extracted with three 250-ml. portions of ether. The aqueous solution was saturated with sodium chloride and again extracted with three 250-ml. portions of ether. The combined ether extracts were washed with 50 ml. of a saturated sodium chloride solution and dried over magnesium sulfate. The ether solution was concentrated to a volume of about 100 ml. and allowed to stand at room temperature for 3 days. The crystalline acid (15 g.) was filtered off and was used without further purification in subsequent preparations. The product melts at 85–90° with resolidification to melt again at 133–135° with gas evolution (anhydride formation).

O-Acetyl-α,α'-dimethyl-β-hydroxyglutaric Anhydride.[5]—*meso*-α,α'-Dimethyl-β-hydroxyglutaric acid (12 g., 0.068 mole) was suspended in 53 g. of acetyl chloride (0.68 mole), and the reaction mixture was swirled occasionally. The mixture was allowed to stand at room temperature for 24 hours; an equal volume of ether was added and the solution was filtered to remove a small amount of undissolved material. The crystalline product which formed on standing at room temperature for 3 hours was filtered off, washed with ether and dried. The filtrate was allowed to stand at 0° for 12 hours and yielded a second crop of crystals. The combined products weighed 5.5 g. and melted at 111.5°.

***meso*-2,4-Dimethylpentane-1,3,5-triol (VIII).** (a) **From *meso*-α,α'-Dimethyl-β-hydroxyglutaric Acid.**—The glutaric acid (7 g., 0.04 mole) was dissolved in 300 ml. of anhydrous ether and this solution was added dropwise to a stirred suspension of 6.8 g. (0.18 mole) of lithium aluminum hydride in 250 ml. of anhydrous ether. The reaction was carried out as described under "*meso*-Triol VIII by Reduction of C_7-Lactone A VI," using 6.5 ml. of water, 5.25 ml. of a 20% sodium hydroxide solution and 25.5 ml. of water. The residue (2.1 g.) obtained was distilled (b.p. 135° at 0.06 mm.), and the distillate was allowed to stand for 10 days at room temperature. The crystalline triol obtained melted at 75–78°; the refractive index of the melted material was $n^{25}D$ 1.4827.

Anal. Calcd. for $C_7H_{16}O_3$: C, 56.73; H, 10.88. Found: C, 56.88; H, 10.70.

(b) From O-Acetyl-α,α'-dimethyl-β-hydroxyglutaric Anhydride.—The glutaric anhydride (5 g., 0.029 mole) was reduced as described above with 11 g. (0.29 mole) of lithium aluminum hydride. The residue obtained crystallized prior to distillation and was identical in all respects with the *meso*-triol as obtained directly from the acid.

***rac*-2,4-Dimethylpentane-1,3,5-triol (XIX) by Reduction of Diethyl α,α'-Dimethyl-β-hydroxyglutarate (XX).**—The diethyl glutarate (32.5 g., 0.14 mole) was reduced with 24 g. (0.63 mole) of lithium aluminum hydride as described above. The residue (8 g.) was allowed to stand with 10 ml. of anhydrous ether at 0° for 2 days. The crystalline material (2.0 g.) was filtered off in a dry atmosphere, and the residue from the filtrate was distilled at 0.08 mm. (bath temperature 180°) to give 2 g. of a distillate from which a further amount (1 g.) of crystalline material was obtained by digestion with ether at 0°. Recrystallization of the crystalline material from ether gave pure triol XIX, m.p. 86–87°; the refractive index of the melted material was $n^{25}D$ 1.4752. The infrared spectrum in acetonitrile, or in chloroform solution, of the crystalline material and of the liquid distillate were identical indicating the *rac*-triol XX to be homogeneous and to contain little, if any, *meso*-triol VIII (Fig. 7).

Anal. Calcd. for $C_7H_{16}O_3$: C, 56.73; H, 10.88. Found: C, 56.57; H, 10.86.

The 1,5-bis-(*p*-toluenesulfonate) of *rac*-triol XIX after recrystallization from ether melted at 111°.

Anal. Calcd. for $C_{21}H_{18}S_2O_7$: C, 55.26; H, 6.18; S, 14.05. Found: C, 55.64; H, 6.21; S, 13.56.

Enanthic Acid by Peroxidation of Enanthaldehyde.—The oxidation was carried out according to the procedure used by Emmons and Lucas[8] for the oxidation of ketones to esters. Trifluoroacetic anhydride (25.4 ml., 0.18 mole) was added with stirring to 4.1 ml. of 90% hydrogen peroxide (0.15 mole) dissolved in 25 ml. of methylene chloride. The temperature of the mixture was maintained at 5 to 9° during the addition and afterwards. The solution of oxidizing reagent was then added dropwise to a stirred suspension of 85 g. of dry, finely powdered disodium hydrogen phosphate in a solution of 90 ml. of methylene chloride and 11.4 g. (0.1 mole) of *n*-heptaldehyde. After all the reagent had been added, the reaction mixture was heated under reflux for 30 minutes and allowed to stand at room temperature overnight. The salts were filtered off and washed with three 50-ml. portions of methylene chloride. The combined methylene chloride solutions were extracted with two 25-ml. portions of a 10% sodium carbonate solution and two 25-ml. portions of a 5% sodium bicarbonate solution. The combined basic extracts were acidified with concentrated hydrochloric acid (*p*H 2.7). The oily layer which formed was separated, and the aqueous layer was extracted with three 75-ml. portions of chloroform. The combined chloroform solutions were dried over magnesium sulfate, and the chloroform was removed under reduced pressure. The residue gave no precipitate with Brady reagent. Titration in 66% dimethylformamide showed a pK'_a of 7.67, apparent molecular weight 128; calculated 130. The infrared spectrum of the distilled product was identical with that of an authentic sample of *n*-heptanoic acid. The yield of *n*-heptanoic acid was 9 g. (70%).

INDIANAPOLIS, INDIANA

COMMUNICATIONS TO THE EDITOR

THE MECHANISM OF "ACTIVE SULFATE" FORMATION[1]

Sir:

The nucleotide-linked "active sulfate"[2,3,4] known to act as sulfate donor in the sulfurylation of phenols has been identified as 3′-phosphoadenosine-5′-phosphosulfate (PAPS).[5] Previous studies from this laboratory have shown that two heat-labile fractions are required for the formation of PAPS.[6] Evidence is here presented that enzyme I, ATP-sulfurylase, catalyzes the formation of adenosine-5′-phosphosulfate (APS) which is then converted into PAPS by enzyme II, adenosine phosphosulfate kinase (APS-kinase), as shown in the reaction sequence

$$ATP + SO_4^{=} \underset{}{\overset{\text{Enz I}}{\rightleftharpoons}} APS + PP \qquad (1)$$

$$APS \xrightarrow[ATP]{\text{Enz II}} PAPS \qquad (2)$$

(1) We are indebted to the National Science Foundation for their support of this work.

(2) R. H. DeMeio, M. Wizerkaniuk and E. Fabriani, *J. Biol. Chem.*, **203**, 257 (1953).

(3) S. Bernstein and R. W. McGilvery, *ibid.*, **199**, 745 (1952).

(4) H. Hilz and F. Lipmann, *Proc. Natl. Acad. Sci.*, **41**, 880 (1955).

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

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

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 (A-MP) and the anion, as shown in reaction 3

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

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).