two phosphates ran further than orthophosphate in this solvent. The butanol–acetic acid solvent was chosen since the R_F values of many muscle constituents in this solvent have already been determined[3].

Many investigators have observed the appearance of two or more spots when orthophosphate was run on paper chromatograms. CURRY[15] investigated the behaviour of orthophosphates and free phosphoric acid on chromatography and concluded that the several spots observed were due to different ionic forms running independently. With the solvents used here, with the exception of butanol–acetic acid, multiple spots of phosphates were not observed. The addition of 1% (w/v) boric acid to the propanol–ammonia and isobutyric acid solvents lowered the R_F values of sugar phosphates containing *cis*-vicinal hydroxyl groups, as described by COHEN AND SCOTT[16] and by HARRAP[17], thus separating them from other compounds of similar R_F not possessing this grouping. An example is the pair G-1-P and G-6-P; addition

* The solvent described by the authors contained 98–100% formic acid but the solvent described here was found to be very satisfactory.

Solvents 1 and 2 were stable for some weeks; others were rejected after 4 to 5 days. In some experiments, 0.6 g of A.R. boric acid was added to 60 ml of the propanol–ammonia or the isobutyric acid solvents.

of boric acid decreased the R_F of G-6-P in propanol–ammonia from 0.26 to 0.16 whereas that of G-1-P was not affected.

The R_F, R_{PO_4} and R_{AMP} values of a number of phosphates in the above solvents were determined by running about 30 compounds on five sheets of paper, simultaneously, in each solvent. Although the absolute values varied from run to run, the relative positions of the compounds remained constant and were checked over a period of two years while this work was in progress, during which time over 600 chromatograms were run. The values found are listed in Table I.

TABLE I

R VALUES OF PHOSPHATES AND RELATED COMPOUNDS

All values determined on Whatman No. 541 paper; values in GW3 twice ($GW3^2$) and GW1 twice ($GW1^2$) are R_{PO_4} values, and in isobutyric acid are $R_{AMP\text{-}5'}$ values. Decomposition in a particular solvent is indicated by "decomp".

Compound	Solvents				
	$GW3^2$ R_{PO_4}	1 $R_{AMP\text{-}5'}$	$GW1^2$ R_{PO_4}	2 R_F	3 R_F
AMP-5′	0.70	1.00	0.00	0.26	0.20
AMP-3′	0.80	1.22	0.00	0.30	0.22
ADP	0.24	0.82	0.00	0.18	0.05
ATP	0.12	0.75	0.00	0.15	0.04
IMP	0.49	0.58	0.00	0.17	0.17
IDP	0.19	0.43	0.00	0.13	0.09
ITP	0.07	0.35	0.00	0.10	0.05
UMP-3′	0.63	0.69	0.03	0.20	0.24
UMP-5′	0.58	0.60	0.01	0.15	0.20
UDP	0.24	0.45	0.00	0.14	0.07
UTP	0.09	0.36	0.00	0.13	0.06
GMP-3′	0.56	0.70	0.00	0.12	0.18
GMP-5′	0.51	0.56	0.00	0.10	0.18
GDP	0.05	0.39	0.00	0.07	0.05
GTP	0.02	0.26	0.00	0.06	0.02
CMP-3′	0.64	0.95	0.01	0.26	0.20
CMP-5′	0.58	0.85	0.00	0.20	0.17
CDP	0.22	0.65	0.00	0.14	0.08
CTP	0.07	0.52	0.00	0.11	0.03
UDPG	0.13	0.25	0.00	0.60	0.05
DPN	0.10	1.02	0.00	decomp.	0.04
DPNH	decomp.	decomp.	0.00	decomp.	decomp.
ADP-R	0.10	0.73	0.00	0.30	0.04
AMPS	0.70	0.62	0.00	0.17	0.25
TPN	0.05	0.71	0.00	decomp.	0.01
TPNH	decomp.	decomp.	0.00	decomp.	decomp.
ATP-R	0.07	0.48	0.00	0.12	0.01
FMN	0.59*	0.67	0.00	0.55*	0.08*
FAD	0.67*	0.75	0.00	0.53*	0.00*
Py-P	1.05	0.80	0.18	0.23	0.26
TPP	0.52	1.26	0.00	decomp.	0.10
PO_4	1.00	0.68	1.00	0.19	0.28
SO_4	0.46	0.44	0.02	0.17	0.08
P_2O_7	0.52	0.52	0.02	0.11	0.11
Cr-P	0.98*	0.86*	0.62*	0.17	decomp.
Ac-P	decomp.	0.86*	decomp.	decomp.	decomp.

(continued on p. 147)

TABLE I (*continued*)

Compound	Solvents				
	GW3[2] R_{PO_4}	1 $R_{AMP-5'}$	GW1[2] R_{PO_4}	2 R_F	3 R_F
Carb-P	0.46*	0.40	decomp.	decomp.	decomp.
GAP	0.72	0.40	0.23	decomp.	0.15
DHAP	0.70	0.68	0.23	decomp.	0.05
PDP	1.19	0.95	1.33	0.33	0.28
PEP	1.06	0.62	0.73	0.26	0.27
3-PGA	0.79	0.45	0.32	0.23	0.18
2-PGA	0.74	0.45	0.20	0.26	0.16
2,3-PPGA	0.54	0.38	0.01	0.04	0.12
Gl-1-P	0.76	0.65	0.52	0.35	0.14
Gl-2-P	0.83	0.65	0.52	0.35	0.14
Et-P	0.81	0.89	0.08	0.30	0.21
Se-P	0.67	0.54	0.02	0.04	0.17
Ch-P	0.99	1.06	0.23	0.33	0.24
GPC	0.67	1.01	0.12	0.49	0.28
R-5-P	0.57	0.53	0.04	0.25	0.18
F-1-P	0.52	0.40	0.01	0.27	0.12
F-6-P	0.50	0.48	0.03	0.31	0.19
F-1,6-PP	0.35	0.37	0.01	0.09	0.08
G-1-P	0.41	0.48	0.02	0.30	0.20
G-6-P	0.41	0.46	0.02	0.26	0.12
6-PG	0.49	0.42	0.02	0.22	0.12
Ga-6-P	0.41	0.46	0.02	0.25	0.12
M-6-P	0.47	0.51	0.02	0.31	0.14
S-7-P	0.45	0.46	0.00	0.28	0.12
Ru-1,5-PP	0.50	0.35	0.00	0.06	0.08
PA	0.07	0.15	0.00	0.01	0.01
G	0.59	0.69	0.16	0.77	0.22
F	0.68	0.77	0.21	0.98	0.26
R	0.86	0.84	0.40	0.78	0.30
Ga	0.55	0.69	0.16	0.73	0.22
Ma	0.32	0.55	0.00	0.65	0.14
Ad	1.40	1.60	0.30	0.72	0.60
Hy	1.15	1.10	0.31	0.66	0.48
Gu	0.78	1.20	0.14	0.45	0.38
Ao	1.00	1.57	0.16	0.84	0.49
I	0.75	0.96	0.16	0.65	0.36

* Partial decomposition but R value of intact portion quoted.

The values in GW3 twice and GW1 twice showed good agreement with those published by GERLACH, WEBER AND DÖRING[14]. The nucleoside triphosphates were stable in all solvents, Cr-P was partially decomposed by all solvents except propanol–ammonia, while DPN, DPNH, TPN, and TPNH were decomposed by this solvent to give a substance fluorescing blue under ultraviolet light. DPNH and TPNH were decomposed by acid solvents forming ADP-R and ATP-R respectively as described by FORREST, WILKEN AND HANSEN[6]; the decomposition of DPNH and TPNH by acid has also been discused by RAFTER, CHAYKIN AND KREBS[18]. FAD and FMN were slowly decomposed by both acid and alkaline solvents as would be expected from their known behaviour in acid and alkali[19] but they seemed to be stable in isobutyric acid. GAP and DHAP were stable in the acid solvents but decomposed by propanol–ammonia.

Two-dimensional chromatography

Different combinations of solvents were found suitable for different purposes. For the separation and identification of the nucleotides, isobutyric acid followed by GW_3 twice was the best combination. It was discovered that when ATP was run with GW_3 as the first solvent, the chromatograms showed an additional spot due to small amounts of ADP. When, however, the order of the solvents was reversed only the single spot of ATP resulted. Since only a single spot resulted when ATP was run in GW_3 alone, it was concluded that the deposition of trichloracetic acid on the paper on drying, after the second run in GW_3 and prior to the run in isobutyric acid, had lowered the pH of the paper to a point where some of the ATP had hydrolysed. Therefore, as a general rule, when the identification of nucleotides was the aim, GW_3 was used as the *second* solvent. However, when reducing sugar phosphates were to be detected with the triphenyltetrazolium reagent, better results were obtained when the solvents were used in the reverse order since the isobutyric acid washed to one side the trichloracetic acid deposited on the paper which otherwise interfered considerably with the colour development of the spots. The combination GW_3 twice/GW_1 twice was found very useful for separating three-carbon phosphates, since the nucleotides, pentose phosphates, and hexose phosphates remain very near the origin in the GW_1 direction. "Maps of the spots" of the phosphates most likely to be found in animal tissues are shown in Figs. 1 and 2.

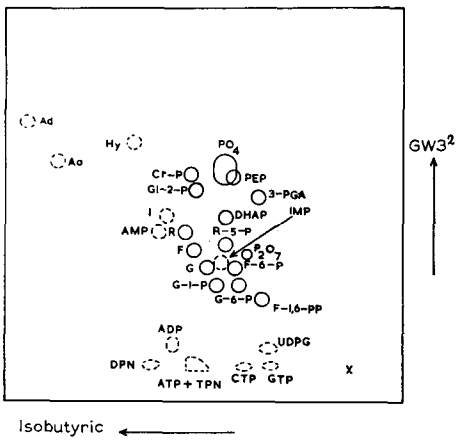

Fig. 1. "Map of the spots" on chromatograms in GW_3 twice followed by isobutyric acid. Origin marked by a cross; spots visible under ultraviolet light are shown in broken outline.

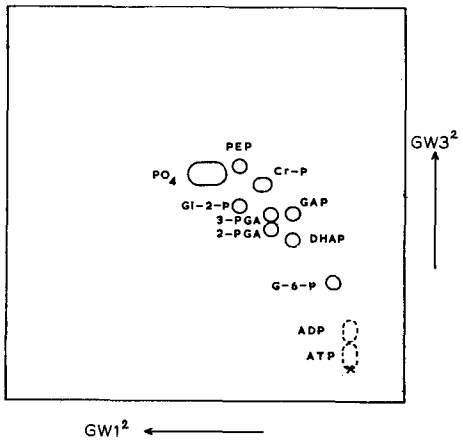

Fig. 2. "Map of the spots" on chromatograms in GW_3 twice followed by GW_1 twice. Origin marked by a cross; spots visible under ultraviolet light are shown in broken outline.

DETECTION OF COMPOUNDS ON CHROMATOGRAMS

Ultraviolet light

Chromatograms were viewed under ultraviolet light from a Hanovia "Chromatolite". Dark spots of purine and pyrimidine derivatives and any fluorescent spots were marked. Guanine derivatives showed a dark blue fluorescence coincident with the

dark spot when acid solvents had been used. To make this more apparent, or when the last solvent was not strongly acidic, the paper was first dipped through a solution of 0.05 ml concentrated hydrochloric acid in 50 ml acetone. After chromatography in GW3 twice/isobutyric acid and leaving the papers overnight in the warm room at 30°, GAP, DHAP, and R-5-P gave faint yellow or blue fluorescent spots under the lamp. Since these spots were not visible directly after the solvent had been removed it was concluded that the fluorescence was due to reaction with the ammonium isobutyrate deposited on the paper to form a fluorescent "Maillard-type" compound[20].

Acid molybdate

The reagent of HANES AND ISHERWOOD[9] as modified by BURROWS, GRYLLS AND HARRISON[21] was used and the spots were developed by exposure to ultraviolet light. After dipping, and before irradiation, orthophosphate and very labile compounds such as Cr-P, Ac-P, Carb-P, GAP, and DHAP, always showed as yellow spots. ATP and other nucleoside triphosphates sometimes appeared as bright yellow spots and ADP and other nucleoside diphosphates were also faintly yellow. This only occurred when GW3 was used as the final solvent; apparently the increased acidity due to the trichloracetic acid deposited on the paper after drying was sufficient to bring about liberation of orthophosphate at room temperature on treatment with acid-molybdate. The effect could be increased by diluting the reagent with a smaller volume of acetone before applying it to the paper. G-1-P sometimes behaved in a similar manner; it could be distinguished from G-6-P, which runs very close to it on two-dimensional chromatograms, by the fact that the blue spot brought up on irradiation always had a yellow-green colour when viewed from the reverse unirradiated side of the paper.

Despite the fact that it was slowly decomposed by the solvents used, quite large spots of Cr-P were found on two-dimensional chromatograms of muscle extracts. At first, it was thought that orthophosphate had split into two spots but a strong reaction with diacetyl showed that the spot was, in fact, intact Cr-P. Pyrophosphate did not give a spot until after irradiation and the spot was blue. The yellow colour of the background produced on irradiation slowly returned to white as moisture was regained from the air. Alternatively, the paper was exposed momentarily to steam; this treatment also increased the brightness of the blue spots. Sulphate ion could give a spurious blue spot if present in great enough quantity so its R values are quoted in Table I.

Adenine compounds

The procedure of GERLACH AND DÖRING[22] showed Ad, Ao, AMP, ADP, ATP, and the pyridine nucleotides, as red spots; other nucleotides gave no colour.

Pyridine nucleotides

These were detected with methyl ethyl ketone and ammonia as described by KODICEK AND REDDI[23].

Thiamine pyrophosphate

TPP was detected by dipping the paper through the reagent described by BLOCK, DURRUM AND ZWEIG[24]. After drying, TPP was revealed as a bright blue fluorescent spot under ultraviolet light. The reaction was not given by the pyridine nucleotides.

Silver nitrate–bromophenol blue

As an additional reagent for purine and pyrimidine compounds this was particularly useful for confirming the presence of inosine and hypoxanthine[25].

Cations

The procedure of SOMMER[10] revealed Mg^{++} and Ca^{++} as fluorescent spots under ultraviolet light.

Diacetyl

The diacetyl reagent of FOSTER AND ASHTON[26] was used to detect creatine and Cr-P. The reagent did not react directly with Cr-P which had to be broken down by heating the papers in an oven for a few minutes at 105° before treatment with the reagent.

Diphenylamine

The reagent described by STEPANENKO AND KUZNETSOV[27] gave a very characteristic rose colour with F-1,6-PP but was of rather low sensitivity.

p-Anisidine

The reagent of PRIDHAM[28] was adapted for dipping. 2.5 g *p*-anisidine hydrochloride and 0.25 g sodium bisulphite were added to 50 ml methanol followed by a few drops of water sufficient to dissolve the bisulphite. Before use the solution was diluted 5 times with acetone. After dipping, the papers were dried and heated at 130° for a few minutes. Glucose, fructose, and their various phosphates gave brown spots while ribose and R-5-P gave red-purple spots.

Orcinol

The reagent described by KLEVSTRAND AND NORDAL[29] was used to detect heptose phosphates. By its use S-7-P was detected in liver extracts.

2,4-Dinitrophenylhydrazine

The paper was drawn rapidly through the surface of a saturated solution of the reagent in 2 *N* hydrochloric acid, taking care not to waterlog the paper. If present in sufficient amount, GAP and DHAP appeared immediately as orange spots on a yellow background.

Pyrophosphate reagent

1.2 g cobalt chloride hexahydrate were dissolved in 12 ml of water, acidified with a few drops of 2 *N* acetic acid, and 28 ml of ethanol added. The papers were dipped

through the reagent, dried, and the background colour removed by two washes in 50% (v/v) ethanol. After drying, the still humid papers were exposed to ammonia fumes and pyrophosphate appeared as a brown spot. Sensitivity: 5 μg P as pyrophosphate.

Triphenyltetrazolium

Before applying the reagent[30] the chromatograms were hung in the air for 6 to 7 days to remove traces of solvent which otherwise caused considerable background colour. The papers were dipped through a freshly prepared solution of 0.1 g 2,3,5-triphenyltetrazolium chloride in 20 ml chloroform, dried, and then passed through a solution of 20% (w/v) aqueous potassium hydroxide diluted to 2% (w/v) with *n*-butanol–ethanol (1:1). The papers were dried in the dark and spots of very strongly reducing sugar phosphates such as R-5-P showed up at room temperature, if present in sufficient amount. The papers were exposed to steam from a boiling water-bath to bring up the reducing sugars or sugar phosphates as red spots on a white or pink background. The background colour could be diminished by careful washing in 50% (v/v) ethanol.

The following substances reacted: glucose, fructose, ribose, galactose, maltose, GAP, DHAP, R-5-P, F-1-P, F-6-P, G-6-P, Ga-6-P, M-6-P, S-7-P, Ru-1,5-PP, DPN, DPNH, TPN, TPNH, ADP-R, ATP-R, and UDPG. G-1-P did not react, and F-1,6-PP gave only a weak reaction.

QUANTITATIVE MEASUREMENT

(a) By measurement of ultraviolet absorption

The spot, as revealed under ultraviolet light, was cut out and placed in a clean dry test-tube. After washing twice with A.R. methanol the tube and paper were drained and dried. 1.2 ml of 0.1 *N* hydrochloric acid were added and the tube was stoppered and left for 18 h with occasional shaking. The optical density of the eluted material was measured in a Beckman spectrophotometer over the range 240 to 290 mμ against a "blank"* prepared from a piece of paper of the same size cut from the corresponding position of a "blank" chromatogram run at the same time. The absorption curve of the eluted material was plotted and the compound identified from the wave-length of its absorption maximum and the position on the chromatogram. The amount present could be calculated from the height of the curve and the published molar extinction coefficient.

In cases where there was not sufficient material in the spot to give a measurable absorption in solution, it was found possible to cut out the spot and measure its absorption directly on the paper, as follows: the spot was moistened with glycerol and caused to adhere to the outside of the Beckman cell filled with water such that it completely covered the aperture in the cell carrier; the same was done with a "blank" prepared as above and the absorption measured over a range of wavelengths

* BRINER[35] has published some useful data on the ultraviolet absorption of material eluted from paper cut from various positions of such chromatograms.

against the blank. Obviously this technique could be adapted to semi-quantitative measurement of the amount present.

(b) By measurement of the phosphorus in a spot

After development with acid-molybdate, spots were cut out and ashed with 0.2 ml of a mixture of concentrated sulphuric acid–10 *N* perchloric acid (1:1 v/v) in a pyrex tube at a temperature not greater than 220°. The acid and washings were transferred to a 10-ml "Quickfit" stoppered test-tube bearing a mark corresponding to 3.5 ml. The solution was made up to the mark and the phosphate determined by MARTIN AND DOTY'S[31] modification of the method of BERENBLUM AND CHAIN[32] as described by LINDBERG AND ERNSTER[33]. The volumes used were as follows:

0.5 ml of 10% (w/v) ammonium molybdate was added (final acid concentration approx 1.1 *N*) and the phosphomolybdic acid was extracted with 3.00 ml benzene–isobutanol (1:1 v/v). 2.00 ml of the supernatant were removed with an all-glass "Tuberculin" syringe, 2.90 ml of 3.2% (v/v) sulphuric acid in ethanol were added followed by 0.10 ml 1% (w/v) stannous chloride and the colour was read at 725 mμ against a blank prepared from 0.2 ml of the digestion acid taken through the same procedure.

It was most important to use fresh stannous chloride which was prepared by dissolving 0.1 g granulated tin in 2 ml concentrated hydrochloric acid and diluting to 20 ml with *N* sulphuric acid. The solution was rejected when more than 5 days old.

A standard curve was prepared over the range of final concentrations 0.10–1.60 μg per ml. The optical densities were found to be quite stable and reproducible from day to day, but standards were always run together with the samples. The standard curve was linear over the above range and up to a final concentration of 8.0 μg per ml. The blank for Whatman No. 541 paper, after being used for phosphate chromatography, was found to be 0.02 μg of phosphorus per cm^2.

DISCUSSION

A large number of published methods for the isolation and separation of organic phosphates were tried and have led to the procedures described here. Many reagents for identifying the separated compounds were tested and those selected were chosen for their specificity and high sensitivity. On two-dimensional chromatograms, spots containing as little as 0.3 μg of phosphorus could be detected by the acid-molybdate reagent, and the triphenyltetrazolium reagent was, if anything, even more sensitive to such compounds as F-6-P and G-6-P.

Large amounts of material, containing a high proportion of orthophosphate, could be chromatographed in the system GW3 twice/isobutyric acid and the phosphates could be detected in extracts applied directly to the paper. However, the best results were achieved by chromatography of the 3 fractions obtained after barium precipitation, since not only were most of the phosphates thereby concentrated, but the occurrence of a compound in a particular fraction aided its identification.

The use of freeze-drying for concentration of the extracts and various fractions was essential; the ease and rapidity with which some sugar phosphates react with amino acids and ammonia has been described by the author[34]. Such reactions, if allowed to take place, would result in the non-detection of some phosphates originally present (particularly R-5-P) and the discovery of "unknown" compounds produced as intermediates in the "Maillard" reaction[20].

The methods described here are of general application and have been applied extensively to the investigation of the acid-soluble phosphates in rat muscle and ox muscle, and also to extracts of whale muscle, rat blood, and rat liver. Diagrams of the chromatograms of extracts of ox muscle will be published elsewhere[34]. It is hoped to present a full report on the compounds found in rat muscle, among which the following have been positively identified: Cr-P, ATP, ADP, GTP, DPN, F-6-P, G-1-P, F-1,6-PP, DHAP.

ACKNOWLEDGEMENT

This work was assisted financially by the Commonwealth Scientific and Industrial Research Organisation of Australia.

SUMMARY

A method for the separation, identification, and quantitative measurement of the acid-soluble phosphates and related compounds in tissues is presented. The R values of 70 compounds in 5 different solvents are listed and "maps of the spots" of those most commonly found in tissues are given. A new reagent for detecting pyrophosphate is described.

REFERENCES

1 C. J. THRELFALL, *Biochem. J.*, 65 (1957) 694.
2 T. WOOD, *J. Sci. Food Agr.*, 7 (1956) 196.
3 T. WOOD AND A. E. BENDER, *Biochem. J.*, 67 (1957) 366.
4 A. E. BENDER, T. WOOD AND J. A. PALGRAVE, *J. Sci. Food Agr.*, 9 (1958) 812.
5 M. C. BAILLY, *Compt. rend.*, 206 (1938) 1902.
6 R. J. FORREST, D. R. WILKEN AND R. G. HANSEN, *Biochim. Biophys. Acta*, 37 (1960) 551.
7 L. MACHO, *Nature*, 180 (1957) 1351.
8 M. ROHDEWALD AND M. WEBER, *Z. physiol. Chem.*, 306 (1956) 90.
9 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
10 G. SOMMER, *Z. anal. Chem.*, 147 (1955) 241.
11 E. GERLACH AND E. WEBER, *Arch. exptl. Pathol. Pharmakol., Naunyn-Schmiedeberg's*, 224 (1955) 496.
12 H. A. KREBS AND R. HEMS, *Biochim. Biophys. Acta*, 12 (1952) 172.
13 I. SMITH, in I. SMITH, *Chromatographic Techniques – Clinical and Biochemical Applications*, Heinemann, London, 1958.
14 E. GERLACH, E. WEBER AND H. J. DÖRING, *Arch. exptl. Pathol. Pharmakol., Naunyn-Schmiedeberg's*, 226 (1955) 9.
15 A. S. CURRY, *Nature*, 171 (1953) 1026.
16 S. S. COHEN AND D. M. SCOTT, *Science*, 111 (1950) 543.
17 F. E. G. HARRAP, *Nature*, 182 (1958) 876.
18 G. W. RAFTER, S. CHAYKIN AND E. G. KREBS, *J. Biol. Chem.*, 208 (1954) 799.
19 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 298 (1938) 150.
20 J. E. HODGE, *J. Agr. Food Chem.*, 1 (1953) 928.
21 S. BURROWS, F. S. M. GRYLLS AND J. S. HARRISON, *Nature*, 170 (1952) 800.

[22] E. GERLACH AND H. J. DÖRING, *Naturwiss.*, 42 (1955) 344.
[23] E. KODICEK AND K. K. REDDI, *Nature*, 168 (1951) 475.
[24] R. J. BLOCK, E. L. DURRUM AND G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd Ed., Academic Press, New York, 1958, p. 401.
[25] T. WOOD, *Nature*, 176 (1955) 175.
[26] M. C. FOSTER AND G. C. ASHTON, *Nature*, 172 (1953) 959.
[27] B. N. STEPANENKO AND A. A. KUZNETSOV, *Biokhimiya*, 24 (1959) 25.
[28] J. B. PRIDHAM, *Anal. Chem.*, 28 (1956) 1967.
[29] R. KLEVSTRAND AND A. NORDAL, *Acta Chem. Scand.*, 4 (1950) 1320.
[30] W. E. TREVELYAN, D. P. PROCTOR AND J. S. HARRISON, *Nature*, 166 (1950) 444.
[31] J. B. MARTIN AND D. M. DOTY, *Anal. Chem.*, 21 (1949) 965.
[32] J. BERENBLUM AND E. CHAIN, *Biochem. J.*, 32 (1938) 295.
[33] O. LINDBERG AND L. ERNSTER, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 3, Interscience, New York, 1956, p. 1.
[34] T. WOOD, *J. Sci. Food Agr.*, 12 (1961) 61.
[35] G. P. BRINER, *J. Chromatog.*, 2 (1959) 418.
[36] C. L. DAVEY, *Nature*, 183 (1959) 995.
[37] H. RUDNEY, *J. Biol. Chem.*, 202 (1953) 263.