209. *Synthesis of Adenosine-5′ Sulphatophosphate. A Degradation Product of an Intermediate in the Enzymic Synthesis of Sulphuric Esters.*

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Adenosine-5′ sulphatophosphate (Ib) has been synthesised from adenosine-5′ phosphate and the pyridine–sulphur trioxide complex in sodium hydrogen carbonate solution. It was identical with a substance obtained by enzymic dephosphorylation of adenosine 3′-phosphate 5′-sulphatophosphate (Ia) (an intermediate formed from adenosine triphosphate and inorganic sulphate during the enzymic synthesis of sulphuric esters).

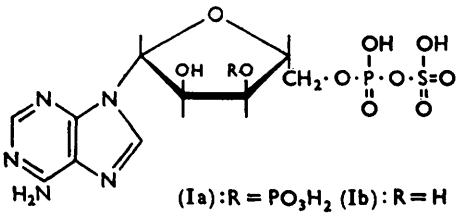
The sulphatophosphate linkage in (Ib) is hydrolysed by an enzyme in rattlesnake venom.

Sulphuric esters are frequently found in Nature as structural materials (*e.g.*, sulphuric esters of carbohydrates), and as excretory products ("ethereal sulphates"). It is believed that phenols and other hydroxy-compounds are converted into their sulphuric esters in the liver by enzymic processes which utilise inorganic sulphate. Adenosine triphosphate (ATP) is required in these reactions [1,2] which involve several stages.[3] The overall process can be separated into an "activation" of sulphate, followed by a transfer of the sulphate group to the phenol.[4] An intermediate was formed when ATP and sulphate were incubated with enzyme preparations in the absence of phenols.

Lipmann and his associates [5] have examined these reactions and in particular the nature of "active sulphate." This intermediate was an adenine derivative bearing a

[1] Bernstein and McGilvery, *J. Biol. Chem.*, 1952, **198**, 195.
[2] DeMeio, Wizerkaniuk, and Fabiani, *ibid.*, 1953, **203**, 257.
[3] DeMeio, Wizerkaniuk, and Schreibman, *ibid.*, 1955, **213**, 439.
[4] Bernstein and McGilvery, *ibid.*, 1952, **199**, 745.
[5] Robbins and Lipmann, *J. Amer. Chem. Soc.*, 1956, **78**, 2652, and personal communication.

sulphate and two phosphate groups. The sulphate was readily removed by acid hydrolysis, a secondary phosphate being liberated. The nucleoside-3′ phosphatase from rye grass removed one phosphate group without loss of sulphate. This and other evidence indicated the structure adenosine 3′-phosphate 5′-sulphatophosphate (Ia) for the active sulphation intermediate. The product of its enzymic dephosphorylation would be adenosine-5′ sulphatophosphate (Ib).



(Ia): R = PO_3H_2 (Ib): R = H

Although it has been claimed [6] that salts of sulphatophosphoric acid are formed when sulphuric acid and alkali-metal phosphates are heated together under suitable conditions, their characterisation is inadequate. Further, esters of sulphatophosphoric acid have not been described previously and it was of interest to attempt the synthesis and chemical study of such compounds.

A total synthesis of adenosine 3′-phosphate 5′-sulphatophosphate (Ia) would present special difficulties in view of the unavailability of adenosine-3′ : 5′ diphosphate or its derivatives. The latter nucleotide is known as an enzymic degradation product of coenzyme A, but its preparation in quantity from this source would be difficult and costly. Consequently, the somewhat simpler synthesis of adenosine-5′ sulphatophosphate (Ib) was undertaken. This product should be identical with that obtained by enzymic dephosphorylation of the 3′-phosphate (Ia), thereby confirming the structure assigned to the latter. The synthetic compound would also be of interest in enzyme studies.

There has been much progress recently on the synthesis of anhydrides of phosphoric acid, mainly confined, however, to the formation of symmetrical and unsymmetrical esters of pyro- and tri-phosphoric acid. Relatively little attention has been given to the synthesis of mixed anhydrides between a nucleotide and another acid. The use of carbodi-imides in the preparation of mixed anhydrides of this type was considered, since these reagents can be used for the synthesis of both pyrophosphates [7] and sulphonic anhydrides [8] from the parent acids. Condensation was attempted between phosphoric and sulphuric acid in the presence of di*cyclo*hexylcarbodi-imide under a variety of conditions. In most cases the reaction products, when examined by paper electrophoresis, were complex, but no good evidence for the formation of mixed anhydrides was obtained.

A general synthesis of mixed anhydrides of carboxylic acids with phosphoric acid and its esters, developed by Avison,[9] involves reaction between phosphates and appropriate anhydrides of carboxylic acids in aqueous pyridine at a low temperature, in which anhydride–pyridine complexes probably participate. The compound obtained by the action of sulphur trioxide on pyridine [10] (" pyridine-*N*-sulphonic acid ") was considered suitable as the anhydride component for the preparation of sulphatophosphates. It was added to a cold aqueous-pyridine solution of adenosine-5′ phosphate, and the mixture was examined by paper chromatography. Much unchanged nucleotide was present, and the only other product which absorbed ultraviolet light on paper was a compound with a higher R_F in basic solvents. Considerably more of the faster-moving nucleotide was obtained by reaction at 40—50° in sodium hydrogen carbonate solution : it was estimated

[6] Cf. Mellor, " A Comprehensive Treatise on Inorganic and Theoretical Chemistry," Longmans Green and Co., 1930, Vol. X, p. 437.
[7] Khorana and Todd, *J.*, 1953, 2254.
[8] Khorana, *Canad. J. Chem.*, 1953, **31**, 585.
[9] Avison, *J.*, 1955, 732.
[10] Baumgarten, *Ber.*, 1926, **59**, 1166.

by ultraviolet spectroscopy that approximately 20% of the total nucleotides was then present as the faster-moving compound. The product was isolated in small amounts by adsorption on charcoal, elution with dilute ammonia, and paper chromatography in two solvent systems. It moved as a single spot on paper and on electrophoresis, and did not contain inorganic sulphate. It had an ultraviolet spectrum typical of an adenosine derivative with no additional substituents on the ring-nitrogen atoms or on the 6-amino-group. It was fairly stable in cold neutral or dilute alkaline solution, but was hydrolysed readily in hot dilute acid and alkali to inorganic sulphate and adenosine-5′ phosphate. These products were identified on paper, the inorganic sulphate being detected by spraying the paper with very dilute barium chloride solution, followed by a solution of rhodizonic acid; sulphate appeared as a white spot against a pink background. The nucleotide was completely hydrolysed in 0·5N-hydrochloric acid at 100° after 15 min. The ratio adenine : sulphate was 1 : 0·95, as determined from the light absorption of its solution at 260 mμ and the amount of sulphate (as barium sulphate) liberated on acid hydrolysis. No substituents were present at position 2′ or 3′, since it gave a strong colour reaction on paper when sprayed with periodate followed by Schiff's reagent. It was concluded that the substance was a sulphatophosphate with the structure (Ib).

Adenosine-5′ sulphatophosphate was isolated as its lithium salt after gradient-elution chromatography on Dowex-2 resin (formate form), elution being effected with ammonium formate solution. The lithium salt was analytically pure, although it contained a trace of adenosine-5′ phosphate, and had an absorption spectrum similar to those of the adenosine phosphates. It consumed one mol. of periodate, thus confirming the absence of substituents at positions 2′ and 3′.

The synthetic material was indistinguishable from the product of enzymic dephosphorylation of the natural compound (Ia) on paper chromatography and on electrophoresis. At pH 5 it migrated towards the anode at a rate very slightly greater than that of adenosine-5′ pyrophosphate, whereas at pH 8 it had a rate similar to that of adenosine-5′ phosphate. These results are consistent with the presence of two primary acidic dissociations in the molecule.

Enzymes present in rattlesnake (*Crotalus atrox*) venom hydrolysed adenosine-5′ sulphatophosphate to adenosine, orthophosphate, and sulphate. The unfractionated venom is known to contain nucleoside-5′ phosphatase, pyrophosphatase, and phosphodiesterase. It is not yet known whether it also contains a specific sulphatophosphatase or whether pyrophosphatases are responsible for the hydrolysis.

Experimental

Adenosine-5′ Sulphatophosphate.—The pyridine–sulphur trioxide complex (400 mg.; m. p. 172°) was added with stirring to a solution of adenosine-5′ phosphate (200 mg.) and sodium hydrogen carbonate (350 mg.) in water (5 c.c.) at 40—50°. After 15 min. at this temperature, stirring was discontinued and the solution was cooled in iced water. A sample of the mixture was examined by paper chromatography in solvent systems A and B (see below). Adenosine-5′ phosphate and the sulphatophosphate were the principal components absorbing ultraviolet light. Only traces of other nucleotides were detected. Spots were eluted from the paper and the optical density of the resulting solution was measured at 260 mμ. On the assumption that the sulphatophosphate has an extinction coefficient at 260 mμ typical of an adenosine-5′ phosphate derivative, about 20% of the starting material had been converted into the sulphatophosphate.

The above mixture was diluted to 250 c.c., adjusted to pH 5·5 with 2M-formic acid, and shaken overnight with Norit A charcoal (10 g.) which had previously been washed with 2M-formic acid (200 c.c.), ethanolic ammonia [water, 100 c.c.; alcohol, 96 c.c.; ammonia (*d* 0·88), 4 c.c.], and finally water (600 c.c.). The charcoal containing the nucleotides was filtered off and washed with water (400 c.c.). The nucleotide content of the filtrate and washings was less than 0·5 mg., as determined by optical density at 260 mμ. Nearly all the inorganic sulphate formed during the reaction was present in this filtrate and washings. Nucleotides were eluted

from the charcoal with ethanolic ammonia (200 c.c.; composition as above). The optical density at 260 mμ of the eluate indicated a recovery of 95%. Without delay, the eluate was concentrated (to 4 c.c.) *in vacuo*, and the sulphatophosphate was separated from adenosine-5′ phosphate and traces of inorganic sulphate by chromatography as a series of spots on Whatman No. 3 paper in the solvent system B. The sulphatophosphate was homogeneous when examined by paper chromatography in solvent systems A and B (yield 18·1%, based on optical density).

A solution of the purified sulphatophosphate was divided into two equal parts. The " adenine " content (3·17 mg.) of one part was determined by optical density at 260 mμ, and the other part was treated with 0·5N-hydrochloric acid at 100° for 30 min. Inorganic sulphate was determined in this part by precipitation as barium sulphate (ratio of adenine : sulphate, 1 : 0·95. Adenosine-5′ sulphatophosphate requires ratio, 1 : 1).

Isolation. The sulphatophosphate (from 200 mg. of adenosine-5′ phosphate and 200 mg. of sulphur trioxide complex) was prepared as above, the reaction being carried out during 30 min. The solution was diluted with water (1 l.) and passed through a column (10 × 3 cm.) of Dowex-2 (formate) resin which was then washed with distilled water (300 c.c.). Elution was carried out with ammonium formate solution (pH 5) in an apparatus designed to give an approximately linear concentration/volume gradient during the delivery of about 3 l. of eluant. The final concentration of ammonium formate was 2M. Elution of adenosine-5′ phosphate was complete after about 1200 c.c. of eluant had been collected. The sulphatophosphate was eluted in the range 2400—3000 c.c.

The fractions containing the sulphatophosphate were bulked and shaken with Norit A charcoal (3 g.) overnight. The charcoal was filtered off and washed with water (300 c.c.), and the nucleotide was eluted with 50% ethanol containing 2% of ammonia. The eluate was concentrated to about 10 c.c., then passed through a column (2 × 1 cm.) of Dowex-50 (lithium form) resin. The column was washed with water until free from material absorbing ultraviolet light, and the combined eluate and washings were freeze-dried. The resulting solid was dissolved in water (2 c.c.), and ethanol (20 c.c.) was added. The precipitated *lithium salt* (11·6 mg.) was collected by centrifugation (Found: P, 6·9. $C_{10}H_{14}O_{10}N_5SPLi_2$ requires P, 7·1%).

The lithium salt had $\lambda_{max.}$ 259 mμ (ε 15,200) and $\lambda_{min.}$ 227 mμ (ε 2620) at pH 7—8. This spectrum corresponded closely with that reported [11] for adenosine-5′ phosphate [$\lambda_{max.}$ 259 mμ (ε 15,400) and $\lambda_{min.}$ 227 mμ (ε 2600) at pH 7]. It consumed 0·98 mol. of periodate, determined spectrophotometrically.[12]

Unchanged adenosine-5′ phosphate was recovered from the above synthesis. The appropriate fractions from the ion-exchange column were bulked and shaken overnight with Norit A charcoal. Elution was carried out as described for the sulphatophosphate, and ammonium ions were removed from the concentrated eluate by passage through a Dowex-50 (H^+ form) resin column. Concentration of the eluate from this column yielded the crystalline phosphate (100 mg.).

Paper Chromatography.—Ascending-front chromatography was carried out on Whatman No. 4 paper, previously washed with dilute acetic acid and water. The following solvent systems were used: (A) *n*-propyl alcohol–ammonia (*d* 0·88)–water (6 : 3 : 1); (B) *iso*butyric acid–0·5N-ammonia (5 : 3). Nucleotides were located by inspection under ultraviolet light, by the perchloric acid–molybdate spray reagent for phosphate, and by the periodate–Schiff reagent for glycols. R_F values are shown in the Table. Synthetic and natural adenosine-5′ sulphatophosphate were indistinguishable in both solvents.

	R_F values in solvent			R_F values in solvent	
Adenosine-5′	A	B		A	B
Phosphate	0·40	0·50	Inorg. sulphate	—	0·20
Pyrophosphate	0·30	0·40	Inorg. phosphate	—	0·25
Sulphatophosphate	0·63	0·32			

Paper Electrophoresis.—Electrophoresis was carried out on Whatman No. 4 paper soaked in 0·05M-ammonium acetate buffers at pH 5·5 and 8·5. Drs. Robbins and Lipmann used a 0·025M-citrate buffer at pH 5·8. Our experiments (see Table) were for 3 hr. with a voltage gradient of 5·4 v per cm. and a current of 7·5 milliamps.

[11] Morell and Bock, 126th Meeting Amer. Chem Soc., New York, 1954, Div. of Biol. Chem., Abs., p. 44 c.

[12] Dixon and Lipkin, *Analyt. Chem.*, 1954, **26**, 1092.

	Distance moved towards cathode (cm.)		
	pH 5·5	pH 8·5	pH 5·8 *
Adenosine-5′ phosphate	4·6	6·4	24·5
Adenosine-5′ pyrophosphate	9·5	10·5	34·0
Adenosine-5′ sulphatophosphate	9·7	8·8	34·5

* Experiments carried out by Drs. Robbins and Lipmann (voltage and time not stated).

Detection of Inorganic Sulphate on Paper Chromatograms.—Chromatograms run as described above were lightly sprayed with a solution of barium chloride (60 mg.) in water (250 c.c.), followed immediately by a solution of rhodizonic acid (5 mg.) in water (20 c.c.). Areas which contained inorganic sulphate appeared white on a pink background.

Action of Crotalus atrox *Venom on Adenosine-5′ Sulphatophosphate.*—A solution of the lithium salt (0·1 mg.) and venom (1 mg.) in a glycine–ammonia buffer (0·1 c.c.) at pH 9 was kept at 37° overnight. Samples were examined by paper chromatography in solvent system B. The products were identified by their R_F values (see Table) and by appropriate spray reagents as adenosine, orthophosphate, and sulphate. In a control experiment to which no enzyme had been added no spontaneous hydrolysis was observed.

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