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*THE ENZYMATIC ACTIVATION OF SULFATE**

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INTRODUCTION

The problem of sulfate activation is somewhat unusual in so far as it represents an ATP-linked activation of an inorganic compound other than phosphate. DeMeio *et al.*[1] and Bernstein and McGilvery[2] showed that the over-all reaction is ATP-linked. Furthermore, a preliminary separation into two fractions has been reported.[3] In view of certain similarities to the carboxyl activation, we have recently turned our attention to this unusual reaction and will report here experiments which are still somewhat preliminary but have already led to a tentative identification of the active sulfate intermediary as an adenyl sulfate.

Lamb liver was found to be a rather good source of the over-all transfer reaction to nitrophenol. The sulfate activation could, furthermore, be studied with *Neurospora* extracts, which were found to contain a strong sulfate-activation system but no transfer enzyme to nitrophenol.

ENZYME PREPARATIONS

Test System for Nitrophenyl Sulfate Synthesis.—Assay mixture: 50 μM imidazole-HCl buffer, pH 7.0; 6.25 μM $MgCl_2$; 0.50 μM nitrophenol; 5 μM cysteine; 5 μM K_4ATP; 5–10 μM K_2SO_4; and enzyme solution in a final volume of 0.50 ml. or the doubled amounts in 1.0 ml.

After incubation at 38° for 60 minutes, the reaction is stopped by the addition of 2 ml. of alcohol, and the mixture is freed of the precipitated protein by centrifugation. To 2.5 ml. of 0.1 *N* KOH is added 0.50 ml. of the supernatant, and, after mixing, the extinction is measured at 420 μM in the Klett-Summerson photometer

and compared with a blank without incubation. Within the range of 10–100 mμM of nitrophenol, the extinction-concentration curve of the nitrophenol anion is practically linear.

For actual determination of nitrophenyl sulfate, an aliquot of a trichloroacetic acid filtrate is heated for 10 minutes at 100° in normal HCl. After neutralization and the addition of alcohol and KOH, the extinction of the hydrolyzate is compared with that of the untreated sample. The difference represents nitrophenyl sulfate formation. Generally, only the disappearance of nitrophenol anion needed to be measured. It was found that the values after hydrolysis corresponded closely to blanks before incubation. This indicates that under the conditions of the test no metabolism of nitrophenol occurred other than sulfurylation.

Lamb Liver Preparation.—Three fresh lamb livers were chilled and minced with a meat grinder. One thousand grams of liver mince were suspended in 3 liters of a solution containing 255 gm. of sucrose and 25 gm. of $KHCO_3$. Suitable portions of this suspension were disintegrated with a Waring Blendor for 20 seconds, and the homogenate spun for 15 minutes at 1,600 rpm. The supernatant containing mitochondria and microsomes was poured through cheese cloth, and one-tenth of its volume of 1.5 *M* KCl was added under vigorous stirring. The mitochondria now were spun off at 5,000 rpm for 1 hour. To obtain clear and more active preparations, the still cloudy supernatant was recentrifuged for 1 hour at 30,000 rpm (Spinco). A suitable acetone powder of this supernate fraction may be prepared in the usual manner, without loss of activity.

Ammonium Sulfate Fractionation of Liver Supernate.—To 350 ml. of the clear liver supernatant are added 110 gm. of ammonium sulfate recrystallized from versene solution (cf. Beisenherz *et al.*[4]), and the solution is centrifuged after 1 hour. The precipitate is dissolved in 15 ml. of water. This preparation, LS 50, is relatively stable when kept frozen.

Separation of the Lamb Liver System into Two Complementary Fractions by Alumina Gel Treatment.—Two hundred and fifty milliliters of clear lamb liver supernatant plus 100 ml. of alumina Cγ gel are stirred for 30 minutes and then centrifuged at 3,000 rpm for 20 minutes. This solution is referred to as "gel supernatant" (10 mg. of protein per milliliter). It contains the sulfate-activating enzyme.

Elution of the Gel.—The gel was extracted with 500 ml. of 0.20 saturated ammonium sulfate solution, pH 7.2. To the extract a solution of 4 *M* (= saturated) ammonium sulfate, pH 7, containing 0.002 *M* versene was added to a final saturation of 0.525, and the precipitate was collected and dissolved in 0.02 *M* tris buffer, pH 7.0, referred to as "EAS 52.5" (36 mg. of protein per milliliter).

Ninety milliliters of this solution were diluted with an equal volume of cold water and refractionated between 0.20 and 0.35 saturation of ammonium sulfate. The precipitate was dissolved in 20 ml. of tris buffer, pH 7.0, and yielded a brownish-green solution referred to as "EAS II 35" (90 mg. of protein per milliliter).

An additional refractionation of this fraction did not increase the specific activity but removed some ATP-ase: 20 ml. of EAS II 35 were diluted with 80 ml. of cold water, and the fraction between 0.30 and 0.40 saturation was collected and dissolved in 10 ml. of water. This is referred to as "EAS III 40" and contained 45 mg. of protein per milliliter. These eluate fractions contain the transfer enzyme from "active" sulfate to nitrophenol.

Removal of Sulfate from Enzyme Solutions.—To free the ammonium sulfate fractions of sulfate, they were dialyzed for 2 hours against an 0.01 *M* tris buffer, pH 7.4 which removed a large amount of the sulfate without great loss in activity. To remove residual sulfate, the preparation was then treated twice with Dowex-1 acetate. It was found necessary to suspend the resin in a little water or buffer and to evacuate before application to the enzyme solution, thus preventing the formation of air bubbles, which caused a partial inactivation of the enzyme. Approximately 100 mg. of semidry Dowex-1 were used for 1 ml. of enzyme solution.

The precipitation of sulfate by the addition of barium acetate at pH 6 resulted in a greater loss of activity. In order to obtain sulfate-free enzyme preparations, attempts were made to purify the system by means other than ammonium sulfate precipitation. Such attempts, however, have failed so far since alcohol or acetone fractionation and acid precipitation caused considerable inactivation.

Enzyme solutions freed of sulfate showed increased sensitivity toward fluoride. These preparations were inhibited by *M*/20 fluoride to 80–100 per cent, whereas the nontreated fractions were only inhibited to 40–50 per cent by the same concentration.

PREPARATION OF NEUROSPORA EXTRACTS

Growth Medium.—Ten grams of Bactopeptone, 40 gm. of sucrose, and 0.10 ml. of saturated ammonium sulfate solution were dissolved in 1 liter of distilled water, sterilized, and inoculated with *Neurospora sitophila*, American Type Culture Collection No. 9278. With adequate aeration, good growth is obtained at 35°. The mycelium was harvested after 24–36 hours by filtering through three layers of cheesecloth.

Extraction of Neurospora Paste.—Two hundred and fifty grams of semidry *Neurospora* mycelial cells were ground with 100 gm. of alumina and 500 ml. of 0.02 *M* tris buffer, pH 7.5, and the mixture was sonorated for 15 minutes in a 10-kc. Raytheon oscillator and centrifuged on a Servall centrifuge. The turbid supernatant, 3 mg. of protein per milliliter, was frozen. This extract was freeze-dried and the residue taken up in 0.02 *M* tris buffer, pH 7.0. The residue of 100 ml. of extract was dissolved in 10 ml. of buffer.

MATERIALS AND METHODS

The compounds used were as follows:

p-Nitrophenol: The commercial product (Eastman Kodak) was purified by sublimation under reduced pressure.

p-Nitrophenyl sulfate: The substance was synthesized from chlorosulfonic acid and *p*-nitrophenol as described by Burkhardt and Wood. Solutions of the compound are stable when kept at 0° and at slightly alkaline pH.[5]

ATP: The crystalline product of the Pabst Laboratories (Milwaukee, Wisconsin) was used in most cases after neutralization with KOH or $KHCO_3$.

PEP: The barium silver salt was converted into the potassium salt by removing the silver with HCl and the barium with Dowex-50 in free acid form in the cold. The Dowex-treated solution was neutralized with potassium hydroxide.

AMP, ADP, TIP, and GDP are commercial products from the Pabst Laboratories.

For a preparation of hexokinase and one of pyruvokinase we are indebted to Dr. John Gregory.

PYROPHOSPHATE DETERMINATION WITH PYROPHOSPHATASE

An aliquot of a trichloroacetic acid filtrate, e.g., 0.1 ml., was taken, to which were added 0.30 ml. of 1 *M* tris buffer, pH 7.5; 0.025 ml. of 0.25 *M* $MgCl_2$; 0.55 ml. of H_2O; 0.25 ml. of a solution of 6-times-recrystallized pyrophosphatase[6] kindly supplied to us by Dr. M. Kunitz, containing 8 μg/ml. A blank was run without pyrophophatase. The protein content of the pyrophosphatase solution may be neglected and phosphate determined without deproteinization.

PAPER ELECTROPHORESIS

The apparatus described by Markham and Smith[7] was used with minor alterations, and for the separation of the nucleotides the procedure described by Berg and Joklik[8] was applied.

EXPERIMENTAL RESULTS

Some Characteristics of the System.—In confirmation of earlier work by other workers,[9] the presence of an active SH-function in the enzyme system is indicated by the increase of activity with cysteine, as shown in Table 1, and by the inhibition with mersalyl, *o*-[(3-hydroxymercuri-2-methoxypropyl)carbamyl]phenoxyacetic

TABLE 1*

EFFECT OF VARIOUS COMPOUNDS ON THE SYNTHESIS OF NITROPHENYL SULFATE

Additions	mμM NPS Formed	Additions	mμM NSP Formed
None	172	0.05 *M* Na_2SO_3	180
0.01 *M* cysteine	220	0.01 *M* Na_2SeO_4	0
0.001 *M* mersalyl	10		

* The complete system contained 100 μM tris buffer, pH 7.1; 12.5 μM $MgCl_2$; 10 μM K_2SO_4; 10 μM crystalline ATP; 1 μM nitrophenol; and purified liver enzyme (LS 50) (4.7 mg. protein) in 1-ml. volume. Incubated at 38° for 1 hour.

acid), which can be reversed by cysteine. It was found that mersalyl inhibits both the sulfate-activation and the transfer reaction. No effect of sulfite was observed; however, selenate is very inhibitory, and this inhibition is not reversed by excess of sulfate. In Table 2 the requirement for divalent ions is shown. Magnesium is most active, but manganese also shows a fair activity. Versene, on the other hand, inhibits at higher concentrations.

TABLE 2*

MAGNESIUM OR MANGANESE REQUIREMENT

Metal Ion	Versene	mμM NPS Formed	Metal Ion	Versene	mμM NPS Formed
None	None	43	Mg^{++}, 0.0125 *M*	0.002 *M*	564
Mn^{++}, 0.02 *M*	None	420	Mg^{++}, 0.0125 *M*	0.04 *M*	64
Mg^{++}, 0.0125 M	None	680			

* The complete system contained 100 μM imidazole-HCl buffer, pH 7.0; 10 μM K_4ATP; 10 μM K_2SO_4; 10 μM cysteine; 1 μM nitrophenol; 6 mg. protein, fraction LS 50 in 1 ml. final volume. Incubated at 38° for 1 hour.

PRELIMINARY IDENTIFICATION OF THE LIVER FRACTIONS

Indications for the separability of the sulfate-activation and sulfate-transfer reaction had been observed previously.[10, 3] Bernstein and McGilvery showed an accumulation of "active" sulfate on preincubation of ATP with sulfate. As described above in the section on liver preparations, a separation of the liver system into two enzymes was obtained by adsorption on alumina gel. The gel adsorbs mainly the sulfate-transferring enzyme (cf. experiments with radioactive sulfate),

which is eluted by stirring with dilute ammonium sulfate solution. As shown in Table 3, after gel absorption the supernate fraction does not form any nitrophenyl

TABLE 3*

MULTIPLE-ENZYME REQUIREMENT FOR NITROPHENYL SULFATE SYNTHESIS (LAMB LIVER)

Additions	mμM NPS Formed	Additions	mμM NPS Formed
Fraction I, gel supernate	0	Fraction I + Fraction II	340
Fraction II, gel eluate, EAS 52.5	182	Fraction I boiled + Fraction II	176

* The complete system contained 100 μM imidazole buffer, pH 7.0; 12.5 μM $MgCl_2$; 10 μM K_2SO_4; 10 μM crystalline ATP; 1 μM nitrophenol; 5 μM cysteine; and purified liver fractions (Fraction I: 1 mg. protein; Fraction II: 3.6 mg. protein) in 1-ml. volume. Incubated at 38° for 1 hour.

sulfate. The gel eluate alone forms some nitrophenyl sulfate, the formation of which is, however, nearly doubled on addition of the supernate fraction. This effect is lost on boiling.

THE SULFATE-ACTIVATING EFFECT OF NEUROSPORA EXTRACT

The liver fractionation has shown a separation into two fractions; the identification of these fractions was simplified by use of extracts from *Neurospora*. It was found that *Neurospora* extracts did not catalyze the esterification of nitrophenol. In conjunction with experiments with radioactive sulfate, the presence of a sulfate-activating enzyme in these extracts appears, however, on combination with the transferring enzyme from liver. As shown in Table 4, the *Neurospora* extracts pre-

TABLE 4*

REPLACEMENT OF LIVER-ACTIVATING FRACTION BY *Neurospora* EXTRACT

Mg. *Neurospora* Protein Added	Liver Fractions	mμM NPS Formed	Mg. *Neurospora* Protein Added	Liver Fractions	mμM NPS Formed
0	II	60	2.5 boiled	II	63
1	II	304	2	I	0
5	II	422	1	None	0
10	II	254			

* The complete system contained 100 μM imidazole-HCl buffer, pH 7.0; 12.5 μM $MgCl_2$; 10 μM K_2SO_4; 10 μM crystalline ATP; 1 μM nitrophenol; 5 μM cysteine; and protein. Fraction I: 1 mg. of protein of gel supernatant; Fraction II: 4 mg. of transferring enzyme preparation, obtained by refractionation of LS 50 between 30 and 35 per cent saturation of ammonium sulfate. Incubated at 38° for 1 hour.

pared in the manner described above are inactive by themselves but become very active for nitrophenol sulfurylation if combined with liver Fraction II, which thus contains the transfer enzyme. On the other hand, this identifies the gel supernate Fraction I (cf. Table 3) with the sulfate-activating enzyme. It should be noted that with the intermediate concentration of *Neurospora* extracts, the yield of nitrophenyl sulfate is highest. This is probably due, at least in part, to the presence of an enzyme in *Neurospora* extract which decomposes the product of the ATP-sulfate reaction. A further characterization of the sulfate-activating enzyme was obtained by the use of radioactive sulfate and will be discussed below.

Phosphate Balance.—For the eventual identification of the mechanism of sulfate activation, it was most important to find out in what manner ATP reacted with the sulfate. In Table 5 the effect of sulfate, and of sulfate plus nitrophenol, on the phosphate balance is shown with various enzyme preparations. For this purpose

TABLE 5*

PHOSPHATE BALANCE IN THE ABSENCE OF FLUORIDE

Enzyme Fraction	Additions	μM P_i Formed	Δ μM P_i	μM NPS Formed
AS III 40	None	2.08	..	..
	Sulfate	2.36	0.28	..
	Sulfate + nitrophenol	2.73	0.64	0.26
AS II 35	None	3.82	..	..
	Sulfate	4.44	0.42	..
	Sulfate + nitrophenol	4.79	0.97	0.50
LS 50	None	11.68	..	..
	Sulfate	12.96	1.28	..
	Sulfate + nitrophenol	13.46	1.80	0.60

* The complete system contained 100 μM imidazole-HCl buffer, pH 7.0 (in the third experiment, pH 6.7); 12.5 μM $MgCl_2$; 10 μM cysteine; 10 μM crystalline ATP; 10 μM K_2SO_4 when indicated; 1.0 *p*-nitrophenol; and enzyme (AS III 40, 6 mg.; AS II 35, 10 mg.; LS 50, 15 mg. protein) in a final volume of 1 ml. Incubated at 38° for 1 hour. All enzyme fractions were freed from sulfate by Dowex treatment as described. Phosphate determination according to C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66** 375, 1925.

the enzyme was freed of sulfate by Dowex treatment, as described above. Unfortunately, the removal of sulfate makes these preparations particularly sensitive to fluoride. Therefore, fluoride could not be used here for an inhibition of the pyrophosphatase activity present in these preparations, and pyrophosphate could not be detected as such but would be split to inorganic phosphate. It was disturbing, furthermore, that a considerable background of inorganic phsophate appeared in the blanks. The data in Table 5 nevertheless, show quite clearly that sulfate addition alone increases phosphate liberation, and the further addition of nitrophenol gives further stimulation of phosphate liberation from ATP. A comparison between nitrophenyl sulfate formed and phosphate liberated above the blank shows that twice as much phosphate as nitrophenyl sulfate is formed. In view of the presence of active pyrophosphatase, these data indicate that pyrophosphate and not phosphate may be the initial product of the ATP-sulfate reaction.

The formation of pyrophosphate is further substantiated by experiments carried out with the addition of fluoride to preserve the pyrophosphate (cf. Jones *et al.*[11]). In this case sulfate was not removed, and therefore the effect of sulfate alone does not appear distinctly. As shown in Table 6, the incubation of this preparation with

TABLE 6*

PYROPHOSPHATE FORMATION

Enzyme Fraction	Additions	μM P_i Formed	μM P_i after Pyrophosphatase	μM PP (P_i/2)	μM NPS
LS 50 (12 mg. protein)	Sulfate	5.70	6.39	0.35	..
	Sulfate + nitrophenol	5.56	6.98	0.71	0.28
AS III 40 (15 mg. protein)	Sulphate	0.91	1.55	0.32; 0.33†	..
	Sulfate + nitrophenol	1.02	1.96	0.47; 0.46†	0.40

* The complete system contained 100 μM imidazole-HCl buffer, pH 7.0; 10 μM ATP; 10 μM K_2SO_4; 12.5 μM $MgCl_2$; 10 μM cysteine; 1 μM *p*-nitrophenol when indicated; 50 μM KF; and protein as indicated, in a final volume of 1 ml. Incubation was at 38° for 1 hour.

† Pyrophosphate figures obtained colorimetrically (see R. M. Flynn, M. E. Jones, and F. Lipmann, *J. Biol. Chem.*, **211** 791, 1954).

ATP alone leads now to liberation of pyrophosphate, the amount of which increased on the addition of nitrophenol. A comparison between the amounts of nitrophenyl sulfate formed and of pyrophosphate liberated is somewhat difficult in view of the fact that the pyrophosphate formed in the absence of nitrophenol cannot be surely identified with the ATP-sulfate reaction because blanks in the absence of sul-

fate were not feasible. There is, nevertheless, proportionality between pyrophosphate formation and nitrophenol esterification. In conjunction with the 2:1 ratio for phosphate liberated to nitrophenyl sulfate formed, shown in Table 5, and the effect of sulfate in the sulfate-free system in the absence of fluoride, it seems justifiable to assume that much of the pyrophosphate formed in the absence of nitrophenol is due to the ATP-sulfate reaction, in which case the data would indicate equivalence between pyrophosphate and nitrophenyl sulfate formation. We consider these results a strong indication that the initial reaction follows the equation ATP + sulfate = AMP-sulfate + pyrophosphate. Further support for such a mechanism will appear from experiments with radioactive sulfate.

As shown in Table 7, the addition of other nucleotide polyphosphates did not cause any increase above the effect of crystalline ATP, which appears to be free of other than adenine polyphosphates. It is therefore assumed that the ATP-sulfate reaction in liver is truly ATP-linked. The same appears to hold for the *Neurospora* system.

TABLE 7*

EFFECT OF VARIOUS NUCLEOTIDIC POLYPHOSPHATES

μM ATP	μM Nucleotides Added	mμM NPS Formed	μM ATP	μM Nucleotides Added	mμM NPS Formed
9	None	166	9	1.25 ITP	144
9	0.50 GDP	160	9	1.25 UTP	200
9	0.50 CTP	130	10	None	190

* The complete system contained 100 μM imidazole-HCl buffer, pH 7.0; 10 μM K_2SO_4; 12.5 $MgCl_2$; 1 μM nitrophenol; 3 mg. protein (liver supernatant); and nucleotides as indicated. Incubation was at 38° for 1 hour.

EXPERIMENTS WITH RADIOACTIVE SULFATE

If the sulfate-activating enzymes of either liver or *Neurospora* are incubated with ATP and radioactive sulfate, and the trichloroacetic acid extracts are submitted to paper electrophoresis, a substance containing radioactive sulfate appears somewhat above the ATP level and considerably below the spot corresponding to inorganic sulfate. The radioautographic tracings overlap with ultraviolet absorption as traced by Mineralite. Such tracings are shown in Figure 1; fluoride does not, but mersalyl does, strongly inhibit the sulfate-activation reaction. In this experiment *Neurospora* extract was used. A further confirmation that the sulfate radioactivity coincides with a compound originating from ATP is shown by an experiment in which carbon-labeled ATP and radioactive sulfate were used in parallel. As shown in Figure 2, the carbon radioactivity coincides in shape and height closely with the sulfur radioactivity. The deeper shading in the sulfate tracing is due to a greater activity of the sulfate used compared with the carbon label in ATP.

The identity of this substance with the "active" sulfate was confirmed in the experiment shown in Figure 3. Here *Neurospora* extract was first incubated in the usual manner with ATP and radioactive sulfate; after incubation the enzyme was inactivated by heating (sample 1), and the sulfate-transfer enzyme from liver, together with nitrophenol, was now added to the heated sample and reincubated (sample 2). Both samples were then deproteinized and submitted to paper electrophoresis, and the chromatogram eventually radioautographed. The tracing in Figure 3 shows that after incubation of ATP + *Neurospora* enzyme, radioactivity and ultraviolet absorption appear as in Figures 1 and 2, above the ATP mark,

EFFECT OF FLUORIDE AND MERSALYL ON THE SULFATE ACTIVATING ENZYME

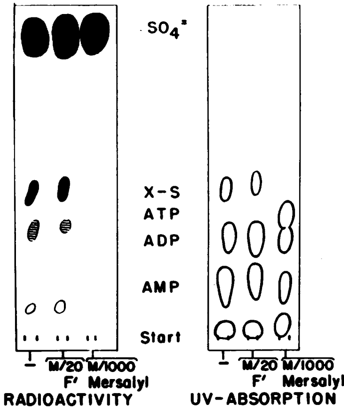


FIG. 1—50 μM tris buffer, pH 8.4; 6 μM $MgCl_2$; 4 μM ATP; 5 μM PEP; 4 μM $K_2S^{35}O_4$ (specific activity 50 MC per gm.); 6 μM cysteine; and 5 mg. lyophilized *Neurospora* protein in 0.5 ml. were incubated at 38° for $2^1/_2$ hours.

Without deproteinization, 0.02 ml. of each sample were applied on Whatman No. 3 paper soaked with 0.1 *M* citrate buffer, pH 5.5, and subjected to electrophoresis at 200 V for 12 hours. It should be noted that radioactivity and ultraviolet absorption coincide. Radioactivity from radioautograph indicated by shaded areas; ultraviolet absorption, traced with Mineralite, indicated by empty areas.

THE DETECTION OF A SULFATE-CONTAINING INTERMEDIATE IN NITROPHENYL SULFATE SYNTHESIS

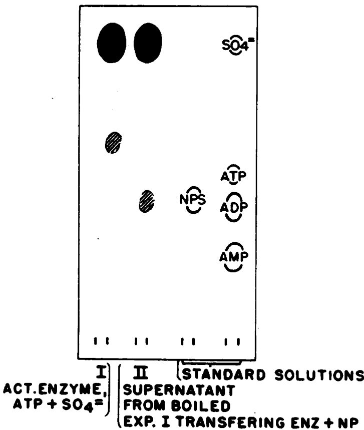


FIG. 3.—0.5 ml. containing 50 μM imidazole-HCl buffer, pH 7.0; 6 μM $MgCl_2$; 5 μM ATP; 5 μM $K_2S^{35}O_4$; 5 μM cysteine; and 1 mg. lyophilized *Neurospora* extract. Incubated for 40 minutes at 38°. The reaction was then stopped by immersion in a boiling water bath for 1 minute. After cooling in ice, 1.5 mg. of protein of the fraction containing the purified transferring enzyme and 2.5 μM nitrophenol were added to sample 2 and a corresponding amount of water to sample 1; both were incubated for another 40 minutes, and the reaction stopped by adding an equal volume of ice-cold 5 per cent TCA.

Electrophoresis: 0.025 ml. of the TCA filtrate of each sample, and standard solutions of NPS and the three adenyl phosphates, are put on a Whatman No. 3 paper strip soaked with 0.1 *M* citrate buffer, pH 5.5, and subjected to electrophoresis at 200 V for 12 hrs. Adenine compounds and NPS were localized by ultraviolet absorption. Radioactivity is indicated by shaded areas.

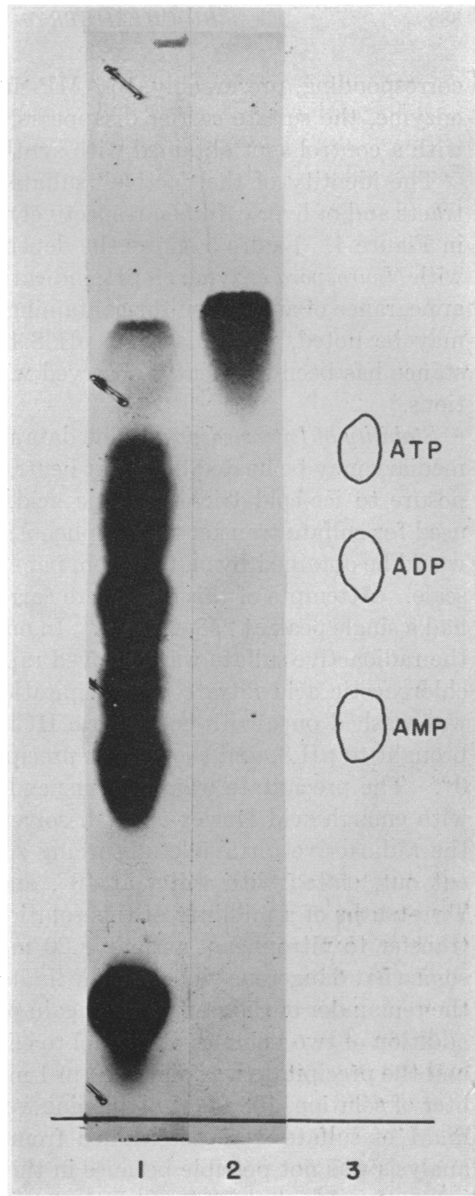


FIG. 2.—Coincidence of carbon label from ATP, and S^{35} label from sulfate, after enzymatic condensation. No. 1: LS 50 incubated with C^{14}-labeled ATP (Schwarz) + sulfate; No. 2: LS 50 incubated with S^{35}-sulfate + ATP; No. 3: ultraviolet tracings of check mixture of ATP, ADP, and AMP electrophoresis with 0.08 *M* citrate of pH 5.8, as usual.

corresponding, presumably, to AMP-sulfate. On incubation with the transferring enzyme, the sulfate carrier disappears, to give rise to a radioactive spot identical with a control spot obtained with synthetic nitrophenyl sulfate.

The identity of the "active" sulfate obtained by incubation of *Neurospora* extracts and of liver extracts, respectively, with ATP and radioactive sulfate is shown in Figure 4. Figure 5 shows the dependence of the formation of the active sulfate with *Neurospora* enzyme on pH, indicating an optimum at pH 8. Furthermore, the appearance of another sulfur-containing material, slightly below the height of ADP, may be noted, particularly at pH 8.4. This unidentified sulfur-containing substance has been frequently observed with *Neurospora* as well as with liver preparations.

Stability of Intermediary.—The data represented in Figure 3 show that the intermediary may be heated briefly at neutral reaction and that it withstands a short exposure to ice-cold trichloroacetic acid. The fact that the heated material was used for sulfate transfer to nitrophenol made it appear hopeful that the intermediary would be obtained by elution from paper after electrophoresis on a somewhat larger scale. Attempts of this type were carried out, and a material was obtained which had a single peak at 258–260 mμ. In one case, in an experiment with liver enzyme, the radioactive sulfate was removed rapidly and with cooling directly from the trichloroacetic acid filtrate by precipitation with barium acetate, and the precipitate was washed once with cold dilute HCl. The combined filtrate and washing were brought to pH 8, and the formed precipiate was centrifuged off after 30 minutes at 0°. The precipitate was then suspended in a small volume of water and shaken with enough acid Dowex-50 to dissolve it. The solution was electrophorized, and the radioactive ultraviolet-absorbing zone corresponding to the intermediary was cut out, eluted with water at 10°, and concentrated to 1 ml. by freeze-drying. Two-tenths of a milliliter of this solution were tested with liver enzyme for sulfate transfer to nitrophenol and gave 20 mμM nitrophenol disappearance, an amount somewhat dangerously close to the limits of the method. For further identification, the remainder of the sample was again precipitated as barium salt at pH 8, with the addition of two volumes of alcohol to eliminate ultraviolet-absorbing contaminants, and the precipitate was dissolved in 1 ml. of 0.10 *M* versene solution. In this milliliter of solution, 106 mμM of adenine were found by ultraviolet absorption, and 103 mμM of sulfate were determined from the isotope content. Reliable phosphate analysis was not possible because in this system the intermediary runs too close to inorganic phosphate. Assuming a complete recovery, the enzymatically transferable sulfate as determined with the initial 0.2 ml. amounts to 75 per cent of the total sulfate.

In another attempt to elute the intermediary again, equimolar amounts of adenine and sulfate were extracted, but this compound had been largely inactivated in the process, yielding only 15 per cent of the expected "active" sulfate on enzymatic reaction with nitrophenol.

CONCLUSION

There are thus various aspects of these experiments which make us still somewhat hesitant to accept them as definite proof for the structure of the active sulfate as an adenyl-P-sulfate—in particular, the small size of the samples obtained and the in-

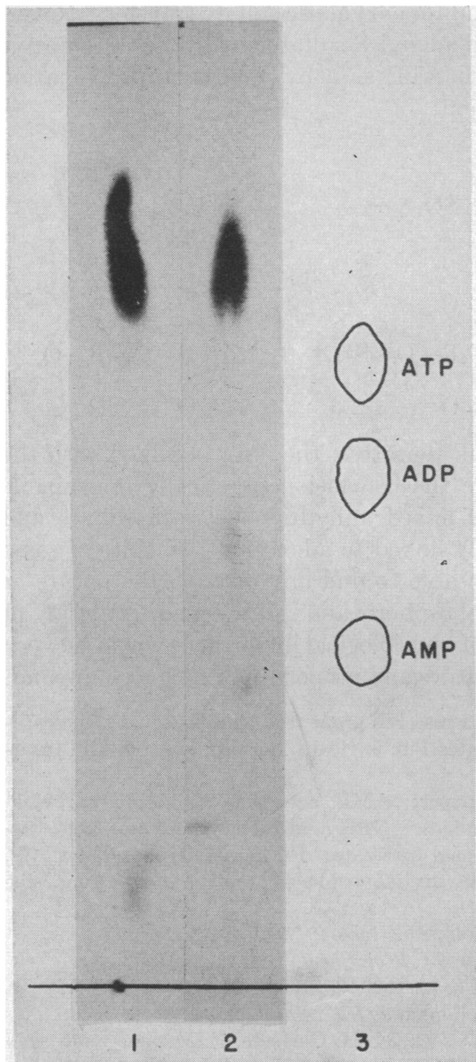


FIG. 4.—Identity of sulfate intermediary formed from ATP + sulfate in *Neurospora* and liver extract. Complete system: 50 μM buffer as indicated; 6 μM $MgCl_2$; 6 μM cysteine; 4 μM ATP; 4 μM S^{35}-sulfate (50 MC per gram) in 0.5 ml. Incubation for 2 hours at 38°.

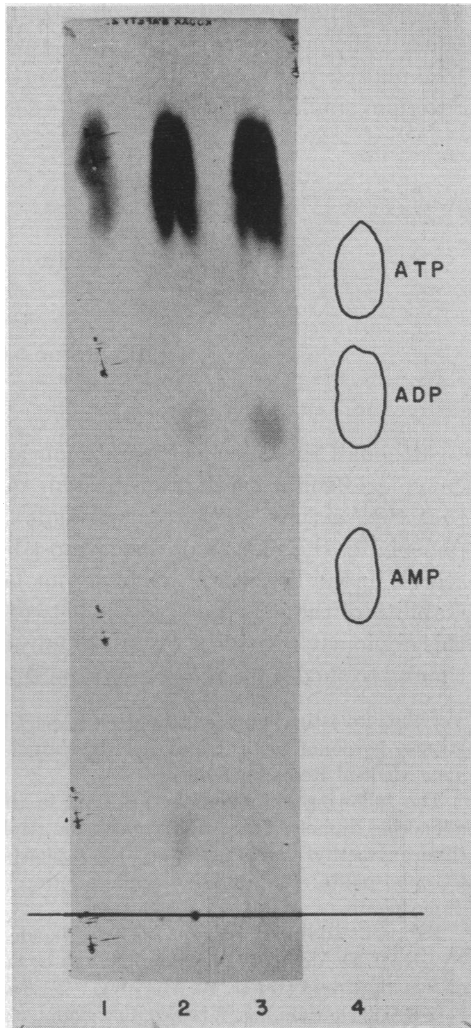
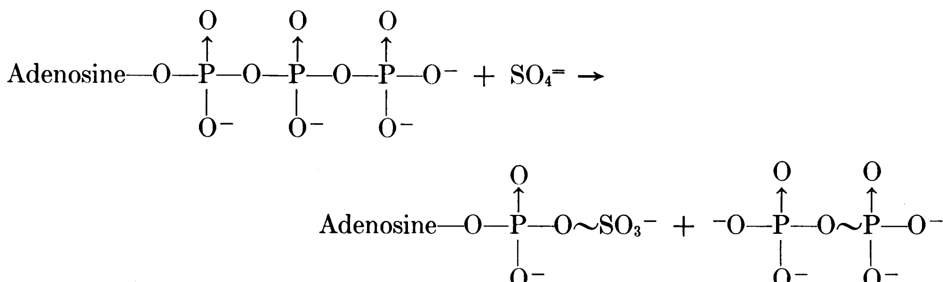


FIG. 5.—Formation of active S^{35}-sulfate in *Neurospora* extract. Radioautogram of electrophorized incubate. Complete system: 50 μM buffer; 6 μM $MgCl_2$; 6 μM cysteine; 4 μM ATP; 5 μM PEP; crystalline pyruvokinase (0.1 ml.); and 4 μM $K_2S^{35}O_4$ of specific activity 50 MC per gram in 0.5 ml. Incubated $2^1/_2$ hours at 37°.

No. 1: lyophilized *Neurospora* enzyme, 2 mg. in tris buffer, pH 7.5; No. 2: lyophilized *Neurospora* enzyme, 2 mg. in tris buffer, pH 8.0; No. 3: lyophilized *Neurospora* enzyme, 2 mg. in tris buffer, pH 8.4; No. 4: ultraviolet tracings of electrophorized mixture of ATP, ADP, and AMP. Electrophoresis with 0.025 ml. of each reaction mixture without deproteinization, 2 hours at 1,500 V. Exposure on film: 3 days.

ability to analyze reliably for phosphate and for enzymatic sulfate transfer. Nevertheless, the present evidence points toward adenyl-P-sulfate and invites a tentative formulation of the sulfate activation with ATP as a pyrophosphate elimination reaction, similar to the ATP-acetate reaction:



Although this specific formulation is still tentative, the data obtained with the enzymatic sulfate-activation system make the conclusion practically inescapable that the "active" sulfate corresponds to a mixed anhydride between sulfate and phosphate, the phosphate being most likely linked to adenosine. In spite of some search in the literature, we have not been able to find any data on the nature or stability of the mixed anhydride between phosphoric and sulfuric acid. A study of this obviously relatively stable anhydride of physiological importance would be very timely, to shed some further light on the nature and properties of such a compound.

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The following abbreviations are used in this paper: AMP, adenosine-5′-phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; NPS, *p*-nitrophenyl sulfate; tris, tris-(hydroxymethyl)aminomethane; PEP, phospho-enol pyruvate; P_i, inorganic phosphate; PP, pyrophosphate; ITP, inosine triphosphate; UTP, uridine triphosphate; and GDP, guanosine diphosphate.

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