and precipitation by ethanol cause degradation, while ultrafiltration does not.

2. The course of purification by ultrafiltration has been followed. It has been confirmed that the protein content of the complex is reduced to a limiting value of 25 %. Electrophoresis is shown not to give products of lower protein content.

3. Analysis of the product of ultrafiltration shows that 93 % of its weight is accounted for by acetyl hexosamine, hexuronic acid, protein and ash. The protein content of the complex is found to be lower than was previously reported.

4. A revised value is given for the composition of mucin, prepared on a small scale.

5. Degradation by hyaluronidase has been shown to leave a considerable fraction of the material in a polymerized state (molecular weight of the order of 10^5).

6. The ultracentrifugal sedimentation of purified hyaluronic acid complex has been re-investigated.

7. Slight modifications of the values of physical quantities are given, based on the revised analytical values and sedimentation constant.

8. Our conclusions about the composition and molecular configuration of the hyaluronic acid complex are summarized.

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Note added in proof. Since this paper was sent in, some of our preparations have been examined by Dr R. Consden, who finds that small amounts of neutral sugars are consistently present.

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Separation of Adenosine Phosphates by Paper Chromatography and the Equilibrium Constant of the Myokinase System

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This paper describes a procedure for the quantitative separation by paper chromatography of adenosinetriphosphate (ATP), adenosinediphosphate (ADP), adenosinemonophosphate (AMP) and inorganic orthophosphate. The technique is a development of those of Hanes & Isherwood (1949) which are satisfactory for many purposes, but are inadequate for the complete separation of ADP and inorganic phosphate. The formic acid *iso*propyl ether solvent of Hanes & Isherwood separates satisfactorily the three adenosine phosphates from inorganic phosphate and the *iso*propanol ammonia solvent separates ATP from ADP, AMP and inorganic phosphate. The imperfect separation of ADP and inorganic phosphate is especially evident when the mixture of phosphates contains $KH_2{}^{32}PO_4$. The latter can be detected by radioactivity when chemical methods are not sufficiently sensitive. Attempts to remove inorganic orthophosphate by precipitation as the magnesium ammonium salt lead to appreciable and variable losses of ATP and ADP by adsorption on to the precipitate.

Satisfactory separation of adenosine phosphates from other phosphates is achieved by a procedure which might be described as a 'two-solvents one-dimensional chromatography'. Two different solvents are used successively to develop the material in the same direction, the first ascending and the second descending on the reversed strip. This technique has the advantage over two-dimensional paper chromatography (of which it is a modification) that smaller pieces of paper are required and that a greater number can therefore be handled simultaneously. A further point is the greater compactness of the developed spots.

The method has been applied to the measurement of the purity of adenosine phosphate preparations and of the equilibrium in the myokinase system.

EXPERIMENTAL

Methods

ATP and ADP. Samples of ATP and ADP prepared from rabbit muscle (see LePage, 1949) and various commercial preparations were used. The barium compounds were dissolved in dilute HCl and barium was precipitated with the calculated quantity of Na_2SO_4. After removal of the precipitate by centrifugation the solutions were neutralized with NaOH. About 15–20% of the ATP was lost by adsorption on $BaSO_4$ (see Bailey, 1949). Calcium salts were treated similarly, sodium oxalate replacing sulphate.

Preparation of filter papers. Spots were more compact, and less 'trailing' occurred after treatment of the paper with ethylenediaminetetraacetic acid (which is expected to remove traces of metal impurities, see Walker & Warren, 1951). Whatman no. 1 papers, 45 × 19 cm., were immersed for 30 min., ten at a time, in a Perspex trough (22 × 60 × 5·5 cm.) in 2 l. 0·2% ethylenediaminetetraacetic acid, neutralized with NaOH to about pH 8·5. The trough was gently rocked at intervals. This was followed by six immersions, of 10 min. each, in 2 l. distilled water. The papers were then dried in a current of hot air.

An 'Agla' micrometer syringe (Burroughs Wellcome and Co.) was used to place 5 μl. ($\pm$0·005 μl.) quantities of the solution to be analysed, 4 cm. apart, on a line 8 cm. from one end of the paper. The spots were dried in a current of warm air from a hair dryer, care being taken to avoid a rise in temperature at the paper surface above 30°. Up to four additional 5 μl. quantities of the solution were added to the dry spot if necessary in order to accumulate 3–10 μg. P. The dry papers were bent at right angles along a line 6 cm. from the end, i.e. 2 cm. from the line of spots, and the other end of the paper was bent at right angles 2 cm. from the end. The 6 cm. margin was required for the second stage of the chromatography.

Preparation of biological material. Tissue extracts or suspensions were deproteinized at 0° by the addition of 0·1 vol. 30% (w/v) trichloroacetic acid and centrifuged at 2–4°. There were no changes in the ADP or ATP content of the supernatant solution after several weeks' storage at − 14°. It was found to be unnecessary to neutralize these solutions for chromatography.

Chromatography. Chromatography was carried out in glass tanks of 40 cm. height and a base area of 30 × 22 cm., fitted with a plate-glass lid. (These are manufactured as jars for stationary accumulators and are available from Oldham and Son Ltd., Derbyshire House, St Chad's Street, London, W.C. 1.) Those used for descending chromatography were equipped with a glass-rod framework supporting two three-quarter cylindrical glass troughs, about 28 cm. long and capable of holding about 70–80 ml. solvent. A glass rod in the trough held the papers down in the solvent.

Two solvents recommended by Hanes & Isherwood (1949), one slightly modified by the addition of ethylenediaminetetraacetic acid, were used: (1) acid solvent consisting of 90 ml. *iso*propyl ether and 60 ml. 90% (v/v) formic acid; (2) alkaline solvent consisting of 240 ml. *n*-propanol, 120 ml. ammonia (sp.gr. 0·880) and 40 ml. 0·002 M-ethylenediaminetetraacetic acid.

The papers were fastened to the plate-glass lid with a strip of adhesive cellulose tape and were allowed to hang in the tank containing a 1–2 cm. layer of the acid solvent, the line of spots being within 2–3 cm. of the surface of the solvent. Up to eight papers could be placed in one jar simultaneously. The solvent was allowed to ascend for 3–4 hr. at 22° without preliminary equilibration, which proved unnecessary. After removal from the tank the papers were dried at room temperature in a fume cupboard by a current of air. The dried papers were serrated with scissors at the upper end and were placed in the reversed position into tanks fitted for descending chromatography. A shallow layer of the alkaline solvent was placed in the bottom of the tank and about 75 ml. into the trough. Four papers could be run simultaneously. The solvent was allowed to descend for 27–30 hr. at 28°. Preliminary equilibration proved again unnecessary. At the end of the run the papers were dried in a current of warm air.

Location of spots. Purines and pyrimidines were located by ultraviolet photography according to Markham & Smith (1949). Phosphate compounds were located by spraying with the molybdate reagents of Hanes & Isherwood (1949), followed by heating for 7 min. at 85° and exposure to H_2S.

Chemical analyses. For the quantitative determination of P an area of the filter paper containing the spots (4–10 sq.cm.) was cut out and wet ashed with 0·5 ml. of a mixture of H_2SO_4 (3 vol.) and $HClO_4$, sp.gr. 1·54 (2 vol.). P was determined in the ashed material by the method of Berenblum & Chain (1938) and colour intensities were measured in a Beckman spectrophotometer at 750 mμ. Some commercial samples of *iso*butanol required for the extraction were found to contain aldehydes and these were found to modify the colour from blue to greenish blue. *iso*Butanol was purified by shaking it with a solution of 2:4-dinitrophenylhydrazine in 10% (w/v) H_2SO_4 and distilling through a fractionating column. Paper blanks gave values of 0·01–0·04 μg. P/sq.cm. The lower limits of the visual detection of molybdate-sprayed spots were of the order of 0·07 μg. P/sq.cm. The ultraviolet technique for the detection of purines was more sensitive, the lower limit being 0·25 μg./sq.cm. adenylic acid, equivalent to about 0·02 μg. P/sq.cm.

To confirm the identity of adenosine phosphates determinations of ribose were made on the spots located in ultraviolet light. After elution of the cut-out paper with 4 ml. water for 1 hr. at 37° a 3 ml. sample was treated with orcinol according to Meijbaum (1939). Paper treated with two solvents gave blanks equivalent to 0·3–0·6 μg. 'ribose'/sq.cm. This was higher than the blanks for untreated paper.

Adenine was not determined. The method of Cohn & Carter (1950) is not applicable to paper containing ethylenediaminetetraacetic acid. Paper blanks were carried out in all determinations on a relatively large piece usually about 15 sq.cm.

Radioactivity measurements. In some experiments solutions containing ^{32}P were analysed. The radioactivity was measured after wet ashing as described above and dilution to 10 ml. A β-counting tube, liquid type M6 of 20th Century Electronics, was used. Small quantities of orthophosphate contaminating the ADP spot can be detected by radioactivity when chemical methods fail.

Separation of phosphates

A diagrammatic representation of the chromatographic behaviour of various physiological phosphates is shown in Fig. 1. Strip *a* shows the separation of the three adenosine phosphates and orthophosphate by the new method. Strip *b* shows the behaviour of five other phosphates under the same conditions. It will be seen that the spots of 3-phosphoglyceric acid, glucose-1-phosphate and glucose-6-phosphate, inorganic pyrophosphate, fructose-1:6-diphosphate overlap partly with the adenosine phosphate spots and these substances may therefore interfere with the analysis of adenosine phosphates. The presence of these interfering substances can be detected by the ascending chromatogram in the acid solvent as shown on strip *c*. In the experiments reported in this paper the interfering substances were not present in significant quantities with the exception of pyrophosphate which overlaps with the ADP spot. The difficulty arising from the presence of pyrophosphate was overcome by separating pyrophosphate with the acid solvent, determining its quantity and making a correction in the analysis of the combined ADP plus pyrophosphate spot.

When both solvents were applied by the descending technique separation was incomplete and $KH_2{}^{32}PO_4$ was found to trail through the adenosine polyphosphate area.

Constancy and control of temperature is essential for satisfactory separation. At lower temperatures the development of the chromatogram is delayed.

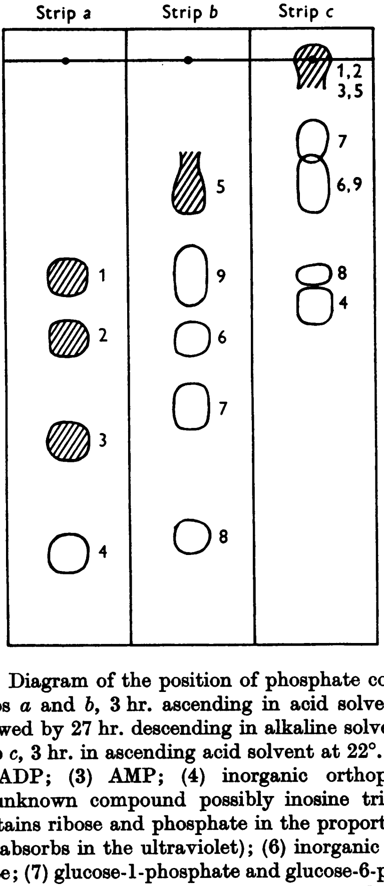

Fig. 1. Diagram of the position of phosphate compounds. Strips *a* and *b*, 3 hr. ascending in acid solvent at 22°, followed by 27 hr. descending in alkaline solvent at 28°. Strip *c*, 3 hr. in ascending acid solvent at 22°. (1) ATP; (2) ADP; (3) AMP; (4) inorganic orthophosphate; (5) unknown compound possibly inosine triphosphate (contains ribose and phosphate in the proportion of 1:3 and absorbs in the ultraviolet); (6) inorganic pyrophosphate; (7) glucose-1-phosphate and glucose-6-phosphate; (8) 3-phosphoglyceric acid; (9) fructose-1:6-diphosphate. Compounds which absorb in the ultraviolet are shaded.

Table 1. *Analysis of ATP and ADP preparations by 'two-solvent-one-dimensional' chromatography*

(Technique as described in text; 3–5 μl. of an 0·02 M solution of the Na salts were analysed; 'laboratory' preparations according to LePage, 1949.)

		Distribution of total P in the final solution (%)					
Preparation	Storage where known	ATP	ADP	AMP	Inorganic pyrophosphate	Orthophosphate	Other compounds
Barium ATP, commercial	—	42·5	39·9	0	7·3	0	10·3
ATP, free acid, commercial	5 months at 6°	68·8	21·8	3·7	0	0	5·7
Barium ATP, laboratory	8 months at − 14°	86·0	11·0	0	0	3·0	0
Calcium ATP, commercial	2 weeks at 6°	32·6	24·3	9·6	7·4	16·2	9·9
ATP solution, commercial	—	30·1	24·6	0·5	0	28·2	16·6
Barium ATP, commercial	—	72·0	24·0	0	0	4·0	0
Barium ADP, laboratory	None	27·2	59·4	2·3	0	0	11·1
Barium ADP, labroatory	None	48·0	52·0	0	0	0	0
Barium ADP, laboratory (first Ba precipitation)	None	34·0	42·7	5·8	7·2	4·5	5·8

Concentrated extracts of biological materials sometimes contained sufficient Mg and Ca to give an additional inorganic phosphate spot located between ADP and AMP when run with the ammonia solvent. This has been prevented by the addition of 5 µl. 0·02 M-ethylenediaminetetraacetic acid to the spot before final drying.

Analysis of ATP and ADP preparations

None of the preparations tested, as will be seen from Table 1, was pure. All ATP preparations were contaminated with ADP and all ADP preparations with ATP. In the best preparations of ATP 11 % of the total P was ADP-P and 3 % inorganic phosphate. The samples of ADP were prepared by the action of adenosinetriphosphatase from pigeon-breast muscle or rat-leg muscle on ATP. The difficulty of complete hydrolysis of ATP has been noted by Bailey (1949).

Separation of radioactive inorganic phosphates

Table 2 shows the result of an experiment in which $KH_2{}^{32}PO_4$ was separated from the three adenosine phosphates. It will be seen that 99 % of the radioactivity was recovered in the orthophosphate spot, the traces found elsewhere being negligible. This degree of separation is satisfactory for experiments in which the incorporation of radioactive phosphate into adenosine phosphates is studied.

Equilibrium constant in the myokinase system

Rough data published by Kalckar (1943) give values of 0·37 and 0·25 for the equilibrium constant K of the myokinase system, at 30° and pH 7·5:

$$\frac{[\mathrm{ATP}^{4-}]\,[\mathrm{AMP}^{2-}]}{[\mathrm{ADP}^{3-}]^2}=K.$$

The methods described in this paper permit a more accurate measurement of the equilibrium constant than was previously possible. The constant was determined because its value is of general interest in connexion with the free energy of hydrolysis of the two pyrophosphate bonds of ATP.

Table 2. *Separation of isotopic orthophosphate from a mixture of adenosine polyphosphates*

(The sample analysed contained 2–10 µg. P of each of the four phosphate compounds. The data refer to the radioactivity found on various areas of a chromatogram paper after running with acid and alkaline solvents consecutively.)

Area of paper	Radioactivity (counts/min.)	Percentage of total radioactivity on the paper
ATP	4	0·05
ADP	12	0·15
AMP	48	0·6
Orthophosphate	7705	99·0
Rest of paper	20	0·2

Myokinase was prepared according to Kalckar (1943), but pigeon-breast muscle was used instead of rabbit muscle.

Table 3. *Myokinase equilibrium at* 25° *and* pH 7·4

(For experimental conditions see text.)

Exp. no.	Period of incubation (min.)	Concentration ($\times 10^{-3}$ M) of ATP	ADP	AMP	$K=\frac{[\mathrm{ATP}]\,[\mathrm{AMP}]}{[\mathrm{ADP}]^2}$
1	0	2·04	0·39	2·96	—
	20	1·14	2·28	2·04	0·446
	60	1·10	2·27	2·08	0·445
	90	1·14	2·26	1·92	0·430
2	0	2·30	0·71	2·06	—
	30	1·54	2·26	1·42	0·428
	90	1·54	2·23	1·28	0·398
3	0	2·68	0·84	4·50	—
	20	1·51	3·22	3·31	0·484
	30	1·48	3·16	3·31	0·490
	40	1·45	3·19	3·29	0·469
	50	1·51	3·32	3·38	0·458
	60	1·55	3·22	3·31	0·498
4	0	2·07	3·90	1·07	—
	50	2·48	3·12	1·57	0·400
	60	2·48	3·09	1·53	0·398
5	0	3·47	5·23	0	—
	20	4·48	3·28	1·06	0·441
	30	4·46	3·30	1·06	0·434
	40	4·50	3·27	1·06	0·446
	60	4·46	3·29	1·06	0·438
				Average	**0·444**

To avoid the presence of inorganic phosphate triethanolamine was used as a buffer. The 0·1 M buffer stock solution of pH 7·4 contained 10 ml. 1 M-triethanolamine and 6·8 ml. 1 N-HCl in 100 ml. A set of parallel tubes was incubated, each containing 0·15–0·6 mg. enzyme protein in a total volume of 2 ml. The final concentration of the sum of the adenosine phosphates was $5\text{–}10 \times 10^{-3}$ M, of $MgCl_2$ 0·01 M and of triethanolamine buffer 0·02 M. The exact quantities of the initial amounts of the adenosine phosphates are given in Table 3. At intervals samples were deproteinized with 0·2 ml. 30% (w/v) trichloroacetic acid and centrifuged at 2–4°. 15–25 μl. of the supernatants were used for chromatography. All analyses were carried out in duplicate which generally agreed within 3–4%, except in the AMP determination of Exp. 5 when the P values were very low. In this case the figure given is the average of eight determinations, the range being $0{\cdot}94\text{–}1{\cdot}18 \times 10^{-3}$ M.

It will be seen that, irrespective of the initial proportions of the adenosine polyphosphates, approximately the same value was obtained for the equilibrium constant, the average being 0·444. Thus the free energy liberated in the hydrolysis of the terminal pyrophosphate group under standard conditions is 480 cal. greater than that of the second pyrophosphate group.

DISCUSSION

Cohn & Carter (1950) separate adenosine phosphates by ion-exchange resins, which leads to large volumes of fluid which are inconvenient for the determination of phosphate and ribose. Alternatively, they use paper chromatography with solvents containing phosphate which make the simultaneous determination of adenosine phosphate and inorganic phosphate impossible. Two other methods were published after the completion of the present work. Bock & Alberty (1951) separate adenosine phosphates by a moving boundary method in the Tiselius apparatus, and Caldwell (1952) uses two-dimensional paper chromatography. The present method is simpler than these.

SUMMARY

1. A method for separating adenosinetriphosphate, adenosinediphosphate, adenosinemonophosphate and inorganic phosphate by paper chromatography is described. Two different solvents are used successively to develop the spots in the same direction.

2. The method has been applied to the analysis of adenosine phosphate preparations and to the determination of the equilibrium constant of the myokinase system. The value of K (as defined on p. 159) was found to be 0·444.

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