Incubation of the sulfurylase with ATP and sulfate in the absence of APS-kinase leads to only a small liberation of pyrophosphate (PP). Sulfurylase activity may, however, be assayed by substitution of certain group VI anions for sulfate. PP is then liberated from ATP by sulfurylase presumably with the transient formation of an unstable anhydride between adenosine monophosphate (AMP) and the anion, as shown in reaction 3

$$\text{ATP} + \text{anion} \overset{\text{Enz I}}{\rightleftharpoons} \text{PP} + [\text{5'-AMP-anion}] \longrightarrow \text{5'-AMP} + \text{anion} \quad (3)$$

Selenate, sulfite, chromate, tungstate and molybdate lead to an increasingly active enzymatic cleavage of PP from ATP (Table I), boiled enzyme controls being inactive. In experiments using sulfite no change in sulfite concentration could be detected. Electrophoresis of sulfite and molybdate reaction mixtures, after prolonged incubation, show quantitative decomposition of ATP to 5'-AMP and PP. Sulfate competitively inhibits PP liberation.

Evidence for the formation of an AMP-anion anhydride has been obtained by measuring the incorporation of P^{32}-labeled PP into ATP. Table I shows that incubation of the sulfurylase with sulfate, sulfite, or selenate results in PP exchange. Similar studies using a liver enzyme and sulfate have been reported.[7] Chromate, molybdate and tungstate, most active in promoting PP liberation from ATP, do not bring about measurable PP exchange. An inverse correlation exists between the efficacy of the anion in promoting PP liberation and its ability to promote PP exchange. We have interpreted these data on the basis of the stability of the AMP-anion anhydride. The stable sulfate anhydride permits extensive PP exchange but little net formation of PP. The unstable sulfite and selenate anhydrides give limited PP exchange and permit a net formation of PP.

TABLE I

PYROPHOSPHATE FORMATION AND EXCHANGE

Addendum	P_i liberated[a] (μM./tube)	PP exchanged[b] (% incorporation into ATP)
NaCl	0.02	0
Na_2SO_4	0.11	4.9
Na_2SeO_4	0.49	1.5
Na_2SO_3	0.82	0.8
Na_2CrO_4	2.86	0
Na_2WO_4	4.96	0
Na_2MoO_4	6.64	0

[a] Each tube contained in μM.: anion 5, ATP 5, Tris HCl 50, EDTA 0.25, $MgCl_2$ 1, pyrophosphatase 9 μg. sulfurylase 100 μg.; total volume 0.5 ml.; *p*H 7.5; incubation 30° for 120 min. Omission of pyrophosphatase results in PP accumulation with no inorganic phosphate (P_i) liberation. [b] Conditions as above except anion concentration 25, P^{32}-labeled PP 1, pyrophosphatase omitted; sulfurylase 200 μg., and incubation 30 min. ATP isolated by combined chromatography and electrophoresis.

Direct evidence for the formation of APS as an intermediate in PAPS synthesis was obtained using S^{35}-labeled sulfate. As shown by the data of Table II, incubation of the sulfurylase with ATP and $Na_2S^{35}O_4$ leads to an accumulation of APS. Combination of the sulfurylase with APS-kinase results in the expected PAPS formation with reduced accumulation of APS.

TABLE II

FORMATION OF APS AND PAPS BY ENZYME FRACTIONS

Enzyme (μg. protein/tube)	Counts per minute APS	Counts per minute PAPS
Sulfurylase (140)	350	13
APS-kinase (600)	10	79
Sulfurylase + APS-kinase (740)	129	1104
Unfractionated enzyme (1340)	142	2615

Each tube contained in μM.: $Na_2S^{35}O_4$ (4 μc./μM.) 25, ATP 5, $MgCl_2$ 1, EDTA 0.25, Tris-HCl 50, pyrophosphatase 9 μg.; total volume 0.5 ml.; *p*H 7.5; incubation 30° for 120 min. 20 μl. aliquots were separated electrophoretically and counted. PAPS and APS were identified by comparison with enzymatically prepared samples having adenine:sulfate:phosphate ratios of 1.3:1.0:2.4 and 1.3:1.0:1.1, respectively.

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THE ENZYMATIC SEQUENCE IN THE BIOSYNTHESIS OF ACTIVE SULFATE[1,2]

Sir:

We recently reported on the identification of 3'-phosphoadenosine-5'-phosphosulfate, PAPS, as the biologically active sulfate donor.[3] The presence of a phosphate in 3'-position, in addition to the 5'-phosphosulfate group, early indicated a two-step reaction. However, the exact mechanism of biosynthesis remained to be determined. Recently, Bandurski, *et al.*, made the observation that the sulfate activation system in yeast could be split into two fractions inactive by themselves but active when combined.[4] Our earlier attempts to fractionate the fairly sensitive liver system were therefore temporarily abandoned and we turned to yeast. We wish to report here on the functional identification of two enzyme fractions representing two consecutive steps in the synthesis of PAPS.

This identification was further facilitated through a recently described synthesis by Baddiley, *et al.*,[5] of adenosine-5'-phosphosulfate, APS, by sulfurylation of adenylic acid with the SO_3 complex of pyridine. Preliminary experiments had indicated APS to be formed initially by sulfurolysis of ATP. In view of recent work on similar systems,[6] it was expected that the reverse reaction, APS + pyrophos-

(1) These abbreviations are used: PAPS, 3'-phosphoadenosine-5'-phosphosulfate; APS, adenosine-5'-phosphosulfate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PP, pyrophosphate; and P_i, inorganic phosphate.

(2) This investigation was supported by research grants from the Cancer Institute of the National Institutes of Health, Public Health Service and the Life Insurance Medical Research Fund.

(3) P. W. Robbins and F. Lipmann, THIS JOURNAL, **78**, 2652 (1956).

(4) L. G. Wilson and R. S. Bandurski, *Arch. Biochem. Biophys.*, **62**, 503 (1956).

(5) J. Baddiley, J. G. Buchanan and R. Letters, *J. Chem. Soc.*, in press.

(6) P. Berg, *J. Biol. Chem.*, **222**, 991 (1956).

(7) H. L. Segal, *Biochim. Biophys. Acta*, **21**, 194 (1956).

phate ⟷ ATP + sulfate, would be most favorable to test for. This reaction was found to be catalyzed by yeast as well as liver extract. In Table I, the separation is shown of the responsible enzyme, ATP-sulfurylase, from APS-kinase, the latter catalyzing the eventual production of PAPS. In addition to the sulfurolysis of ATP, the yeast extract, as shown in the third column of Table I, contains a different enzyme that catalyzes sulfurolysis of ADP, a reaction somewhat analogous to Ochoa's nucleotide diphosphate-linked ribonucleic acid synthesis. Since in the equilibrium

$$\text{ATP} + \text{sulfate} \rightleftarrows \text{APS} + \text{pyrophosphate} \quad (1)$$

TABLE I

SEPARATION OF ATP-SULFURYLASE AND APS-KINASE

Separation of Yeast Enzymes.—National bakers' yeast was used and extracts were prepared essentially according to the method of Jones, *et al.*[7] The formation of ATP from APS was measured by following pyrophosphate disappearance or by measuring ATP formation with hexokinase and glucose-6-phosphate dehydrogenase.[8] PAPS formation was followed by transfer to nitrophenol or by PAP assay. The PAP assay depends on the catalytic activity of PAP in the transfer of sulfate from *p*-nitrophenol to phenol. The rate of nitrophenol formation is measured at 400 mμ in the Beckman DU spectrophotometer.[9] The reaction between APS and P_i was routinely followed by measuring the disappearance of P_i with chromatographic checks on ADP formation.

Fraction	ATP-sulfurylase (APS + PP → ATP) −PP, μM./mg./hr.	APS-kinase (APS + ATP → PAPS) PAPS, μM./mg./hr.	ADP-sulfurylase (APS + P_i → ADP) $-P_i$, μM./mg./hr.
Dialyzed extract, I	2.7	...	1.8
NaCl precipitate, II	10.1	0.55	5.8
17–23% EtOH, III	22.1	3.5	3.0
pH 5.4 precipitate, IVa	85.0	0.6	0
Supernate + 10% EtOH, IVb	0.5	4.1	
40–50% $(NH_4)_2SO_4$, V	0	12.5	

thermodynamically the reverse reaction is favored, the forward reaction as well as the over-all synthesis of PAPS is considerably enhanced by the removal of pyrophosphate by pyrophosphatase. As shown in Table II, in the absence of the APS-kinase, APS can form only on addition of pyrophosphatase. Even so, the yield is very low when compared with the ease with which APS and pyrophosphate reverse to ATP (Table I).

The removal of APS through APS-kinase

$$\text{APS} + \text{ATP} \longrightarrow \text{PAPS} + \text{ADP} \quad (2)$$

likewise favors sulfurolysis, yielding PAPS also in the absence of pyrophosphatase. But the addition of pyrophosphatase still considerably enhances the overall reaction (Table III). Pyrophosphate inhibits strongly (*cf.* also Segal[10]). The equilibrium

(7) M. E. Jones, S. Black, R. M. Flynn and F. Lipmann, *Biochim. Biophys. Acta*, **12**, 141 (1953).

(8) A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.*, **193**, 481 (1951).

(9) J. D. Gregory, unpublished method.

(10) H. L. Segal, *Biochim. Biophys. Acta*, **21**, 194 (1956).

TABLE II

ENZYMATIC FORMATION OF APS FROM ATP

The complete incubation mixture contained: 100 μM. tris-(hydroxymethyl)-aminomethane buffer, *p*H 7.5; 5 μM. ATP; 5 μM. $MgCl_2$; 20 μM. K_2SO_4; 10 γ crystalline pyrophosphatase; and 1.0 mg. Fraction IVa, Table I, ATP-sulfurylase, in a final volume of 1 ml. Incubation was for 30 minutes at 37°. The reaction was stopped by heating in a boiling water-bath for 90 seconds. After removing the denatured protein, aliquots were taken for APS assay. APS was determined by conversion to PAPS with APS-kinase in the presence of ATP and $MgCl_2$.

System	APS, μM./ml.
Complete	0.23
No sulfurylase	0
No pyrophosphatase	0.01
No $SO_4^=$	0

TABLE III

PYROPHOSPHATE INHIBITION OF OVER-ALL ACTIVATION REACTION,

$$2\text{ATP} + SO_4^= \longrightarrow \text{PAPS} + \text{ADP} + \text{PP}$$

The basic incubation mixture contained: 100 μM. tris-(hydroxymethyl)-aminomethane buffer, pH 8.5; 10 μM. ATP; 5 μM. $MgCl_2$; 20 μM. K_2SO_4; 100 γ Fraction IVa, Table I, ATP-sulfurylase; and 100 γ Fraction V, APS-kinase, in a final volume of 1 ml. Incubation at 37°. The reaction was stopped by heating in a boiling water bath for 90 seconds. PAPS concentration was determined by means of the PAP assay, as described in Table I.

	PAPS, μM./ml./hr.
Basic	0.27
Basic + 10 γ pyrophosphatase/ml.	0.81
Basic + 1 μM. pyrophosphate/ml.	0.02

concentration of APS in the over-all system should therefore be rather low. Accordingly, the affinity of the second enzyme for APS was found to be high. It may be significant that the highest rate of PAPS formation is observed with 10^{-5} *M* APS while higher concentrations cause inhibition. Both enzymes, ATP-sulfurylase and APS-kinase, require as cofactors Mg^{++} or other divalent cations.

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A ROLE OF BIOTIN IN THE INTERACTION OF ORNITHINE AND CARBAMYL PHOSPHATE

Sir:

The loss in ability of rat liver homogenates to convert ornithine, bicarbonate and ammonium ion to citrulline as a result of biotin deficiency has been reported to be restored by supplements of N-carbamylglutamate but not by biotin.[1,2] N-Carbamylglutamate has a role in the conversion of carbonate and ammonium ion to a compound X which interacts with ornithine to form citrulline.[3] Evidence that compound X is carbamyl phosphate

(1) P. R. MacLeod, S. Grisolia, P. P. Cohen and H. A. Lardy, *J. Biol. Chem.*, **180**, 1003 (1949).

(2) G. Feldott and H. A. Lardy, *ibid.*, **192**, 447 (1951).

(3) S. Grisolia and P. P. Cohen, *ibid.*, **198**, 561 (1952).