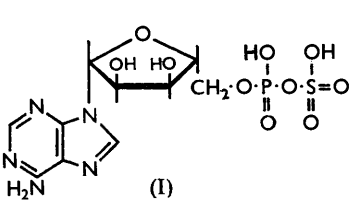
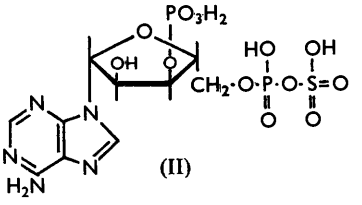
343. *Synthesis of "Active Sulphate" (Adenosine 3′-Phosphate 5′-Sulphatophosphate).*

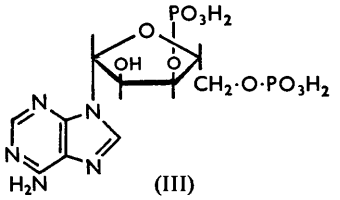
By J. Baddiley, J. G. Buchanan, R. Letters, and A. R. Sanderson.

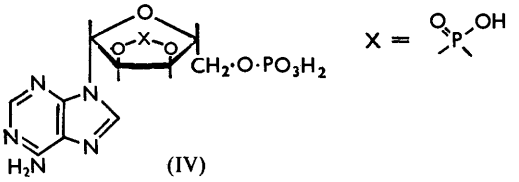
The pyridine–sulphur trioxide complex reacts with adenosine-3′ : 5′ diphosphate (III) in sodium hydrogen carbonate solution to give a mixture containing the cyclic phosphate (IV) and adenosine 3′-phosphate 5′-sulphatophosphate (II). The sulphatophosphate was indistinguishable, both chemically and enzymically, from "active sulphate."

The biosynthesis of sulphuric esters occurs, at least in a number of cases examined so far, by a sequence of enzymic reactions during which inorganic sulphate is converted into a sulphuric–phosphoric anhydride known as "active sulphate." These reactions have been studied by Lipmann and his collaborators,[1,2] and by Wilson and Bandurski [3] who have shown that adenosine triphosphate (ATP) reacts first with sulphate to give adenosine-5′ sulphatophosphate (I) (APS) which is then phosphorylated at the 3′-position by a second mol. of ATP to give adenosine 3′-phosphate 5′-sulphatophosphate (II) (active sulphate, PAPS). This intermediate is able to transfer its sulphate group to a variety of substrates in the presence of the appropriate enzyme.


(I)


(II)


(III)


(IV)

The sulphatophosphate (I) was synthesised in these Laboratories [4] by reaction between adenosine-5′ phosphate and the pyridine–sulphur trioxide complex in sodium hydrogen carbonate solution, and independently by Reichard and Ringertz [5] who condensed inorganic sulphate with adenosine-5′ phosphate in the presence of di*cyclo*hexylcarbodiimide. An advantage of the former method lies in the ease of separation of the product. This synthesis was of much value in making available authentic adenosine-5′ sulphatophosphate required for the elucidation of the enzymic reaction sequences.[2,3]

A synthesis of active sulphate itself would require either the phosphorylation of adenosine-5′ sulphatophosphate or the sulphation of the 3′ : 5′-diphosphate (III) of adenosine. Whereas the latter method seemed promising, the necessary diphosphate (III) was not readily available when this work was started. It had been obtained by enzymic degradation of coenzyme A,[6] and more recently it has been identified as a co-factor in certain sulphate transfer reactions.[7] Preliminary experiments on the synthesis of "active

[1] Hilz and Lipmann, *Proc. Nat. Acad. Sci., U.S.A.*, 1955, **41**, 880.
[2] Robbins and Lipmann, *J. Biol. Chem.*, 1957, **229**, 837; 1958, **233**, 681, 686.
[3] Wilson and Bandurski, *Arch. Biochem. Biophys.*, 1956, **62**, 503; *J. Biol. Chem.*, 1958, **233**, 975.
[4] Baddiley, Buchanan, and Letters, *J.*, 1957, 1067.
[5] Reichard and Ringertz, *J. Amer. Chem. Soc.*, 1957, **79**, 2025.
[6] Wang, Shuster, and Kaplan, *J. Biol. Chem.*, 1954, **206**, 299.
[7] Gregory and Lipmann, *ibid.*, 1957, **229**, 1081.

sulphate" were carried out on a small sample of the diphosphate obtained by Dr. J. D. Gregory from natural sources. Larger quantities of a mixture of adenosine-2′ : 5′ and -3′ : 5′ diphosphate were prepared by direct phosphorylation of adenosine,[8] and exploratory synthetical experiments were carried out with this mixture. For a synthesis of active sulphate it was desirable to separate these isomers before sulphation, in view of the difficulties expected in separating the mixture of sulphatophosphates and other products which would be formed in the reaction with pyridine–sulphur trioxide. The 2′ : 5′- and 3′ : 5′-diphosphate were separated by ion-exchange chromatography;[9] pure 3′ : 5′-diphosphate obtained in this way was used in the present work.

Adenosine-3′ : 5′ diphosphate was treated with pyridine–sulphur trioxide in sodium hydrogen carbonate solution under conditions similar to those used in the synthesis of the sulphatophosphate (I). Paper chromatography and electrophoresis of the reaction mixture demonstrated the presence of several nucleotides. The principal components of this mixture were unchanged diphosphate and two new nucleotides. Both had higher R_F values than the diphosphate in the *n*-propyl alcohol–ammonia solvent but only one contained sulphate, as was indicated by acid-hydrolysis *in situ* followed by the barium–rhodizonic acid spray.*

The sulphur-free product was identified as adenosine 2′ : 3′-phosphate 5′-phosphate (IV) by its behaviour on paper chromatography and electrophoresis, and by acid- or alkali-hydrolysis to a mixture of adenosine-2′ : 5′ and -3′ : 5′ diphosphate. The formation of this cyclic phosphate is readily explained on the assumption that an initial reaction product was adenosine 5′-phosphate 3′-sulphatophosphate. Such a mixed anhydride would be unstable, decomposing spontaneously to inorganic sulphate and a 2′ : 3′-cyclic phosphate. In support of this it was shown that a mixture of adenosine-2′ and -3′ phosphate was converted in part into the 2′ : 3′-cyclic phosphate of adenosine by reaction with the pyridine–sulphur trioxide reagent in sodium hydrogen carbonate solution.[4] This ready cyclisation of mixed anhydrides at the 2′- or 3′-position in a nucleoside is also observed in the action of trifluoroacetic anhydride on nucleotides, where intermediate trifluoroacetic–phosphoric anhydrides are believed to be formed.[10]

The sulphur-containing nucleotide obtained in the above reaction was identified as "active sulphate" by comparison with authentic material. It had the same R_F value in several solvents and a characteristically high rate of migration on paper electrophoresis in a citrate buffer at pH 5·8. It was isolated by charcoal adsorption and ion-exchange chromatography under conditions similar to those used by Brunngraber [11] for its isolation from natural sources. The final product contained a trace of adenosine-3′ : 5 diphosphate, possibly arising through the known great acid-lability of the sulphatophosphate. The absence of phosphate migration during synthesis and isolation was demonstrated by alkali-hydrolysis of the product to adenosine-3′ : 5′ diphosphate and inorganic sulphate No 2′ : 5′-diphosphate was detected in the hydrolysate.

Enzymic assay of the synthetic material was carried out by Dr. J. D. Gregory in Dr. Lipmann's laboratory at the Rockefeller Institute. Difficulty was experienced through the partial decomposition of samples of the lithium salt during transit between this country and America. This was indicated by the low activity of these samples in the enzymic transfer of sulphate to *m*-aminophenol. The occurrence of considerable decomposition was confirmed by paper electrophoresis of material before dispatch and after receipt. A solution of the lithium salt containing a trace of ammonia was more stable. Special precautions were observed during the direct transport of this preparation by air between Newcastle and New York, and material examined by Dr. Gregory had a purity of 75—76%.

* A preliminary account of this work has been given (Baddiley, Buchanan, and Letters, *Proc. Chem. Soc.*, 1957, 147).

[8] Cramer, Kenner, Hughes, and Todd, *J.*, 1957, 3297.
[9] Baddiley, Buchanan, and Letters, *J.*, 1958, 1000
[10] Brown, Magrath, and Todd, *J.*, 1952, 2708.
[11] Brunngraber, *J. Biol. Chem.*, 1958, **233**, 472.

The activity of this preparation in the enzyme test was about 67% of what would be expected from chromatographic data. This rather low value is understandable, since the enzyme system used gives low values with active sulphate.

The experimental conditions for this synthesis were chosen in order to minimise the possible introduction of more than one sulphate group into the nucleotide. If both phosphate groups in adenosine-3' : 5' diphosphate were to react with the pyridine–sulphur trioxide reagent, the resulting di(sulphatophosphate) would decompose spontaneously to give adenosine 2' : 3'-phosphate 5'-sulphatophosphate. Only traces of a compound with the paper-chromatographic properties expected for this cyclic phosphate were detected in the reaction mixture. This product increased in amount when a larger excess of sulphation reagent was employed, but it was not studied further.

Experimental

Adenosine-3' : 5' Diphosphate.—The nucleotide was separated from its 2' : 5'-isomer by concave gradient elution chromatography on Dowex-1 (×2) resin (chloride form; 200—400 mesh), with calcium chloride and hydrochloric acid as eluting agents.[9] The eluate was adjusted to pH 7 with calcium hydroxide solution, then passed through a column of Dowex-50 (lithium form) resin. The product was isolated by freeze-drying, then extracting lithium chloride from the residue with acetone–ethanol (4 : 1) (4 × 100 c.c.). The *lithium salt* of adenosine-3' : 5' diphosphate had $\lambda_{max.}$ 256·5 mμ (ε 15,600) and $\lambda_{min.}$ 228 mμ (ε 3000) (Found: C, 25·0; H, 3·9; P, 12·7. $C_{10}H_{13}O_{10}N_5P_2Li_4,2H_2O$ requires C, 24·6; H, 3·5; P, 12·7%).

Adenosine 3'-Phosphate 5'-Sulphatophosphate.—Preliminary experiments were carried out on a micro-scale to determine the optimal conditions of synthesis. Products were separated by chromatography in solvent system *A* (see below), and estimated by elution from the paper and measurement of the optical density of the resulting solution at 257·5 mμ. Provided that adenosine 3'-phosphate 5'-sulphatophosphate has an extinction coefficient at 257·5 mμ typical of an adenosine-3' : 5 diphosphate derivative, the maximum yield was 12%, under the conditions subsequently described.

The pyridine–sulphur trioxide complex (800 mg.) was added, with stirring, to a solution of the lithium salt of adenosine-3' : 5' diphosphate (200 mg.) and sodium hydrogen carbonate (1·2 g.) in water (12 c.c.) at 45°. After 40 min. at this temperature, the solution was cooled, diluted with iced water (500 c.c.), adjusted to pH 6 with N-formic acid, and passed through a column (7 × 5 cm.) of Norit A charcoal–Supercel silica (4 : 3), at a rate of 4 c.c./min. The charcoal had previously been washed with N-hydrochloric acid, N-ammonia, water, ethanol, and ether, then dried at 110° for 16 hr. The column was washed with water until the washings were free from sulphate and their optical density at 257·5 mμ was less than 0·1.

Nucleotides were eluted (1·5 c.c./min.) from the column with 50% aqueous ethanol which contained 1% of N-ammonia. The optical density (257·5 mμ) of the eluate indicated a recovery of 87%. The eluate was then passed through a column (7 × 4 cm.) of Dowex-1 (×2) resin (chloride form; 200—400 mesh), which was washed with water (3 l.). Concave gradient elution was performed using 2N-lithium chloride solution (2 l.) as eluant in the reservoir, and water (3 l.) in the mixing flask. Fractions (25 c.c.) were collected at a rate of 10 c.c./min., and the optical density at 260 mμ of each fraction was measured. Two sharp peaks were observed, the first between fractions 38 and 60, and the second between fractions 79 and 120. The former corresponded to adenosine-3' : 5' diphosphate, probably containing a trace of adenosine 2' : 3'-phosphate 5'-phosphate (IV), while the latter corresponded to adenosine 3'-phosphate 5'-sulphatophosphate. The yield of sulphatophosphate, calculated from ultraviolet measurements, was about 10%. The fraction containing the desired nucleotide was passed through a charcoal–Supercel column (2 × 5 cm.), similar to that mentioned previously, at a rate of 10 c.c./min. After being washed with water (2 l.), the nucleotides were eluted with 50% aqueous ethanol (300 c.c.) which contained 1% of N-ammonia. The optical density of the eluate at 257·5 mμ indicated a recovery of 88%. The eluate was concentrated under reduced pressure below 35° and passed through a column (1 × 1 cm.) of Dowex-50 (×8) resin (lithium form; 200—400 mesh). Evaporation of the eluate and washings yielded a light-brown solid (19 mg.). A sample (5 mg.) of the lithium salt was chromatographed on paper in solvent *A*. The appropriate ultraviolet-absorbing area was eluted from the paper. [The ratios

adenosine : P : ribose : S : N (total) : N (ammonium) were 1·00 : 2·02 : 0·96 : 0·96 : 8·95 : 3·95. The calculated ratios for the tetra-ammonium salt ($C_{10}H_{27}O_{13}N_9P_2S$) are 1 : 2 : 1 : 1 : 9 : 4.] Adenosine was determined spectroscopically, phosphorus by Allen's method [12] ribose by Mejbaum's method,[13] sulphur as inorganic sulphate after hydrolysis,[14] and the two forms of nitrogen by Johnson's method.[15]

A sample of the product was almost homogeneous when examined by paper chromatography and paper electrophoresis; about 5% of adenosine-3′ : 5′ diphosphate was present. The diphosphate content slowly increased on storage.

Hydrolysis with Alkali.—Adenosine 3′-phosphate 5′-sulphatophosphate (lithium salt; 1 mg.) was heated at 100° in 0·1N-sodium hydroxide, and the products were examined by paper chromatography in solvent *A*, after the removal of metal ions with Dowex-50 (ammonium form) resin. Hydrolysis was incomplete even after 2 hr. A sample of the resulting diphosphate was isolated by elution from the paper and, after evaporation of eluant, was re-chromatographed in solvent *B*. The diphosphate had the same R_F value as adenosine-3′ : 5′ diphosphate, no trace of the 2′ : 5′-isomer being observed.

Adenosine 2′ : 3′-*Phosphate* 5′-*Phosphate.*—The nucleotide with R_F 0·23 (solvent *A*), prepared in a small-scale sulphation experiment, was eluted from a paper chromatogram. It was homogeneous when examined on paper in M-ammonium acetate (pH 7·5)–ethyl alcohol (30 : 75) and *iso*butyric acid–0·5N-ammonia (10 : 6), in which it had R_F 0·2 and 0·34 respectively. When kept overnight at pH 12 and room temperature, it was converted into a mixture of the 2′ : 5′- and 3′ : 5′-diphosphates of adenosine.

Paper Chromatography.—Chromatography was carried out on Whatman No. 4 paper, which had previously been 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*, Saturated ammonium sulphate–0·1M-ammonium acetate–*iso*propyl alcohol (79 : 19 : 2).[16] Nucleotides were located by inspection under ultraviolet light (see Table).

	R_F in solvent			R_F in solvent	
	A	*B*		*A*	*B*
Adenosine-5′ phosphate	0·28	0·36	Adenosine-5′ triphosphate	0·17	—
Adenosine-2′ phosphate	0·33	0·31	Adenosine 2′ : 3′-phosphate 5′-phosphate	0·23	—
Adenosine-3′ phosphate	0·33	0·21			
Adenosine-2′ : 5′ diphosphate ...	0·10	0·48	Adenosine 3′-phosphate 5′-sulphatophosphate	0·18	0·29
Adenosine-3′ : 5′ diphosphate ...	0·11	0·40			
Adenosine-5′ pyrophosphate ...	0·10	0·50			

Paper Electrophoresis.—Electrophoresis was carried out for 6 hr. on Whatman No. 1 paper soaked in 0·025M-sodium citrate buffer at pH 5·8, with a voltage gradient of 10 v per cm. (see Table).

	Distance moved towards anode (cm.)		Distance moved towards anode (cm.)
Adenosine-5′ phosphate	18·7	Adenosine-5′ pyrophosphate	29·6
Adenosine-2′ phosphate	20·2	Adenosine-5′ triphosphate ...	37·4
Adenosine-3′ phosphate	20·2	Adenosine 3′-phosphate 5′-sulphatophosphate	44·1
Adenosine-2′ : 5 diphosphate	34·0		
Adenosine-3′ : 5′ diphosphate	34·1		

We thank Drs. J. D. Gregory and F. Lipmann for their helpful co-operation in carrying out the enzyme tests, and B.K.S. Air Transport for special facilities in connection with the transport of samples. This work was carried out during the tenure of Earl Grey and Salters Fellowships (by R. L. and A. R. S. respectively).

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

[*Received, December 16th,* 1958.]

[12] Allen, *Biochem. J.*, 1940, **34**, 858.
[13] Mejbaum, *Z. physiol. Chem.*, 1939, **258**, 117.
[14] Iwasaki, Utsumi, Hagino, Tarutani, and Ozawa, *Bull. Soc. Chem. Japan*, 1957, **30**, 847.
[15] Johnson, *J. Biol. Chem.*, 1941, **137**, 575.
[16] Markham and Smith, *Biochem. J.*, 1951, **49**. 401.