Thus phloretic acid, 3-iodophloretic acid and 3,5-diiodophloretic acid could not be separated from each other satisfactorily by chromatography. In solvents 1, 2 and 3 phloretic acid and 3-iodophloretic acid have almost identical R_F values and in solvents 5 and 6, the two iodinated phloretic acids move together. High voltage electrophoresis achieves an excellent separation of these three substances. Similarly, 3,5-diiodo-4-hydroxyphenylglycolic acid and 3,5-diiodo-4-hydroxyphenylglyoxylic acid can be much better separated by electrophoresis than by chromatography (Fig. 2 and Table II).

The present investigation demonstrates that the non-enzymic conversion of analogs of diiodotyrosine to the corresponding analogs of thyroxine is extremely sensitive to small structural changes in the aliphatic side chain. In the incubation of the propionic acid analog (diiododesaminotyrosine) the corresponding analog of thyroxine is obtained in particularly good yield and only small amounts of side products are formed. This should make the propionic acid analog of diiodotyrosine an excellent model substance for the study of the mechanism of the conversion of diiodotyrosine to thyroxine.[48]

(48) The fate of the aliphatic side chain that is lost in the model reaction with diiodophloretic acid will be reported in a forthcoming paper.

BETHESDA, MARYLAND

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY I AND THE INSTITUTE OF CELL RESEARCH AND GENETICS, KAROLINSKA INSTITUTET]

Chemical Synthesis of Adenosine 5′-Phosphosulfates[1]

BY PETER REICHARD AND N. R. RINGERTZ

RECEIVED MAY 27, 1958

Adenosine 5′-phosphosulfate was prepared by the carbodiimide route in a final yield of 20–25% (based on adenosine 5′-phosphate). By-products of the reaction, containing sulfate groups attached to ribose, were separated by zone electrophoresis on a cellulose column and characterized. Snake venom (*Crotalus adamanteus*) and extracts of human prostate were able to cleave phospho–sulfate but not ribose–sulfate linkages. Sulfate groups attached to the ribose of adenosine 5′-phosphate inhibited the phosphatase activity of snake venom but not that of the prostatic extract.

Introduction

It was originally demonstrated by Bernstein and McGilvery[2] and De Meio, *et al.*,[3] that sulfate must be "activated" with ATP before further participating in enzymic reactions. The mechanism of the activation was recently clarified by Robbins and Lipmann[4] and Bandurski, *et al.*,[5] who showed that a two-step reaction is involved. Thus during the first step adenosine 5′-phosphosulfate (APS) is formed from ATP[6] and sulfate (ATP sulfurylase)

$$\text{sulfate} + \text{ATP} \rightleftharpoons \text{adenosine 5′-phosphosulfate} + \text{pyrophosphate}$$

The second step involves the conversion of APS to the biologically active sulfate donor, adenosine 3′-phosphate-5′-phosphosulfate (adenosine-phosphosulfate kinase).

The present paper is concerned with the chemical synthesis of APS and some compounds closely related to it. Chemical synthesis of APS has recently been carried out by sulfurylation of AMP with the SO_3 complex of pyridine by Baddiley, *et al.*[7] In preliminary reports[8] we have described a one-step synthesis of APS and other compounds containing both AMP and sulfate by treating a mixture of AMP and concentrated sulfuric acid in aqueous pyridine with an excess of dicyclohexylcarbodiimide (DCC). This paper is a complete report of these studies.

Carbodiimide has previously been used for the synthesis of biologically important anhydrides containing pyrophosphate groups[9] and also for the synthesis of sulfonic acid anhydrides.[10] Therefore, it seemed likely that mixed anhydrides between a phosphate group and sulfuric acid could be synthesized with the aid of DCC. We found that the reaction took place only when the volume of the reaction mixture was kept very small. However, other compounds were also formed, which contained sulfate esterified to the ribose of AMP. Thus, a mixture of AMP-monosulfates, AMP-disulfates and AMP-trisulfates was obtained. However, by choosing the proper conditions it was possible to transform about 50% of the AMP to APS with the formation of only small amounts of sulfate esters. After preparative zone electrophoresis[11] APS was obtained in a final yield of 20–25%.

(1) This paper describes the synthesis of compounds containing sulfate attached to the ribose and/or phosphate moieties of adenosine 5′-phosphate (AMP). Such compounds are named AMP-sulfates. The term adenosine 5′-phosphosulfates is restricted to compounds, specifically containing an anhydride linkage between phosphate and sulfate.

(2) S. Bernstein and R. W. McGilvery, *J. Biol. Chem.*, **198**, 195 (1952); **199**, 745 (1952).

(3) R. H. DeMeio and L. Tkacz, *ibid.*, **195**, 175 (1952); R. H. DeMeio, M. Wizerkaniuk and E. Fabriani, *ibid.*, **203**, 257 (1953).

(4) P. W. Robbins and F. Lipmann, THIS JOURNAL, **78**, 2652 (1956); **78**, 6409 (1956); *J. Biol. Chem.*, **229**, 837 (1957).

(5) R. S. Bandurski, L. G. Wilson and C. C. Squires, THIS JOURNAL, **78**, 6408 (1956).

(6) The following abbreviations are used: AMP and ATP for adenosine-mono- and triphosphate, APS for adenosine-5′-phosphosulfate, SAP for adenosine 2′-(and -3′-)-sulfate-5′-phosphate, DCC for dicyclohexylcarbodiimide and tris-(hydroxymethyl)-aminomethane.

(7) J. Baddiley, J. G. Buchanan and R. Letters, *J. Chem. Soc.*, 1067 (1957).

(8) (a) P. Reichard and N. R. Ringertz, THIS JOURNAL, **79**, 2026 (1957). (b) N. R. Ringertz and P. Reichard, *Acta Chem. Scand.*, **11**, 1081 (1957).

(9) H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954); G. W. Kenner, A. R. Todd and R. F. Webb, *J. Chem. Soc.*, 2843 (1954).

(10) H. G. Khorana, *Can. J. Chem.*, **31**, 585 (1953).

(11) J. Porath, *Biochem. Biophys. Acta*, **22**, 151 (1956).

Experimental

Electrophoretic Separations.—The different AMP-sulfates could be separated from each other and from AMP and sulfate by paper electrophoresis. The separation was carried out on Whatman No. 3 filter papers using the apparatus described by Markham and Smith.[12] Good separation was obtained in one hour runs (14 v./cm.) using 0.05 *M* ammonium formate, ammonium acetate or tris buffer. The nucleotides were located by inspection under ultraviolet light (Mineralight lamp model V 41, Ultra-Violet Prod. Inc., San Gabriel, Calif.). Quantitative determinations were carried out by eluting the nucleotides with 0.1 *N* HCl and reading the absorbance at 260 mμ. Alternatively the radioactivity of the various spots was measured directly on the paper strips and the amounts were expressed as per cent of the total radioactivity counted in all spots. The former procedure was found to be more reliable than the latter. Measurements of the electrophoretic mobilities of the various AMP-sulfates demonstrated that APS was separated from adenosine 2'- (and 3')-sulfate-5'-phosphate at *p*H values above 6, where the latter compounds acquire an additional acid group through the dissociation of the secondary phosphate hydroxyl. AMP, AMP-disulfates and AMP-trisulfates were completely separated from AMP-monosulfates at *p*H 3–9.

The information obtained from the paper electrophoretic separations was applicable to the preparative separation of AMP-sulfates using zone electrophoresis on ethanolized cellulose columns according to Porath.[11]

The separations were carried out with either 0.05–0.1 *M* ammonium formate buffer, *p*H 3 or 0.05 *M* ammonium acetate buffer, *p*H 9.5. Complete separation of AMP, sulfate, AMP-monosulfate, -disulfate, -trisulfate and one additional unidentified compound at the acid *p*H required only 18 hr. at 3 v./cm. Such a separation has been described earlier.[8a,13] The AMP-monosulfates were not separated from each other at this *p*H.

At *p*H 9.5, however, APS was separated from the other AMP-monosulfates, and this *p*H was chosen for preparing larger samples of APS.

For this purpose the lyophilized material obtained after elution from charcoal (corresponding to 1120 μ*M* of adenine; see below under larger scale preparation of APS) was dissolved in a total volume of 22 ml. of water. The *p*H of the solution was adjusted to 9–10 with ammonia, and the material was added to the top of a cellulose column (5 × 50 cm.). Ammonium acetate buffer, *p*H 9.5 (0.05 *M*) containing 0.0005 *M* ethylenediaminetetraacetic acid was used for the separation. Zone sharpening[11] was accomplished by the application of barrier zones of distilled water (15 ml.) before and behind the zone containing the AMP-sulfates. In order to obtain good separation the electrophoresis had to be run for 44 hours at 3 v./cm. The electrophoretic migration of the different AMP-sulfates toward the anode was counteracted by a hydrodynamic counterflow of about 20 mm. of buffer in the anode compartment. This pressure was usually sufficient to balance the electric migration of APS. However, this balance depended, on several factors, especially the packing of the column. Consequently it was advantageous to include a colored zone with known mobility during the electrophoresis. Phenol red, which has a mobility very close to APS, was used for this purpose. The phenol red zone was applied separately and displaced to 20 cm. from the bottom of the column before the introduction of the AMP-sulfates. During electrophoresis the hydrodynamic counterflow was then adjusted in such a way that the colored zone moved very slowly or not at all toward the anode. The column was cooled with an alcohol–water mixture (0°) which circulated through a cooling jacket.

When the electrophoresis was completed the column was directly eluted with ammonium acetate buffer. The fractions (*ca.* 10 ml.) were collected with a fraction collector and analyzed for radioactivity and absorbancy at 260 mμ. Appropriate fractions were pooled and lyophilized twice in order to remove all the ammonium acetate buffer. The separation in the experiment described below is plotted in Fig. 1. Of the 1120 μ*M* of adenine introduced into the column 4.1% were recovered as AMP-disulfates, 3.8% as adenosine 2'(and 3')-sulfate-5'-phosphate, 67.0% as APS and 8.2% as AMP.

Paper Chromatography.—Ascending chromatography was carried out on Whatman No. 1 filter paper. The following solvent systems were used: (I) 5% Na_2HPO_4–amyl alcohol (2:1)[14] and (II) 0.1 *M* sodium phosphate *p*H 6.8–ammonium sulfate–1-propanol (100 ml.:60 g.:2 ml.).[15] The nucleotides were located with a Mineralight lamp. As after paper electrophoresis quantitative evaluations were based on measurements of absorbancy at 260 mμ or on radioactivity measurements. R_f values are given in Table I. The table also includes data of the electrophoretic mobilities on paper at *p*H 3.0 and 8.8.

TABLE I

R_f VALUES OF SOME AMP SULFATES

	Solvent I	Solvent II	Mobility[b] at *p*H 3	Mobility[b] at *p*H 8.8
Adenosine 5'-phosphate	0.65	0.26	30	66
Adenosine 2'-sulfate[a]	.48	..	32	47
Adenosine 3'-sulfate	.61			
Adenosine 5'-phosphosulfate (A)	.62	.26	66	80
Adenosine 2'-sulfate-5'-phosphate[a] (A)	.71	.22	66	97
Adenosine 3'-sulfate-5'-phosphate (A)	.74	.34		
AMP-disulfates (B)	.82	.29	88	110
Adenosine-2',3'-disulfate-5'-phosphosulfate (C)	.74	.24	116	128

[a] No attempts have been made to establish which of the two isomers gives which R_f value. [b] Electrophoretic mobilities (mm./hr.) were obtained at 14 v./cm. No correction has been made for endosmosis.

Small Scale Synthesis of AMP-sulfates.—When studying the influence of time on the yield of the different AMP-sulfates, small scale experiments of the following type were performed: AMP (50 mg.), 0.02 ml. of concentrated H_2SO_4[16] and 250 mg. of dicyclohexylcarbodiimide were dissolved in 0.05 ml. of pyridine and 0.03 ml. of water and shaken vigorously at room temperature. After 5, 10, 15 and 20 hr., respectively, 250 mg. of DCC and 0.15 ml. of pyridine were added. At various time intervals small samples were removed, diluted with water and immediately analyzed by paper electrophoresis. Vigorous shaking was essential since the mixture solidified during the experiment. The influence of the H_2SO_4 to AMP ratio on the formation of AMP-sulfates was studied in experiments of the same type but with varying amounts of concentrated H_2SO_4.

Synthesis on a Larger Scale.—AMP (1 g. = 2.6 mmoles) was dissolved in 1 ml. of pyridine and 0.5 ml. of water, and DCC (5 g. = 24 mmoles) was added. Then 0.2 ml. of concentrated H_2SO_4 (3.7 mmoles) was added slowly with vigorous stirring. The mixture was then shaken in a 100-ml. stoppered, thick walled glass tube containing about 20 glass beads. After 2 and 4 hr., respectively, 5 g. of DCC was added. At various time intervals small samples of the mixture were removed, diluted with water and analyzed by paper electrophoresis. After 5.5 hr. the mixture had almost completely solidified and the results of the paper electrophoresis indicated that 48% of the AMP had been transformed to APS. After 6.5 hr. the synthesis was stopped by extraction of the mixture with equal parts of water and ether. This was done by homogenizing the semi-solid mass with water and ether in a Waring Blendor. The solution was filtered on a büchner funnel and the precipitate was rehomogenized 6 times with more water and ether. A total of 500 ml. of water and 500 ml. of ether was used. The pooled filtrates were extracted 10 times with together 10 volumes of ether. The water phase (500 ml.) was then lyophilized to a volume of *ca.* 200 ml. The efficiency of the whole extraction procedure as judged by the recovery of absorbancy at 260 mμ was 95–100%.

(12) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(13) By mistake a voltage gradient of 14 v./cm. was given in reference 8a.

(14) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950).

(15) Pabst Laboratories, Circular OR-10 (1956).

(16) S^{35}-labeled H_2SO_4 (0.1–1.0 mc./mmole) was used in order to facilitate characterization of the synthetic products.

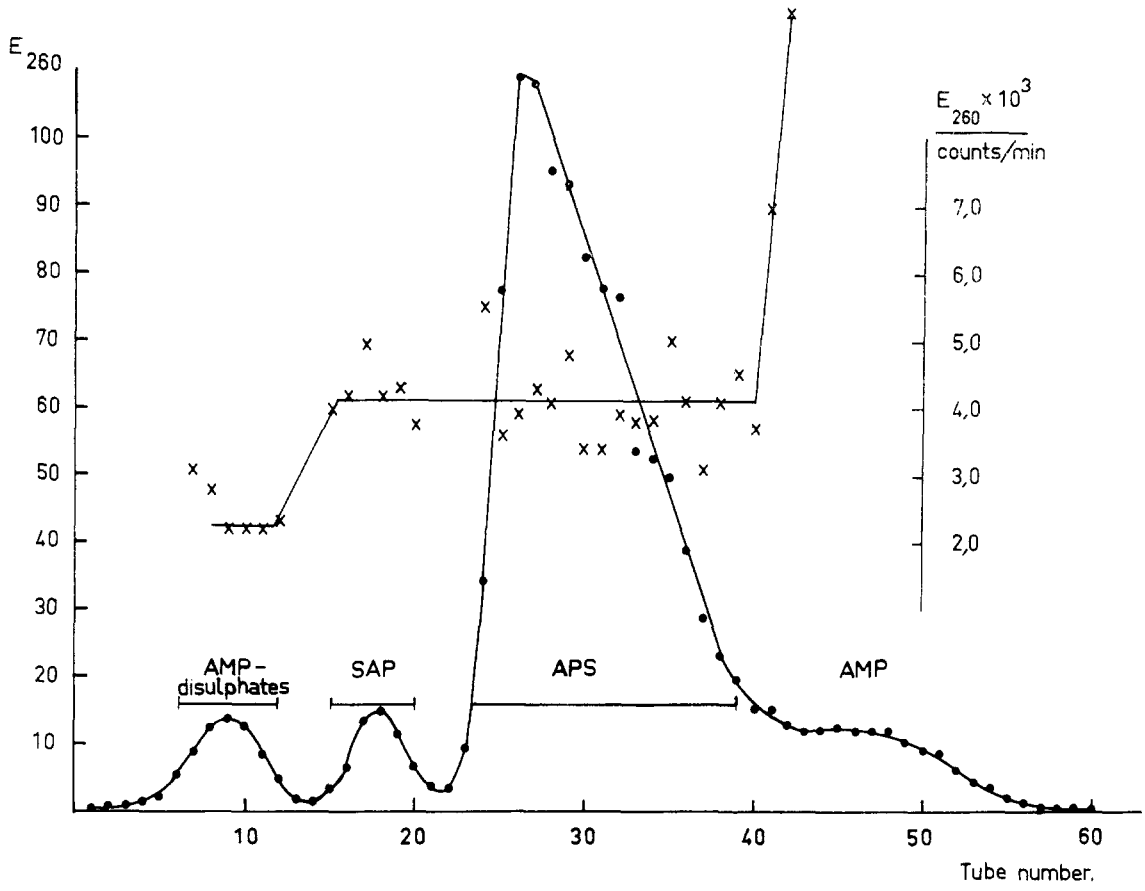

Fig. 1.—Electrophoretic separation at *p*H 9.5 of the material from an APS synthesis after treatment with Dowex 50 and Norit F.

The water solution obtained after extraction of the semi-solid reaction mixture contained AMP, sulfate and AMP-sulfates. In order to facilitate the electrophoretic separation of the different AMP-sulfates, AMP and sulfate were first removed. The solution (200 ml.) was rapidly (*ca.* 30 minutes) passed through a Dowex 50 column (H^+ form, 200–500 mesh, 2 × 6 cm.) and the column was further washed with 50 ml. of cold water. Forty per cent of the absorbance at 260 mμ (equivalent to 1160 μ*M* of adenine) passed through the column. Paper electrophoresis showed that AMP was completely removed by the passage through the column.

Charcoal (Norit F, 3 g.)[17] was then immediately added to the eluate until at least 97% of the absorbance at 260 mμ was removed from the supernatant after centrifugation. The supernatant was discarded and the charcoal was washed twice with 50 ml. of water. The AMP-sulfates were then eluted from the charcoal with ethanol: 0.4% NH_3 (1:1) in 50-ml. portions. After 6 elutions most of the ultraviolet absorbing material was eluted. The solution was evaporated *in vacuo* to about 100 ml. and then lyophilized. The subsequent separation of the AMP-sulfates was carried out by zone electrophoresis as described earlier.

Analytical Methods.—Ultraviolet measurements were carried out in a Beckman spectrophotometer model DU. For quantitative determinations of the amount of adenine in the different compounds the solutions were diluted with 0.1 *N* HCl. For the calculations a value of 14.500 was used as the molar absorbancy at 260 mμ of adenine in these compounds. Sulfate determinations were based on radioactivity measurements as labeled sulfate of known specific activity was used in the synthesis. Ribose was determined by the orcinol method[18] with AMP as a standard. The methods of Fiske–Subbarow[19] and Lowry–Lopez[20] were used for phosphate determinations. Periodate titrations were carried out according to Dixon and Lipkin[21] and deaminations as described by Kleinzeller.[22]

Hydrolysis Curves.—All hydrolyses were performed on 2–12 m*M* solutions. Acid hydrolysis of the different nucleotides was carried out in 0.4 *N* $HClO_4$, alkaline hydrolysis in 0.4 *N* KOH at 100°. The solutions were neutralized with KOH and $HClO_4$, respectively, before paper electrophoresis. Hydrolysis[23] with CaO was carried out by adding 10 mg. of CaO per ml. solution and boiling at 100°. The rate of sulfate liberation was determined by radioactivity measurements after paper electrophoresis. The degradation products formed were further analyzed by paper chromatography.

Enzymatic Degradations.—The effect of crude snake venom and prostatic phosphatases on some AMP-sulfates was studied in the following experimental systems: (A) 1 mg. of snake venom phosphatase,[24] 0.3–1.6 μ*M* of nucleotide, 20 μ*M* of tris buffer, *p*H 8.4, and 2 μ*M* of $MgSO_4$ in a total volume of 0.35 ml. were incubated in a water-bath at 37° for 1 hr.; (B) 1 mg. of prostatic phosphatase,[25] 0.3–1.6 μ*M* of nucleotide, 15 μ*M* of ammonium acetate buffer, *p*H 4.3, in a total volume of 0.3 ml. were incubated in a water-bath at 37° for 1 hr.

(17) Norit F was pretreated by boiling under reflux with 6 *N* HCl for 6 hr. and washed free from HCl with water (14 days) and dried at 150°.

(18) W. Mejbaum, *Z. physiol. Chem.*, **258**, 117 (1939).

(19) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

(20) O. H. Lowry and J. A. Lopez, *ibid.*, **162**, 421 (1946).

(21) J. S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954).

(22) A. Kleinzeller, *Biochem. J.*, **36**, 729 (1942).

(23) K. Lohmann, *Biochem. Z.*, **233**, 460 (1931).

(24) *Crotalus adamanteus* venom was purchased from Ross Allen's Reptile Farm, Silver Springs, Florida, USA.

(25) Surgically enucleated hypertrophic prostates were homogenized in a Waring Blendor with 10 volumes of cold distilled water and filtered through a thin layer of Celite. The resulting cloudy solution was lyophilized and the powder was used for the experiments.

After incubation the tubes were cooled to 0°, deproteinized by the addition of 0.02 ml. of 4 *N* $HClO_4$ and centrifuged. Samples were removed for the determination of inorganic phosphate. The deproteinized supernatant was neutralized to *p*H 6 with 4 *N* KOH. The amount of sulfate liberated and the degradation products formed were determined by radioactivity measurements after paper electrophoresis. The identity of the degradation products was further checked by paper chromatography.

Results and Discussion

The material from a small scale synthesis was separated by paper electrophoresis at *p*H 3 into four spots containing both adenine (as indicated by ultraviolet absorption) and sulfate (as indicated by radioactivity). After zone electrophoresis on a cellulose column at the same *p*H, sizeable amounts of the four compounds were obtained and analyzed with the results shown in Table II.

TABLE II

ANALYTICAL DATA OF AMP-SULFATE FRACTIONS AFTER ELECTROPHORESIS AT *p*H 3

Compound	Sulfate/ phosphate	Ribose/ phosphate	Adenine/ phosphate
D[a]	3.45	1.01	1.00
C[a]	2.75	0.98	0.91
B[a]	1.88	0.92	.88
A[a]	0.99	1.02	.99

[a] *Cf.* Table I and ref. 8a.

The most rapidly moving component (D) probably consists of a mixture of AMP-sulfates (sulfate/phosphate = 3.45). This compound was generally obtained only after very prolonged synthesis or with a large excess of sulfate. It was not further investigated.

The data for the other compounds fit best with the formulation of C as an AMP-trisulfate, B as an AMP-disulfate and A as an AMP-monosulfate. Compound A could be further separated into two different monosulfates by electrophoresis at *p*H 7–10. The amounts of adenine in compounds A–D were determined by measurements of absorbancy at 260 mμ. The light absorption curves of these compounds at *p*H 1 and *p*H 6.8 showed maxima and minima at the same wave lengths as AMP. Also the shapes of the curves between 220 and 320 mμ were almost identical to that of AMP.

It will be shown below that at *p*H 7–10 during electrophoresis the slowest moving monosulfate corresponded to adenosine 5'-phosphosulfate while the more rapid monosulfate was found to be a mixture of adenosine 3'-sulfate-5-phosphate and adenosine 2'-sulfate-5'-phosphate. These compounds could also be separated from each other by paper chromatography in systems I and II. The same solvents did not further resolve the di- and trisulfate peaks.

A study of the influence of time on the formation of the different AMP-sulfates revealed that the dominating primary compound was adenosine-5'-phosphosulfate. Prolongation of the reaction time (Fig. 2) resulted in extensive formation of compounds containing more than one sulfate group per molecule of AMP. Increase of the molar ratio of sulfuric acid to AMP also increased the formation of these latter components.

Since in the first place the synthesis of the biologically interesting APS was attempted the optimal conditions for its formation without the simultaneous formation of other compounds were studied. The method described in the experimental section was found satisfactory in this respect. Optimal formation of APS occurred at slightly varying time points in different experiments and it was therefore of advantage to follow the course of the synthesis by paper electrophoresis. The reaction was terminated at a point where a 40–50% conversion of AMP to APS had occurred.

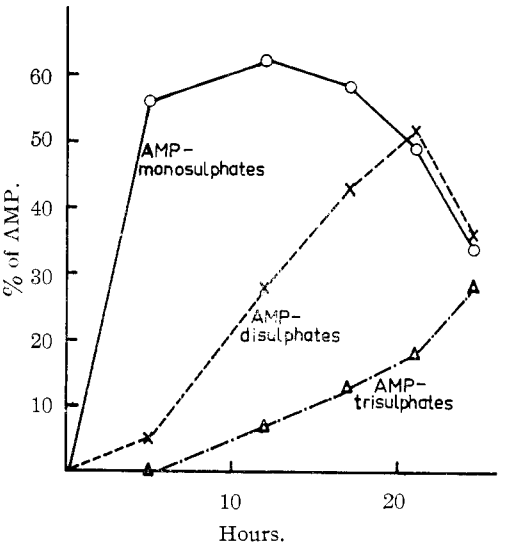

Fig. 2.—Time curve for the formation of AMP-sulfates. The calculations are based on S^{35}-determinations.

After treatment with Dowex 50 and charcoal more than 80% of the ultraviolet absorption could be accounted for as APS and less than 10% as other AMP-sulfates, the remainder being AMP which arose from the degradation of APS after the Dowex 50 treatment. For many purposes such a preparation might be sufficiently pure.

Complete freedom from other AMP-sulfates was obtained after zone electrophoresis. After this step and the subsequent lyophilization the preparation contained about 5% of AMP. However, it was possible to obtain preparations completely free from AMP by passing a concentrated (0.1–0.2 *M*) solution of APS through small columns of Dowex-50-H^+ (0.4 × 5 cm.) and immediately neutralizing the effluent. Because of the lability of the anhydride linkage, pure APS-solutions should be stored in the frozen state.

Characterization of Different AMP-sulfates. Adenosine 5'-Phosphosulfate (APS) was characterized by the following properties.

It contained equimolar amounts of adenine, ribose, phosphate and sulfate. One mole of APS consumed 1.04 moles of periodate without liberation of sulfate, demonstrating the presence of two hydroxyl groups attached to adjacent carbon atoms.

After deamination, a compound was obtained which still contained sulfate and moved faster than APS during electrophoresis at pH 3. At this *p*H the mobility of the compound (as determined by ultraviolet and radioactivity detection) was almost identical with that of the AMP-disulfates, while at *p*H 5 the mobility was almost the same as that of

TABLE III

ENZYMIC DEGRADATION OF VARIOUS AMP-SULFATES BY SNAKE VENOM AND PROSTATIC PHOSPHATASES

	% phosphate liberated	% sulfate liberated	UV-absorbing product[b]
Snake venom phosphatase			
Adenosine 5'-phosphosulfate	100	100	Adenosine
Adenosine 2'-(or 3'-) sulfate-5'-phosphate	0–30[a]	10–10[a]	Adenosine 2'-(or 3') sulfate-5'-phosphate
Adenosine 2',3'-disulfate-5'-phosphosulfate	5	31	Adenosine 2'-, 3'-disulfate-5'-phosphate
Prostatic phosphatase			
Adenosine 5'-phosphosulfate	74	94	Adenosine
Adenosine 2'-(or 3'-) sulfate-5'-phosphate	96	8	Adenosine 2'-(or 3'-) sulfate
Adenosine 2',3'-disulfate-5'-phosphosulfate	88	38	Adenosine 2',3'-disulfate

[a] Two different experiments were performed. [b] These compounds were identified by their paper electrophoretic mobilities at *p*H 3 and 9.5 and by paper chromatography in solvents I and II.

APS. This showed that the new compound at *p*H 3 contained one more negative charge than APS, while at *p*H 5 the charges were identical. Such a behavior is explained by the absence of a free amino group in the new compound. When this compound was prepared by zone electrophoresis it was found to give an ultraviolet spectrum which was very similar to that of inosine 5'-phosphate. The compound was treated with snake venom phosphatase. The products of the reaction were sulfate, phosphate and a third substance which moved like inosine during paper chromatography (1-butanol saturated with water). The results are thus in agreement with a formulation of the deaminated compound as inosine 5'-phosphosulfate and demonstrate that the sulfate group before deamination was not attached to the amino group of AMP.

Acid hydrolysis and CaO hydrolysis[23] very rapidly liberated inorganic sulfate from APS while alkaline hydrolysis resulted in a slower liberation of sulfate (Fig. 3).

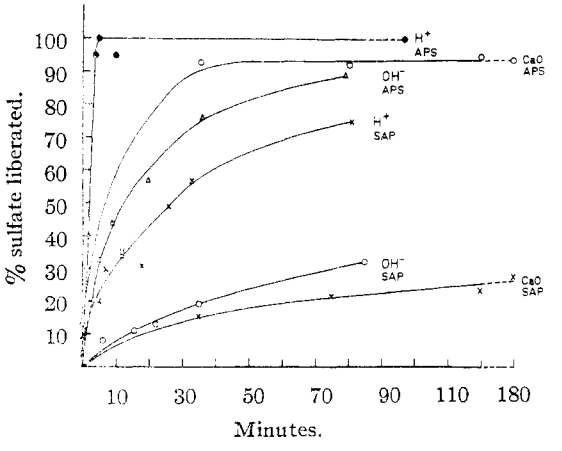

Fig. 3.—Time curves for acid, alkaline and CaO hydrolysis of APS and SAP.

Crude snake venom and prostatic phosphatases liberated equimolar amounts of phosphate and sulfate (Table III) and a compound which on paper chromatography (solvents I and II) and paper electrophoresis (*p*H 3.0) behaved like adenosine. Obviously both enzyme preparations used in our experiments contained an enzyme which was able to split the phosphosulfate linkage with the formation of free sulfate + AMP. Subsequently AMP was dephosphorylated.

It was also of interest to investigate the ability of the synthetic APS to act as substrate for the enzymes described by Lipmann and co-workers.[4] It was found that synthetic APS reacted with pyrophosphate in the ATP-sulfurylase reaction and formed ATP + sulfate.[26]

The **other AMP-monosulfate** was separated from APS by electrophoresis at *p*H values above 6.6. This behavior indicated that the dissociation of a secondary phosphate group was involved and that the sulfate group therefore was not bound to phosphate.

Deamination of this monosulfate gave similar results as with APS and showed that the amino group of the compound was free and not involved in the binding of sulfate.

During periodate titration a maximum of 0.08 mole of periodate was consumed per mole of compound. This indicated the absence of vicinal OH-groups in the ribose moiety and showed that at least one of the OH-groups in the 2'- or 3'-positions of AMP was esterified with sulfuric acid.

The relative stability toward acid, alkaline and especially CaO hydrolyses (Fig. 3) also speaks strongly against an anhydride or sulfamate linkage but is in accordance with the behavior of a sulfate ester.

Prostatic phosphatase completely dephosphorylated the compound without releasing any sulfate. The electrophoretic behavior of the resulting adenosine sulfates is recorded in Table I. However, very little liberation of sulfate or phosphate took place when the digestion was carried out with snake venom (Table III). It was earlier found by Kornberg and Pricer[27] that adenosine 2'(or 3')-5'-diphosphate is not attacked by crude snake venom phosphatase. Our results show that a sulfate group on carbon 2 or 3 of the ribose likewise constitutes a hindrance for the action of snake venom phosphatase. The results are thus further evidence for a ribose–sulfate linkage. The prostatic phosphatase was not inhibited in its action. None of the enzyme preparations attacked sulfate ester linkages.

The experiments reported so far demonstrate that the compound is either adenosine 2'-sulfate-5'-phosphate or adenosine 3'-sulfate-5'-phosphate or a mixture of these two sulfate esters. Theoretically one would expect esterification to take

(26) We wish to thank Dr. P. W. Robbings for testing the enzymic activity of our APS.

(27) A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.*, **186**, 557 (1950).

place with about equal ease in both the 2′- and 3′-position of AMP. Evidence for this was obtained by paper chromatography during which the compound separated into two spots of about equal radioactivity and ultraviolet absorption (Table I). Furthermore, after dephosphorylation with prostatic phosphatase, the resulting adenosine sulfates again separated into two spots of about equal radioactivity and ultraviolet absorption (Table I).

Adenosine 2′-Sulfate-5′-phosphosulfate, Adenosine 3′-Sulfate-5′-phosphosulfate and Adenosine 2′,3′-Disulfate-5′-phosphate.—The data on the AMP-monosulfates showed that sulfate groups during the synthesis were attached to both the ribose and phosphate moieties of AMP. It was therefore expected that the disulfate peak after zone electrophoresis at *p*H 3 would be a mixture of three isomers. We have not separated the three disulfates from each other by chromatography or electrophoresis, but the analytical results confirmed our assumption.

Attempts at acid, alkaline and CaO hydrolysis gave irregular results with different preparations. Between 20–60% of the sulfate was rapidly liberated while further liberation always occurred at a much slower rate. The degradation products from CaO hydrolysis on paper chromatography behaved like sulfate and adenosine 2′-sulfate-5′-phosphate and adenosine 3′-sulfate-5′-phosphate. Digestion with prostatic phosphatase partially degraded the mixture to compounds that moved like adenosine sulfates, adenosine 2′-sulfate-5′-phosphate and adenosine 3′-sulfate-5′-phosphate.

Deamination could be carried out without liberation of sulfate. The material consumed no periodate.

Adenosine 2′,3′-Disulfate 5′-Phosphosulfate.—The trisulfate peak from the *p*H 3 electrophoresis moved as a single compound during chromatography and electrophoresis and contained sulfate bound to both phosphate and the 2′- and 3′-positions of AMP.

Consistent results were obtained with different preparations on hydrolysis with acid, alkali and CaO. When treated with 0.4 *N* $HClO_4$, the hydrolysis curve showed a rapid phase in the beginning followed by a much slower liberation of sulfate (Fig. 4). During early stages the formation of an AMP-disulfate could be demonstrated by paper electrophoresis. Further decomposition by splitting of the adenine–ribose linkage occurred at the same time. During alkaline and CaO hydrolysis about one out of the three sulfate groups was rapidly liberated with the simultaneous formation of an AMP-disulfate (adenosine 2′, 3′-disulfate-5′-phosphate) which was quite stable toward further hydrolysis (Fig. 4).

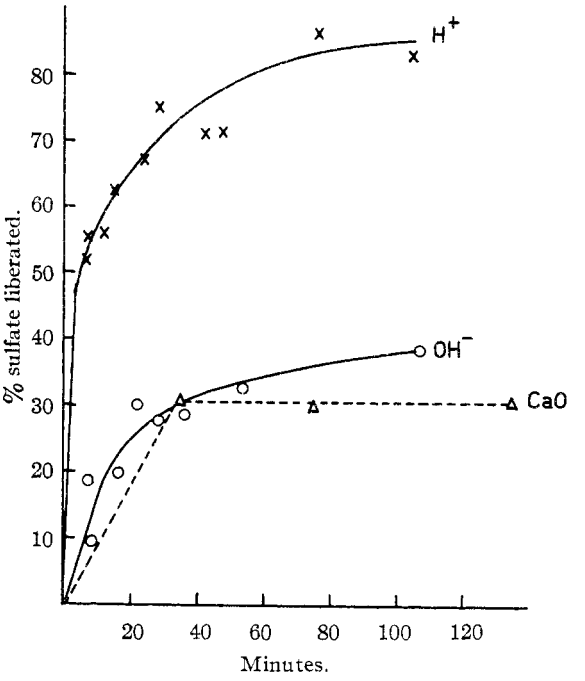

Fig. 4.—Time curves for acid, alkaline and CaO hydrolysis of adenosine 2′,3′-disulfate-5′-phosphosulfate.

Snake venom phosphatase produced AMP-disulfate and sulfate in equimolar amounts, demonstrating that the enzymic hydrolysis stopped after breakage of the phospho–sulfate linkage and did not further dephosphorylate the formed adenosine 2′, 3′-disulfate-5′-phosphate. Prostatic phosphatase, however, attacked both the phospho–sulfate and the ribose–phosphate bonds giving adenosine 2′ 3′-disulfate, phosphate and sulfate (Table III). This difference in the effect of snake venom and prostatic phosphatases is in agreement with the effects recorded above on APS and adenosine 2′ (and 3′) - sulfate -5′-phosphate: a sulfate group on the2′- or 3′-position of AMP constitutes a steric hindrance for the action of snake venom phosphatase but not for prostatic phosphatase.

During deamination of the trisulfate no liberation of sulfate took place. No periodate was consumed by the compound.

In summary the evidence clearly indicates that the AMP-trisulfate is identical with adenosine 2′,3′-disulfate-5′-phosphosulfate.

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