200. *The Separation and Characterisation of the 2′ : 5′- and 3′ : 5′-Diphosphate and the 2′ : 3′ : 5′-Triphosphate of Adenosine.*

By J. Baddiley, J. G. Buchanan, and R. Letters.

The 2′ : 5′- and 3′ : 5′-diphosphate of adenosine have been separated from each other by paper chromatography and by ion-exchange chromatography, employing concave gradient elution with calcium chloride and hydrochloric acid. The phosphates were characterised by enzymic and chemical hydrolysis.

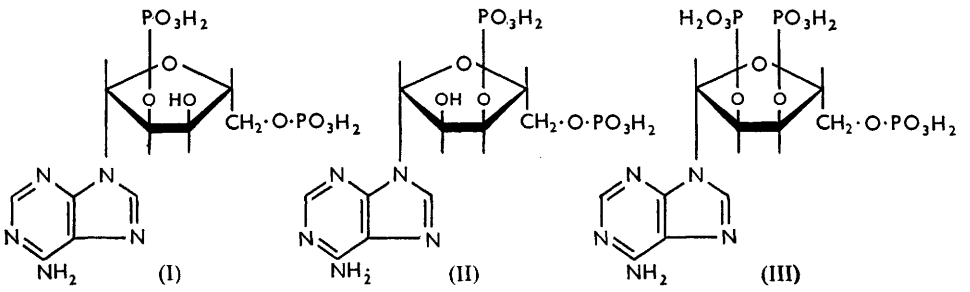
The value of hydrolysis at pH 4 for the location of phosphate groups in polyphosphates is discussed.

Adenosine-2′ : 3′ : 5′ triphosphate has been prepared by phosphorylation of adenosine with an excess of dibenzyl phosphorochloridate, followed by removal of protecting groups. The action of phosphatases on this ester has been studied.

The isomeric diphosphates of adenosine are important structural units in several nucleotide coenzymes. The adenosine-2′ : 5′ diphosphate (I) structure occurs in triphosphopyridine nucleotide, whereas the isomeric 3′ : 5′-diphosphate (II) is a component of coenzyme A and "active sulphate". Although unequivocal syntheses of these diphosphates have not been achieved, their structures have been established by enzymic methods.[1] A mixture

[1] Wang, Shuster, and Kaplan, *J. Biol. Chem.*, 1954, **206**, 299.

containing the two isomers has been obtained by phosphorylation of adenosine with an excess of dibenzyl phosphorochloridate followed by removal of benzyl groups.[2] Todd and his collaborators were unable to resolve this mixture, but its value in the synthesis of certain nucleotide coenzymes was indicated. It was suggested that most of the known methods for the synthesis of pyrophosphates from such a phosphate would inevitably cause migration of the phosphate at the 2′- or 3′-position, and subsequent steps would always produce a mixture of phosphates.



In a recent synthesis of "active sulphate" (adenosine 3′-phosphate 5′-sulphatophosphate) we showed that adenosine-3′ : 5′ diphosphate (II) (isolated from natural sources) reacts with the pyridine–sulphur trioxide complex in a buffer solution to give the desired 3′-phosphate 5′-sulphatophosphate.[3] Although some cyclisation of the phosphate at the 3′-position occurred the two products were separated from each other quite readily by paper chromatography or by electrophoresis. It is unlikely that phosphate migration would occur in such a synthesis. It follows therefore that a laboratory-scale separation of the isomeric diphosphates of adenosine, although it may present great difficulty, would be of value for synthetic work. Such a separation would also provide an alternative to the enzymic identification of these compounds in studies on the structure of nucleotide coenzymes.

A method has been developed in these laboratories for the resolution of complex mixtures of nucleotides by ion-exchange chromatography on Dowex-1 resin of low cross-linkage, employing an eluting solution of calcium chloride in an apparatus which ensures a continuous, concave concentration gradient.[4] This method, which is particularly effective in the separation of the more acidic nucleotides, has the additional advantage of ease of isolation of the pure products. Evaporation of solvent from the eluate, and addition of an alcohol–ether mixture to the residue, yields the calcium salts of the nucleotides as insoluble precipitates. The technique has now been applied successfully to the separation and isolation of the isomeric adenosine diphosphates.

The synthesis of the mixed diphosphates was carried out by a method similar to that used by the Cambridge workers, and we are grateful to them for information in advance of publication. They found it necessary to remove benzyl groups and destroy cyclic phosphates in the neutral ester mixture in three separate operations, first by anionic debenzylation with lithium chloride, then by catalytic hydrogenolysis, and finally by alkaline hydrolysis. We have confirmed this observation and simplified the preparation by reversal of the last two stages. In this way the hydrogenolysis occurs more smoothly.

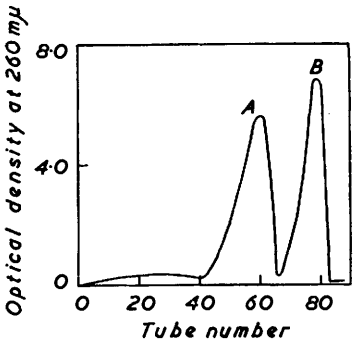
Separation of products was first attempted by chromatography on Dowex-2 resin in the formate form, resolution being achieved by concave gradient elution with ammonium formate. The 2′ : 5′- and the 3′ : 5′-diphosphate were not completely separated from each other by this method and reproducibility was not good. Excellent separation

[2] Cramer, Kenner, Hughes, and Todd, *J.*, 1957, 3297.
[3] Baddiley, Buchanan, and Letters, *Proc. Chem. Soc.*, 1957, 147.
[4] Pontis and Blumsom, in the press.

occurred (see Fig. 1) on the chloride form of Dowex-1, by concave gradient elution with a solution of calcium chloride and hydrochloric acid. A concentration range from 0·03M-calcium chloride and 0·0045N-hydrochloric acid, up to 0·07M and 0·007N respectively, was chosen. The diphosphates were isolated from appropriate fractions as their calcium salts. Adenosine-2′ : 5′ diphosphate was eluted from the column in advance of the 3′ : 5′-diphosphate. This was in agreement with the order of elution of monophosphates of adenosine, where the 2′-phosphate precedes the 3′-phosphate.[5]

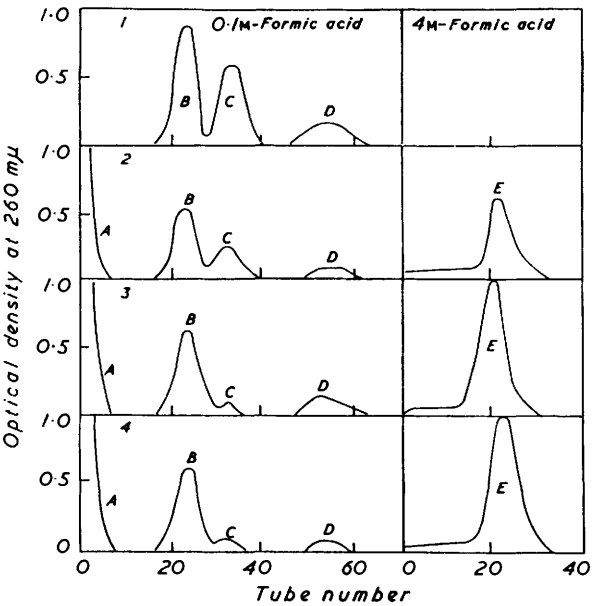
The diphosphates were characterised by their behaviour towards hydrolysis with a specific phosphatase from germinating rye grass. This enzyme, which was purified by Shuster and Kaplan's method,[6] is known to hydrolyse a phosphate group at the 3′-position

FIG. 1. *Ion-exchange separation of adenosine-2′ : 5′ and -3′ : 5′ diphosphates.*



A, Adenosine-2′ : 5′ diphosphate.
B, Adenosine-3′ : 5′ diphosphate.

FIG. 2. *Ion-exchange analysis of products from hydrolysis of nucleotides at* pH 4.



1, *Authentic nucleotides for calibration of column.* 2, *Hydrolysate of adenosine-2′ : 5′ diphosphate.* 3, *Hydrolysate of adenosine-3′ : 5′ diphosphate.* 4, *Hydrolysate of adenosine-2′ : 3′ : 5′ triphosphate.*
A, Adenosine. B, Adenosine-5′ phosphate. C, Adenosine-2′ phosphate. D, Adenosine-3′ phosphate. E, Adenosine diphosphates.

in adenosine and its phosphates, but is without action on phosphate groups in other positions. In accordance with the earlier observations, it was shown that adenosine-3′ : 5′ diphosphate (II) was readily hydrolysed by this enzyme to adenosine-5′ phosphate and inorganic phosphate, whereas the 2′ : 5′-diphosphate (I) was unaffected (see Table 1). Our enzyme preparation contained a trace of a nucleoside-5′ phosphatase, and consequently a very small amount of hydrolysis of the adenosine-5′ phosphate, liberated from the 3′ : 5′-diphosphate, was observed.

An alternative proof of structure of the diphosphates was obtained by partial hydrolysis at pH 4. The mechanism of phosphate hydrolysis at this pH differs from that in stronger acid and is known to involve phosphorus–oxygen fission.[7] It follows that hydrolysis at

[5] Cohn, *J. Amer. Chem. Soc.*, 1949, **71**, 2285.
[6] Shuster and Kaplan, *J. Biol. Chem.*, 1953, **201**, 535.
[7] Desjobert, *Compt. rend.*, 1947, **224**, 575; *Bull. Soc. chim. France*, 1947, **14**, 809; Butcher and Westheimer, *J. Amer. Chem. Soc.*, 1955, **77**, 2420.

the higher pH, although quite rapid, should be accompanied by little or no phosphate migration even in those esters which contain an adjacent hydroxyl group. The validity of these views is illustrated by the behaviour of glycerophosphates [8] and other polyol phosphates [9] towards hydrolysis at pH 4. In both cases removal of phosphate occurs readily without migration.

In order to evaluate hydrolysis at pH 4 as a method for determining the structure of nucleotides, controlled conditions were employed with the 2′- and 3′-phosphate of adenosine. After 4 hr. at 100° in an ammonium formate buffer (pH 4) the products from the two nucleotides were examined by ion-exchange chromatography. Previously calibrated columns were used under standard conditions for the separation and quantitative determination of the three isomeric monophosphates. These experiments (see Table 2) showed that substantial hydrolysis can be effected with little migration. Approximately 46% of the adenosine-3′ phosphate was hydrolysed to adenosine, only 4% being converted into the 2′-phosphate. Adenosine-2′ phosphate was slightly more labile than the 3′-isomer. Under comparable conditions about 52% of this isomer was hydrolysed to adenosine, and 7% was converted into the 3′-phosphate. Although the differences in rates of hydrolysis and migration of the two isomers are not large, they are probably significant.

This method of analysis was applied to hydrolysates of adenosine-2′ : 5′ and -3′ : 5′ diphosphate. The results (Table 2, Fig. 2) show that phosphate groups at positions 2′ and 3′ are more labile than that at 5′. Slight migration of phosphate between the 2′- and 3′-position was observed, and it is noteworthy that migration from 2′ to 3′ is somewhat greater than that in the reverse direction. The amount of migration which occurs at pH 4 is slight in all cases and in no way invalidates structural determinations based on such hydrolyses. Adenosine-2′ : 5′ diphosphate gave adenosine-2′ and -5′ phosphate with a trace of the 3′-phosphate (about 3%). Similarly, the 3′ : 5′-diphosphate gave the 3′- and 5′-phosphate, and very little 2′-phosphate. No evidence was obtained for the presence of adenosine-2′ : 3′ diphosphate in the nucleoside diphosphate preparations under examination. It would be unnecessary normally to carry out an ion-exchange separation of products, since the characterisation of these nucleoside diphosphates can be effected rapidly and accurately from the qualitative information given by paper chromatography of the hydrolysates.

From the experiments on nucleotides described here, and from similar investigations on polyol phosphates, it seems that hydrolysis at pH 4 can be of considerable value in structural determination. The outstanding advantage of this over many other hydrolytic methods is the comparatively small amount, or even complete absence, of group migration. The ready hydrolysis of glycerophosphates and their derivatives at pH 4 has been known for a considerable time, and the absence of phosphate migration under these conditions is also well known. However, even in the phospholipid field, only occasional use has been made of these facts. It is interesting that, although no migration of the phosphate group was observed with ribitol 1(5)-phosphate at pH 4, a small amount of migration occurred under comparable conditions with adenosine-2′ and -3′ phosphate, and with the diphosphates. Migration was detectable even at pH 6 with these nucleotides. We conclude that the extremely favourable steric disposition of the phosphate and adjacent hydroxyl group in the ribofuranose ring of nucleotides accounts for these differences. It has been suggested that the conjugate acid which occurs as an intermediate in the hydrolysis of phosphoric esters in strongly acidic solution is also an intermediate in phosphate migration.[9,10] The small amount of phosphate migration which occurs with nucleotides at pH 4 may be related to the concentration of conjugate acid at that pH. It should be noted that the presence of a hydroxyl group in very close proximity to the

[8] Bailly, *Bull. Soc. chim. France*, 1942, **9**, 340.
[9] Baddiley, Buchanan, and Carss, *J.*, 1957, 4058.
[10] Brown, Magrath, Neilson, and Todd, *Nature*, 1956, **177** 1124.

phosphoric ester linkage is likely to enhance the amount, and modify the structure, of the conjugate acid present at relatively high pH values.

Todd and his collaborators [2] were unable to detect a triphosphate of adenosine in the mixture obtained by phosphorylation of the nucleoside with an excess of dibenzyl phosphorochloridate. In our hands, however, the method gave appreciable amounts of adenosine-2′ : 3′ : 5′ triphosphate (III). This compound was eluted from an ion-exchange column after the diphosphates, and had a lower R_F than these on paper chromatograms run with basic solvent mixtures. It had an absorption maximum at 260 mμ and was deaminated with nitrous acid to inosine-2′ : 3′ : 5′ triphosphate. This demonstrated the absence of a phosphate group on the 6-amino-substituent of the adenine residue. The unlikely possibility of the presence of a 5′-pyrophosphate group was eliminated by the failure of *Crotalus atrox* venom pyrophosphatase to attack the triphosphate. The crude venom is known to hydrolyse pyrophosphates, and it has been shown that the 5′-phosphatase in a similar rattlesnake venom is without action on nucleoside-5′ phosphates which bear a substituent at the 2′- or 3′-position.[11] The triphosphate was also unaffected by the 3′-phosphatase from rye grass. It seems that substitution of all hydroxyl groups in the ribose residue of a nucleoside by phosphoric ester groups prevents the action of both 3′- and 5′-phosphatase.

The triphosphate was hydrolysed at pH 4 under conditions comparable to those used with the diphosphates. Paper chromatography showed that all the triphosphate had been hydrolysed. The principal products were the diphosphates and adenosine-5′ phosphate. Smaller amounts of adenosine-2′ and -3′ phosphate were detected; these were present in approximately equal proportion. Although quantitative results were not obtained, it is clear that the triphosphate is hydrolysed at pH 4 in the manner expected from the results described on the mono- and di-phosphates of adenosine.

Experimental

Adenosine-2′(3′) : 5′ Diphosphate and Adenosine-2′ : 3′ : 5′ Triphosphate.—The method used is essentially that described by Cramer *et al.*[2] but with certain modifications.

Adenosine (5 g.; dried at 110°/1 mm.) was dissolved in boiling anhydrous pyridine (250 ml.). The solution was cooled to −40° (without solidifying) and added to dibenzyl phosphorochloridate (from 26 g. of dibenzyl phosphite). The mixture was allowed to warm slowly to 0°, and kept at this temperature for a further 18 hr. before addition of 2-ethoxyethanol (13 ml.). The pyridine hydrochloride, which had separated within 30 min., was collected and the filtrate was evaporated. After addition of 2-ethoxyethanol (50 ml.) and subsequent evaporation, the syrup was dissolved in ethoxyethanol (100 ml.) containing anhydrous lithium chloride (12 g.), and kept at 100° for 3 hr. with exclusion of moisture. Addition of ether (500 ml.) to the cooled solution precipitated a gum which solidified when shaken with ethanolic acetone, a cream-coloured powder (12·6 g.) being obtained. The solid was treated with N-sodium hydroxide (300 ml.) at room temperature. After 40 hr. the solution was neutralised to pH 7 with dilute hydrochloric acid, extracted with ether (2 × 50 ml.) and concentrated to 150 ml. The solution was passed through a column (20 × 5 cm.) of Dowex-50-(× 8) resin (lithium form), which was washed with water. When no more light-absorbing (at 260 mμ) material was eluted, the solution was evaporated. The residual syrup was shaken with a mixture of acetone (400 ml.) and ethanol (100 ml.), and the resulting solid was collected by centrifugation. It was washed with a second portion of ethanolic acetone, then dried. The solid (8·7 g.) was dissolved in water (150 ml.), acetic acid (2 ml.) added, and the solution hydrogenated with palladium black (from 0·2 g. of palladium chloride). After removal of catalyst, the solution [containing 3·86 g. of nucleotides calculated as adenosine-2′(3′) : 5′ diphosphate, based on optical density at 260 mμ] was passed through a column (20 × 5 cm.) of Dowex-2-(× 10) resin (chloride form) and the column was washed with water (1 l.). Monophosphates were removed by passing a solution of

[11] Kornberg and Pricer, *J. Biol. Chem.*, 1950, **186**, 557.

0·003N-hydrochloric acid (2 l.) through the column; 0·025N-acid (4 l.) removed the adenosine-2′(3′) : 5′ diphosphate. Adenosine-2′ : 3′ : 5′ triphosphate was removed (in 1·5 l.) with 0·05N-hydrochloric acid which was 0·2N with respect to calcium chloride. The solution of adenosine-2′ : 3′ : 5′ triphosphate was adjusted to pH 7 with a suspension of calcium hydroxide, evaporated to 20 ml., and freeze-dried. The resulting solid was washed with alcoholic ether (3 × 50 ml.; 1 : 1) and finally with ether (3 × 50 ml.) until free from chloride. The residue (0·8 g.) was centrifuged and dried. It was dissolved in water (50 ml.) and passed through a column (10 × 3·2 cm.) of Dowex-50-(× 8) resin (hydrogen form), which was washed with water (1 l. until the eluate was free from light-absorbing (260 mμ) material. The eluate was freeze-dried and the residue redissolved in water (5 ml.). Addition of dry acetone (50 ml.) gave a white precipitate (300 mg.) of *adenosine-2′ : 3′ : 5′ triphosphate*, $\lambda_{max.}$ 257—258 mμ (ε 14,850) (Found: C, 22·4; H, 3·7; P, 17·5. $C_{10}H_{16}O_{13}N_5P_3,2H_2O$ requires C, 22·2; H, 3·7; P, 17·1%). The adenosine-2′(3′) : 5′ diphosphate (2·1 g.) was isolated in a similar manner and compared with an authentic specimen (kindly provided by Dr. G. W. Kenner) by paper chromatography and paper electrophoresis.

Inosine-2′ : 3′ : 5′ Triphosphate.—Adenosine-2′ : 3′ : 5′ triphosphate (25 mg.) was dissolved in 2N-acetic acid (10 ml.), and a solution of sodium nitrite (1 g.) in water (4 ml.) was added dropwise. After 36 hr. at room temperature the mixture was adjusted to pH 7 with 2N-sodium hydroxide, and barium acetate (50 mg.) was added. The *barium salt* of inosine-2′ : 3′ : 5′ triphosphate (35 mg.) was collected by centrifugation, washed with a small amount of water, and dried (Found: P, 9·5. $C_{10}H_{15}O_{14}N_4P_3Ba_3$ requires P, 9·9%). The barium salt had $\lambda_{max.}$ 249 mμ (ε 10,850) and $\lambda_{min.}$ 224 mμ (ε 3570) at pH 7·5. This spectrum corresponded closely with that reported for inosine [12] [$\lambda_{max.}$ 248·5 mμ (ε 12,250) and $\lambda_{min.}$ 223 mμ (ε 3400) at pH 6]. The inosine-2′ : 3′ : 5′ triphosphate was also characterised by paper electrophoresis (see Table 4).

Separation of Adenosine-2′ : 5′ Diphosphate and Adenosine-3′ : 5′ Diphosphate.—(a) *Formate system at* pH 4. A mixture of the isomeric diphosphates (15 mg.) was absorbed on a column (45 × 1·2 cm.) of Dowex-2-(× 10) resin (formate form; 200—400 mesh). Elution was carried out with ammonium formate solution (pH 4) in an apparatus designed to give a concave concentration–volume gradient. The concentration range was from 1·5M- to 3M-ammonium formate. The isomeric diphosphates were not completely separated in this sytem, but paper chromatography showed that adenosine-2′ : 5′ diphosphate was eluted before adenosine-3′ : 5′ diphosphate.

(b) *Chloride system.* Adenosine-2′(3′) : 5′ diphosphate (40 mg.) was adsorbed on a column (60 × 1·05 cm.) of Dowex-1-(× 2) resin (chloride form; 200—400 mesh) which was then washed with water (100 ml.). Elution was carried out with calcium chloride and hydrochloric acid solutions, in an apparatus which gave a concave concentration–volume gradient, similar to that used in (a). The concentration range was from 0·03M-calcium chloride and 0·0045N-hydrochloric acid, to 0·07M-calcium chloride and 0·007N-hydrochloric acid. The isomeric nucleotides were completely separated by this system, adenosine-2′ : 5′ diphosphate being eluted first from the column. The solutions of the nucleotides were adjusted to pH 7 with a suspension of calcium hydroxide, and the calcium salts were isolated as previously described. The elution diagrams are shown in Fig. 1. The *calcium salt* (26 mg.) of adenosine-2′ : 5′ diphosphate had $\lambda_{max.}$ 258 mμ (ε 14,250) and $\lambda_{min.}$ 227 mμ (ε 2875) (Found: C, 18·9; H, 4·2; P, 9·2. $C_{10}H_{11}O_{10}N_5P_2Ca_2,8H_2O$ requires C, 18·7; H, 4·2; P, 9·6%). The *calcium salt* (19 mg.) of adenosine-3′ : 5′ diphosphate had $\lambda_{max.}$ 258 mμ (ε 14,050) and $\lambda_{min.}$ 227 mμ (ε 2850) (Found: C, 20·8; H, 3·2; P, 9·9. $C_{10}H_{11}O_{10}N_5P_2Ca_2,5H_2O$ requires C, 20·3; H, 3·55; P, 10·5%).

Action of Rye Grass Enzyme on Adenosine Phosphates.—Rye grass enzyme was prepared by Shuster and Kaplan's method [6] and purified by ammonium sulphate precipitation and adsorption on C_γ alumina. The action of the enzyme on the various nucleotides was determined, the following system being used: the nucleotide (0·4 mol.) in tri(hydroxymethylamino)methane buffer (0·2 ml.) at pH 7·5 was incubated overnight with enzyme solution (0·04 ml.) at 37°. The reaction was stopped by the addition of 20% trichloroacetic acid solution (0·2 ml.), and inorganic phosphate was determined by Fiske and Subbarow's method.[13] Control experiments were carried out on the same system to which trichloroacetic acid solution had been added at zero time. An experiment with adenosine-5′ phosphate indicated that a 5′-nucleotidase was

[12] Chargaff and Davidson, " Nucleic Acids," Academic Press, New York, Vol. I, p. 508.
[13] Fiske and Subbarow, *J. Biol. Chem.*, 1925, **66**, 375.

present in the purified preparation. The results of the enzyme experiments are shown in Table 1.

TABLE 1. *Enzymic hydrolysis of adenosine phosphates.*

Substrate	Mols. of phosphate liberated per mol. of nucleotide	Hydrolysis (% of total phosphate)
Adenosine-5′ phosphate	0·15	15
Adenosine-2′ phosphate	0	0
Adenosine-3′ phosphate	0·95	95
Adenosine-2′ : 5′ diphosphate	0	0
Adenosine-3′ : 5′ diphosphate	1·13	56·5
Adenosine-2′ : 3′ : 5′ triphosphate	0	0

Action of Crotalus atrox *Venom on Adenosine-2′ : 3′ : 5′ Triphosphate.*—Adenosine-2′ : 3′ : 5′ triphosphate (0·4 mol.) in a 0·25M-glycine–ammonia buffer (0·2 ml.; pH 9) was kept at 37° overnight. No inorganic phosphate was liberated. Under the same conditions, 1 mol. and 2 mols. of inorganic phosphate were liberated from adenosine-5′ phosphate and -5′ pyrophosphate respectively.

Anion-exchange Analyses.—In the following experiments a column (7·3 × 1·05 cm.) of Dowex-1-(× 2) resin (formate form; 200—400 mesh) was used with a flow rate of 0·3—0·5 ml./min. The same column was used throughout the investigation, without any apparent change in its adsorption characteristics. The adenosine monophosphates were eluted with 0·1N-formic acid, and the adenosine diphosphates with 4N-formic acid. Fractions (5 ml.) were collected by means of an automatic fraction collector. Nucleotides were determined quantitatively from the absorption of their solutions at 260 mμ.

Hydrolysis of Adenosine Phosphates at pH 4.—The nucleotide sample (2—3 mg.) was heated at 100° for 4 hr. with 0·4M-ammonium formate buffer (0·3 ml.; pH 4). Paper chromatographic examination of the hydrolysate with solvent systems A and B established the nature of the reaction products. Identification was confirmed and yields were determined by ion-exchange chromatography on a Dowex-1 column prepared in the above manner. The hydrolysis mixture was adjusted to pH 7·5 with N-ammonia and run on the column. Adenosine was recovered by washing the column with water (2 × 3 ml.) and nucleotides were eluted as described above. Results are given in Table 2 and Fig. 2.

TABLE 2. *Hydrolysis of adenosine phosphates at* pH 4.

		Nucleotide recovered (%) as			
	Hydrolysis to adenosine (%)	2′-phosphate	3′-phosphate	5′-phosphate	diphosphates
Adenosine-2′ phosphate	52	41	7	—	—
Adenosine-3′ phosphate	46	4	50	—	—
Adenosine-2′ : 5′ diphosphate	18	13·3	3·1	25·5	38
Adenosine-3′ : 5′ diphosphate	23·5	1	7	28	40
Adenosine-2′ : 3′ : 5′ triphosphate	21·5	2·2	2·8	23·5	50

Paper Chromatography.—Ascending-front chromatography was carried out on Whatman No. 4 paper, previously washed with 2N-acetic acid. The following solvent systems were used: (A) *n*-propyl alcohol–ammonia (*d* 0·88)–water (11 : 7 : 2); (B) saturated ammonium sulphate–0·1M-ammonium acetate–*iso*propyl alcohol [14] (79 : 19 : 2). Nucleotides were located by

TABLE 3. *Paper chromatography of nucleotides.*

	R_F in solvent A	R_F in solvent B		R_F in solvent A	R_F in solvent B
Adenosine	0·92	0·22	Adenosine-3′ : 5′ diphosphate	0·29	0·50
Adenosine-5′ phosphate	0·42	0·43	Adenosine-2′ : 3′ : 5′ triphosphate	0·14	—
Adenosine-2′ phosphate	0·51	0·39	Inorganic phosphate	0·38	—
Adenosine-3′ phosphate	0·51	0·29	Inosine-2′ : 3′ : 5′ triphosphate	0·12	—
Adenosine-2′ : 5′ diphosphate	0·29	0·57			

inspection under ultraviolet light and by the perchloric acid–molybdate spray reagent. R_F values are shown in Table 3.

[14] Markham and Smith, *Biochem. J.*, 1951, **49**, 401.

Paper Electrophoresis.—Electrophoresis was carried out for 16 hr. on Whatman No. 4 paper soaked in 0·1M-ammonium acetate buffer at pH 6, with a voltage gradient of 1 v per cm. and a current of 2·5 milliamps.

TABLE 4. *Electrophoresis of nucleotides.*

	Distance moved towards anode (cm.)		Distance moved towards anode (cm.)
Adenosine-5′ phosphate	15	Adenosine-2′ : 3′ : 5′ triphosphate	47
Adenosine-2′(3′) : 5′ diphosphate	34	Inosine-2′ : 3′ : 5′ triphosphate...	56

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KING'S COLLEGE, UNIVERSITY OF DURHAM,
NEWCASTLE UPON TYNE.

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