An Analytical Procedure for the Acid-Soluble Phosphorus Compounds in Rat-Skeletal Muscle

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Few attempts have been made at the identification and estimation of all the phosphorus compounds present in the acid-soluble fraction of skeletal muscle. Recent methods may be divided into two main groups. The first achieves fractionation of the phosphate esters by their precipitation as alkaline-earth salts under conditions of controlled pH and differing ethanol concentrations (LePage, 1948, 1949). Clear-cut separations are not, however, always obtained, and one compound may appear in more than one fraction (Wade & Morgan, 1955). The levels of the esters in each fraction are determined either by the estimation of the carbohydrate moiety or by the increase in inorganic orthophosphate on controlled hydrolysis. Both of these types of estimation are carried out in the presence of similar compounds whose contribution to the final answer is difficult to assess.

Chromatography has provided an alternative approach. Hanes & Isherwood (1949) separated synthetic mixtures by paper chromatography, and Caldwell (1953) utilized their solvent systems for the direct paper chromatography of the acid-soluble extract. Identification of the spots on the chromatograms was made by comparison with known compounds before their estimation by determination of the inorganic phosphate present in the spot after wet ashing. It is difficult, however, to find solvent systems capable of separating adequately the many compounds present, and the detection of trace amounts of esters is only possible when large amounts of extract are applied. This causes overlapping of the smaller spots by compounds present in very much higher concentration, and irregular running of the chromatograms owing to high salt concentrations.

Ion-exchange chromatography has been used widely for separating compounds of a particular class. Cohn & Carter (1950) and Bergkvist & Deutsch (1953, 1954*a*) have successfully separated the nucleotide components but no comprehensive scheme covering all the phosphate esters has been forthcoming.

Wade & Morgan (1955) have elaborated a scheme which appears capable of separating most of the phosphate esters occurring in muscle. This combines paper chromatography with paper electrophoresis but has not yet been applied to biological material. The criticism directed at Caldwell's technique may well apply in this case also. Wade & Morgan point out the difficulty of accommodating the acid-labile phosphates in an analytical scheme involving acid solvents.

Heald (1956) has applied a combination of the fractionation by barium precipitation and the ionophoretic technique of Wade & Morgan (1955) to the analysis of the acid-soluble extract of brain. The difficulties of fractionation into well-defined groups by the barium-precipitation method are apparent, and the separation of the phosphate esters by the ionophoretic method is not complete. Further, some phosphorus compounds remain unidentified and the difficulty of accommodating phosphocreatine in an analytical scheme of this nature is emphasized.

The object of the present method is to overcome these difficulties by first fractionating the phosphate compounds into well-defined and reproducible groups before applying the technique of quantitative paper chromatography.

A preliminary account of this work was given at the Third International Congress of Biochemistry (Brussels, August 1955).

EXPERIMENTAL

Materials

Creatine phosphate (CP) was a synthetic sodium salt obtained from Dr L. A. Stocken, Biochemistry Department, University of Oxford. Adenosine diphosphate (ADP) and adenosine triphosphate (ATP) were 'Chromatographically Pure' sodium salts from Schwarz Laboratories Inc., Mount Vernon, U.S.A., and contained traces of adenosine 5-phosphate (AMP) and ADP respectively. Barium 2- and 3-phosphoglycerates and silver barium phosphopyruvate were gifts from Mr R. Hems, Biochemistry Department, University of Oxford. Inosine mono-, di- and tri-phosphates were prepared as barium salts from AMP, ADP and ATP respectively by the method of Klienzeller (1942).

Diphosphopyridine nucleotide (DPN) was prepared from yeast according to Kornberg & Pricer (1953) as described by Aldridge & Cremer (1955). Reduced DPN (DPNH) was prepared according to Lehninger (1952). Triphosphopyridine nucleotide (TPN) was prepared from cow liver by an adaptation of the methods of LePage & Mueller (1949) and Kornberg & Horrecker (1953). Reduced TPN (TPNH)

was prepared in solution by the reduction of TPN with sodium dithionite.

Glucose 6-phosphate, fructose 6-phosphate, fructose diphosphate, ribose 5-phosphate, glucose 1-phosphate and AMP were commercial preparations.

Cations were removed from all phosphate compounds used (except CP) by treatment with Amberlite IR-120 (H). Norit SX 30 Special was a gift from Haller and Phillips Ltd., 14, Wool Exchange, Basinghall Street, London, E.C. 2. Celite 535 was obtained from Johns Manville Co. Ltd., London. *iso*Butanol, *iso*butyric acid and propan-1-ol were B.D.H. Ltd. laboratory reagents. Other reagents were B.D.H. Ltd. A.R. quality.

Methods

Preparation of extracts. Normal muscle samples were obtained as previously described (Stoner & Threlfall, 1954). The muscle sample from the fore-limb was composed mainly of the pectoral muscles and triceps from each side. The hind-limb muscle samples contained the rectus femoris, adductores and gastrocnemius from each side. In both limbs the muscles were rapidly dissected free from fat before freezing in liquid nitrogen. After rapid weighing and the addition of 6 vol. of cold 3% perchloric acid the samples were homogenized in a Nelco 10 blender (MSE, London) for 3 min. and filtered through a Whatman no. 1 filter paper. These operations were carried out at 2°. The blender will homogenize 3–6 g. of muscle satisfactorily; smaller amounts of muscle (1–3 g.) are not dealt with satisfactorily and were ground by hand in a mortar.

Determination of inorganic phosphate (IP) and creatine phosphate. A volume (1 ml.) of the filtered extract was rapidly neutralized with 0·4 N-NaOH and diluted to 50 ml. The free and bound creatine were determined on 2·5 ml. samples by the method of Eggleton, Elsden & Gough (1943) as described by Ennor & Rosenberg (1952), with the omission of the *p*-chloromercuribenzoic acid. To obtain consistent results the colour was developed in a water bath at 20° for 30 min.

Creatine phosphate was also determined by the modified method of Berenblum & Chain (1938) as the difference between the IP present in the neutralized extract and the IP present after standing for 25 min. at 20° in the presence of acid molybdate (Ennor & Stocken, 1950), 2·5 ml. samples being used in each case. The procedure was as described below, and 0·9 ml. of 10 N-H_2SO_4 was used instead of the ashing mixture.

Phosphorus determinations. Total phosphorus in the initial extract, in the extract after Norit-column treatment and in the pyridine eluate from the Norit column was determined by the method of Fiske & Subbarow (1925). Total phosphorus in the chromatographic spots was determined after ashing the paper (previously treated with acid molybdate mixture as described under chromatography) with 0·5 ml. of sulphuric–perchloric acid mixture (Hanes & Isherwood, 1949); the following modified procedure of Berenblum & Chain (1938) was used. The ashed sample was diluted with 3 ml. of water, cooled and transferred with two washings of 2 ml. of water to a 100 ml. separating funnel containing 2·5 ml. of 2·5% (w/v) ammonium molybdate and 8 ml. of *iso*butanol. The funnels were shaken for 30 sec. on a Microid flask shaker (Baird and Tatlock, London). The organic phase was then shaken twice more with 5 ml. of N-H_2SO_4 for 15 sec., after the lower layer had been run off. The *iso*butanol layer was run into a graduated tube, made up to 10 ml. with ethanol washings from the funnel; reduction was carried out with 0·1 ml. of stannous chloride solution (40%, w/v) as described by Bartley (1953), and readings were made with 1 cm. cells at 725 mμ. on a Unicam SP. 500 spectrophotometer.

Preparation of Norit–Celite columns. Norit SX 30 Special (100 mg.) was mixed with an equal weight of Celite 535 and introduced into a glass column 3–4 mm. in diameter and 8 cm. high sealed with a glass-wool plug at the lower end. To the upper end a wider piece of glass tubing was attached to hold 10 ml. of liquid. The charcoal was packed by tapping the column on the bench and 5 ml. of 8% (v/v) *sec.*-octanol in ethanol forced down the column by compressed-air pressure (4 lb./in.^{2}) followed by 1 ml. of water to remove ethanol. Pretreatment of the Norit–Celite with *sec.*-octanol in ethanol was found to be essential to inactivate the Norit somewhat, otherwise quantitative elution of the nucleotides is not possible with aqueous pyridine.

Preparation of nucleotide fraction. A sample of perchloric acid extract of muscle (5 ml.) was forced down the column followed by 0·5 ml. of water. The adsorbed nucleotides were eluted with 3 ml. of 10% (v/v) aqueous pyridine and this was used, after evaporation to dryness under a current of air and solution in 0·5 ml. of water, for chromatography. Trichloroacetic acid extracts of tissues cannot be used as then the nucleotides are only slowly eluted by aqueous pyridine.

Removal of IP and CP phosphorus. The Norit-treated perchloric acid extracts from several columns were combined and transferred to a 20 ml. separating funnel; the IP and CP phosphorus were removed by the addition of 10% (w/v) ammonium molybdate and 0·5 vol. of pentanol. The two phases were allowed to stand for 25 min. at room temperature with occasional shaking. After a final vigorous shake the organic layer was removed and the aqueous phase shaken twice more with pentanol to remove traces of phosphomolybdic acid complex. The volume of ammonium molybdate solution used was determined empirically as the least necessary to remove the IP and the IP formed during the catalytic breakdown of the CP as determined initially. This was found to be 0·4 ml. of 10% (w/v) ammonium molybdate for 500 μg. of P in 5 ml. of extract. Separate experiments had shown that perchloric acid could replace H_2SO_4 in the breakdown of CP catalysed by acid molybdate.

Excess of molybdate was removed by passing a vigorous current of H_2S through the cold solution for 20 min., to precipitate the molybdate as molybdenum sulphide, and allowing to stand overnight at 2°. After filtration at 2° through Whatman no. 42 filter paper the solution was aerated to remove H_2S.

The removal of IP and CP phosphorus was found to be essential to obtain good chromatograms of the sugar phosphates, since the presence of IP in high concentrations tended to obscure the other constituents and cause uneven running of the chromatograms.

Preparation of extract for chromatography. The remaining phosphates were precipitated from the ice-cold extract, after aeration, by the addition of finely divided baryta until the solution was markedly alkaline to phenolphthalein, 4 vol. of ethanol was then added (Sacks, 1949) and the mixture allowed to stand at −15° overnight. The barium salts were centrifuged at −5° and washed once with cold

ethanol. Water (5 ml.) was added to the precipitate, followed by Amberlite cation exchanger IR-120(H) to remove barium. The aqueous phase was pipetted off and the resin washed twice with 2 ml. of water. The combined washings were adjusted to 10 ml., and after addition of more dry resin were shaken and filtered through Whatman no. 42 filter paper. After neutralization with N-NH_3 soln. a measured volume was freeze-dried in a 25 ml. round-bottomed flask. The small amount of white solid obtained was dissolved in 0·2 ml. of water for chromatography.

Chromatography. Chromatography was carried out on Whatman no. 1 papers (50 cm. × 23 cm.) treated with ethylenediaminetetraacetic acid as described by Eggleston & Hems (1952) at 25° in an all-glass apparatus. Application of material to the paper was made with an Agla micrometer syringe (Burroughs Wellcome and Co., London); successive spots (5 μl.) were dried with a stream of cold air from a hair-drier.

The concentrated pyridine eluate from the Norit–Celite columns were applied and developed in the *iso*butyric acid–ammonia solvent of Krebs & Hems (1952) for 20 hr. Good separations of inosine monophosphate, ATP, ADP and AMP were obtained. Guanosine and uridine triphosphates which are present in trace amounts (Bergkvist & Deutsch, 1953, 1954*b*, *c*) were not separated from each other but could be separated in the ammonium sulphate–propan-2-ol solvent system of Bergkvist & Deutsch (1953). Nucleotide spots were located by viewing under ultraviolet light (Hanovia Chromatolite) and the absence of other spots containing non-nucleotide phosphorus confirmed by the molybdate-spraying technique of Hanes & Isherwood (1949).

The solution of the freeze-dried intermediates (50–100 μl. corresponding to 0·25–0·5 g. of muscle) was chromatographed for 16 hr. in the *tert.*-butanol–picric acid solvent of Hanes & Isherwood (1949), in an upward direction along the minor axis of the paper; the two short sides of the paper were secured together with cellulose self-adhesive tape (Sellotape) to form a cylinder. Inside the chromatographic tank H_2S was generated in a beaker containing barium sulphide and H_2SO_4. After drying at room temperature the paper was developed in a downward direction for 48 hr. along its major axis in the propanol–ammonia system of Hanes & Isherwood (1949). Phosphorus-containing spots were located by the acid molybdate-spraying technique as described above and estimated as previously described.

Compounds were identified by co-chromatography with known markers.

RESULTS

Results presented by Ennor & Rosenberg (1952) suggest that the phosphocreatine content of tissues determined by the two methods employed here does not always coincide. Data presented in Table 1 suggest that in rat-skeletal muscle, at least, good agreement is obtained by the two methods, indicating that no other phosphate compounds labile to acid molybdate are present.

The efficiency of the Norit–Celite columns for the retention of nucleotides is shown in Table 2 and the recovery with 10% aqueous pyridine of the adsorbed nucleotide is seen to be quantitative. Table 3 shows that non-nucleotide phosphate esters are not retained by the columns but are recovered quantitatively in perchloric acid medium after passing down the column.

Table 4 shows that inorganic phosphate can be removed from the phosphorylated glycolytic intermediates by the molybdate–pentanol technique, leaving the organically bound phosphorus (except for CP) in the aqueous phase in quantitative yield.

Table 1. *Estimation of creatine phosphate*

Creatine phosphate (6·2 μmoles) was dissolved in 1 ml. of cold 3% perchloric acid; 0·5 ml. was rapidly neutralized with NaOH and diluted to 25 ml. Portions (2·5 ml.) were taken for estimations of IP and CP phosphorus and for free and total creatine. Muscle was extracted with 6 vol. of cold 3% perchloric acid, filtered, neutralized and diluted (1:50). Portions (2·5 ml.) were taken as for synthetic CP. Each pair of muscle values is from a separate animal.

	Phosphate	Creatine
Synthetic CP (μmoles/ml.)	6·16	6·30
Muscle CP (μmoles/g.)	15·6	14·9
	17·0	16·7
	17·3	17·5
	15·85	15·7
	16·7	16·5
	13·8	13·6
	16·5	16·3
	15·7	15·9
Mean ± standard error of mean	16·05 ± 0·35	15·9 ± 0·42

Table 2. *Adsorption and recovery of nucleotides from Norit columns*

Nucleotides were dissolved in 5 ml. of 3% perchloric acid and passed down a Norit column (100 mg.); elution was with 3 ml. of 10% (v/v) aqueous pyridine.

Nucleotide applied to column (μmoles)		Ultraviolet absorption of perchloric acid effluent at 250–270 mμ.	Nucleotide recovered in pyridine eluate (μmoles)
ATP	5·70	+ + +	4·0
	4·10	+	3·9
	2·87	–	2·74
	0·40	–	0·40
	0·18	–	0·18
	0·09	–	0·09
ADP	0·53	–	0·52
AMP	4·80	–	4·90
Inosine monophosphate	4·30	+ + +	2·60
	0·48	–	0·48
Uridine triphosphate	0·78	+ + +	0·59
	0·35	+	0·33
	0·22	–	0·22
DPN	1·41	–	1·42
	4·70	–	4·69
DPNH	4·18	–	4·29
TPN	4·62	–	4·75
TPNH	4·80	–	4·75

Recoveries of some typical phosphate esters during the barium-precipitation stage are shown in Table 5. Glycogen, which is stated (LePage, 1949) to interfere with the precipitation of these compounds, is adsorbed on the Norit and is not eluted with 10% aqueous pyridine.

Table 6 shows the distribution of the total phosphorus in the acid-soluble fraction of rat-skeletal muscle into its major fractions together with the distribution of metabolites within these fractions. The figures contain the values from the fore- and hind-limb muscle of each animal, the only significant difference being in the IP and CP fraction. The fore limb has a mean IP of 9·47 μmoles/g. and a mean CP of 16·33 μmoles/g., and the hind limb has values of 11·33 and 14·98 μmoles/g. for IP and CP respectively. This is doubtless because the fore limb was always sampled first, the CP in the hind limb probably breaking down to a greater extent *in situ* during the removal procedure.

For the estimation of DPNH the muscle samples were extracted with perchloric acid at room temperature to convert DPNH into a compound which is stable towards acid. Otherwise DPNH is slowly transformed during the chromatographic separation of the nucleotides in the *iso*butyric acid solvent and gives rise to two spots with trailing between them. The procedure is otherwise unaltered. It was found that elevated IP (13·6 μmoles/g.) and lowered CP (11·34 μmoles/g.) values were obtained for normal muscle by this modification. The values for the nucleotides were little altered by running the chromatograms at right angles in the acetone–water system of Rafter, Chaykin & Krebs (1954) or in the propanol–ammonia system used for the sugar phosphate separations, except that in the latter system the DPN is completely destroyed as would be expected from its known lability in alkaline media (Schlenk, Euler, Heiwinkel, Gleim & Nyström, 1937). The acetone–water system has been shown to separate ATP from TPN and DPNH which have the same R_F values in the *iso*butyric acid solvent system; the results indicate that TPN and DPNH are not present in any appreciable quantity in normal resting muscle.

Contrary to expectation a spot corresponding to either 2- or 3-phosphoglycerate was not found with the *tert.*-butanol–picric acid/propanol–ammonia solvent systems, although a consistently occurring unknown spot was found, second only in quantitative significance in the glycolytic intermediate fraction. Synthetic 2- or 3-phosphoglycerates added to a perchloric acid extract of muscle before manipulation did not produce a spot coincident with this unknown spot.

Glucose- and fructose-6-phosphate were not sufficiently well separated to allow their separate estimation, but with a longer run in the picric acid–*tert.*-butanol solvent system this could probably be achieved.

A spot corresponding to the position occupied by synthetic phosphopyruvic acid was not found with the *tert.*-butanol–picric acid/propanol–ammonia solvent systems.

Table 3. *Recovery of non-nucleotide phosphates in perchloric acid after passing through Norit columns*

Compounds were dissolved in 5 ml. of 3% perchloric acid and passed through the column.

	μmoles of P/5 ml. of perchloric acid	
	Before column	After column
Orthophosphate	0·72	0·72
Pyrophosphate	0·77	0·77
Creatine phosphate	0·50	0·50
Glucose 1-phosphate	0·72	0·72
Glucose 6-phosphate	0·53	0·51
Fructose 6-phosphate	0·23	0·23
Fructose diphosphate	0·39	0·37
β-Glycerophosphate	0·76	0·75
Barium 3-phosphoglycerate	0·55	0·58

Table 4. *Recovery of glycolytic intermediates after removal of IP*

Compounds were dissolved in 3% perchloric acid and 0·4 ml. of 10% ammonium molybdate (w/v) and 0·5 vol. of pentanol were added. The two phases were shaken in a separating funnel and the aqueous phase was re-extracted twice with pentanol. Excess of molybdate was removed with H_2S.

	μmoles of P/5 ml. of perchloric acid			
	Before treatment		After treatment	
Compound	Inorganic P	Organic P	Inorganic P	Organic P
Fructose 6-phosphate	8·1	1·34	0·00	1·36
Glucose 6-phosphate	8·1	1·35	0·01	1·40
Glucose 1-phosphate	8·1	1·50	0·01	1·50
Fructose diphosphate	8·1	1·50	0·00	1·46
Barium 3-phosphoglycerate	8·1	1·82	0·03	1·84
β-Glycerophosphate	8·1	1·55	0·01	1·60

DISCUSSION

The results presented indicate that 95% of the phosphorus present in a perchloric acid extract of rat-skeletal muscle can be consistently allocated among the known constituents. It is essential to estimate the CP separately in a scheme involving acid-extraction procedure and solvent systems because of its lability under these conditions.

The charcoal-column technique is based on observations of Crane & Lipmann (1953) and Fiske (1934), who showed that adenine nucleotides could be separated from IP and the glycolytic intermediates by shaking with Norit charcoal. This has proved successful in obtaining a clear-cut separation of the nucleotides present in the perchloric acid extract from the other phosphate compounds. No substances absorbing in the region 250–270 mμ. are present in the perchloric acid extract after Norit treatment, nor are non-nucleotide phosphate esters adsorbed by the Norit. It is seen in Table 2 that 100 mg. of Norit columns will retain 4–5 μmoles of nucleotides containing the adenine moiety, but that substitution of the amino group of adenine by the hydroxyl group, as in inosine monophosphate, decreases considerably the amount retained, and that uridine triphosphate, containing no amino group but two hydroxyl groups on a pyrimidine moiety, is only retained to a small extent by the Norit columns. This technique has been successfully applied to liver extracts by increasing the amounts of Norit and Celite by 50%, keeping the diameter of the column the same and by using 5 ml. of a similar extract. The increased amount of Norit was made necessary by the higher concentrations of hydroxylated nucleotides found in liver.

The absence of AMP in muscle agrees with the recent findings of Mommaerts (1955) and indicates that in resting muscle all the adenine mononucleotide occurs as either the di- or tri-phosphate.

The absence of a spot corresponding to phosphoglycerate and the appearance of another spot not co-chromatographed with any of the glycolytic intermediates used is not readily explained, but Caldwell (1953) has shown that in the propanol–ammonia system phosphoglycerate has an R_F value of 1·7 relative to IP, which compares well with that found for the unknown spot. Also, LePage (1948) has reported the presence of phosphoglycerate in

Table 5. *Precipitation of phosphate esters with baryta*

The compounds were dissolved in 10 ml. of 3% perchloric acid. Solid baryta was added until the solution was pink to phenolphthalein and followed by 4 vol. of ethanol. The solution was set aside for 24 hr. at −15°, centrifuged at −5°, treated with IR-120 (H) and adjusted to 10 ml.

	μmoles of P/10 ml. of perchloric acid	
	Before treatment	After treatment
β-Glycerophosphate	0·82	0·86
Fructose diphosphate	0·94	0·90
Glucose 6-phosphate	1·21	1·24
Barium 3-phosphoglycerate	1·90	1·80
Fructose 1-phosphate	1·13	1·12
Glucose 1-phosphate	1·32	1·34
Mixture of above compounds	3·48	3·48

Table 6. *Distribution of phosphorus compounds in perchloric acid extract of rat-skeletal muscle*

Values (μmoles of P/g. wet wt.) are means ± standard error of mean. Number of observations in each case is shown in parentheses.

	Determined constituent	Calculated totals	Determined total
(1) Determined immediately			
IP	10·4 ±0·35 (20)	25·9	—
CP	15·5 ±0·29 (20)		
(2) Pyridine eluate			
ATP	18·52±0·26 (20)	22·28	22·0±0·40 (14)
ADP	2·10±0·45 (20)		
DPN	1·14±0·03 (20)		
Guanosine and uridine triphosphates	0·52±0·03 (20)		
(3) Perchloric acid extract after Norit–Celite column			
Fructose diphosphate	0·45±0·03 (19)	29·26	30·0±0·43 (15)
Glucose 6-phosphate + fructose 6-phosphate	1·83±0·15 (20)		
Glucose 1-phosphate	0·38±0·04 (11)		
Phosphoglycerate*	0·70±0·14 (20)		
IP + CP	25·9±0·45 (20)		
(4) Total phosphorus	53·6±0·71 (15)	51·54 (by addition of (2) and (3))	52·0

* Not positively identified: see text.

Table 7. *Comparison of results obtained with those of LePage* (1948)

	Present results (μmoles/g. wet wt.)	Results of LePage (μmoles/g. wet wt.)
Total acid-soluble P	53·6	50·7
IP	10·4	7·48
CP	15·48	16·30
ATP	6·17	5·42
ADP	1·05	0·59
AMP	0	1·55
DPN	0·59	0·17
Guanosine and uridine triphosphates	0·47	0
Glucose 6-phosphate + fructose 6-phosphate	1·83	2·83
Glucose 1-phosphate	0·38	0·80
Fructose diphosphate	0·23	0·14
Phosphoglycerate*	0·70	1·40
Ribose 5-phosphate	0	0·22

* See text.

rat-skeletal muscle, and it seems possible therefore that this unknown spot is phosphoglycerate and that the compounds used may not have been the naturally occurring acids.

Comparison of results found here with those of LePage (1948) is made in Table 7. There is good agreement between the two differing methods. LePage's technique cannot differentiate between the various nucleotide di- and tri-phosphates or between inosine monophosphate and AMP, both of which can occur in muscles under pathological conditions. LePage gives concentrations of AMP and ribose 5-phosphate which would easily have been detected by the present method if present. In normal muscle the amount of nucleotides other than the adenine nucleotides is small, but in other organs such as liver this is not so; for analysis in such cases the procedure outlined is superior.

SUMMARY

1. A method is presented for the quantitative estimation of 95% of the phosphate compounds present in a perchloric acid extract of tissues.

2. The nucleotide components are adsorbed on Norit charcoal and eluted with aqueous pyridine before their separation by paper chromatography.

3. After removal of the inorganic phosphorus and creatine phosphate-phosphorus as the phosphomolybdic acid complex, the glycolytic intermediates are precipitated as barium salts. After removal of barium with cation-exchange resin the solutions are lyophilized and the compounds separated by paper chromatography.

4. Results are presented showing the application of this technique to rat-skeletal muscle.

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