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OCCURRENCE OF A 3′-PHOSPHOADENOSINE 5′-PHOSPHOSULPHATE SYNTHESIZING SYSTEM IN TWO *OCHROMONAS* SPECIES

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Abstract—The presence of an enzyme capable of incorporating $^{35}SO_4^{2-}$ into 3′-phosphoadenosine 5′-phosphosulphate has been demonstrated in *Ochromonas danica* and *O. malhamensis*. This system probably includes the enzymes ATP sulphate adenyltransferase, E.C. 2.7.7.4 and ATP adenylsulphate 3′-phosphotransferase, E.C. 2.7.1.25.

INTRODUCTION

THE ALGA *Ochromonas danica*, a member of the Chrysophyceae, synthesizes considerable quantities of a hitherto unknown class of polar lipids, the chlorosulpholipids.[1–5] There are two series of chlorosulpholipids; the most abundant consists of docosane-1,14-disulphate and its chlorinated derivatives having from 1 to 6 chlorine atoms replacing hydrogen atoms on the aliphatic chain whilst the least abundant consists of tetracosane-1,15-disulphate and its mono- to hexa-chlorinated derivatives.

It has been suggested[6] that the chlorosulpholipids are synthesized by way of docosane-1,14-diol and tetracosane-1,15-diol, the primary and secondary hydroxyl groups of which are then sulphated by "active sulphate" (3′-phosphoadenosine 5′-phosphosulphate; PAPS). In order to prove that PAPS is the donor of the sulphate in chlorosulpholipid biosynthesis it is necessary to show that *O. danica* is capable of, (a) forming PAPS from sulphate, and (b) transferring the sulphate moiety of PAPS to a chlorosulpholipid precursor. The purpose of this paper is to show that *O. danica* and the closely related *O. malhamensis*, which also appears to synthesize sulpholipids of the type found in *O. danica*,[7,8] are capable of forming PAPS from sulphate.

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[6] MOONEY, C. L., MAHONEY, E. M., POUSADA, M. and HAINES, T. H. (1972) *Biochemistry* **11**, 4839
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[8] HAINES, T. H. (1965) *J. Protozool* **12**, 655

The formation of PAPS from inorganic sulphate and adenosine 5′-triphosphate (ATP) in bacteria, fungi, animals,[9] *Chlorella pyrenoidosa*,[10] and *Euglena gracilis*[11] and higher plants[12] requires the activity of three enzymes, ATP sulphurylase (ATP:sulphate adenyltransferase, E.C. 2.7.7.4), APS kinase (ATP.adenylsulphate 3′-phosphotransferase, E.C. 2.7.1.25) and pyrophosphatase (pyrophosphate phosphohydrolase, E.C. 3.6.1.1) which catalyse reactions (1), (2) and (3) respectively.

$$ATP + SO_4^{2-} \rightleftharpoons APS \text{ (adenosine 5′-phosphosulphate)} + PP_i \quad (1)$$

$$APS + ATP \rightleftharpoons PAPS + ADP \quad (2)$$

$$PP_i + H_2O \rightleftharpoons 2P_i \quad (3)$$

The equilibrium of reaction (1) is very unfavourable for APS synthesis, being about 10^{-8} for the yeast enzyme at 37° and pH 8. The reaction is however pulled in the direction of APS synthesis *in vivo* by the rapid removal of the products of the reaction, APS by reaction (2) and inorganic pyrophosphate by reaction (3).[9]

RESULTS AND DISCUSSION

Incorporation of $^{35}SO_4^{2-}$ *into APS and PAPS by cell-free preparation of* Ochromonas (a) danica *and* O. malhamensis

Aliquots (1 ml) of an 86000 *g* supernatant fraction of logarithmic phase cells of *O. danica* and *O. malhamensis* were incubated under anaerobic conditions and in the absence of light for 2 hr at 30° with 50 μmol ATP, 25 μmol $MgCl_2$, 25 μmol mercaptoethanol, 150 μCi $^{35}SO_4^{2-}$, one unit of pyrophosphatase and 50 μmol of either Tris buffer, pH 8.5, or phosphate buffer, pH 7.5. Incubation under anaerobic conditions and in the presence of mercaptoethanol protected the cell-free system from air oxidation; the same conditions were shown to be required for PAPS formation by cell-free extracts of *Chlorella pyrenoidosa*.[10] Incubations were carried out in darkness to prevent any possible degradation of PAPS to PAP and sulphite by the light-dependent reaction reported by Asahi.[13] Radioactive substances formed during incubation were separated from each other and from residual $^{35}SO_4^{2-}$ by high voltage paper electrophoresis. Radioscans of the electrophoretograms showed two zones with mobilities, relative to $^{35}SO_4^{2-}$, similar to those reported for APS and PAPS.[14] Their identity was confirmed by comparison of their electrophoretic behaviour with those of authentic AP^{35}S and PAP^{35}S (see radioscan 5, Fig. 1) formed biosynthetically with a rat liver enzyme system.[15] Radioactive zones corresponding to APS and PAPS were cut from the electrophoretograms, eluted and assayed for radioactivity by liquid scintillation counting. Table 1 shows the results of a number of such experiments. The "complete incubation mixture" (incubation 1, Table 1) always produced a radioactive zone corresponding to PAPS and less frequently one corresponding to APS. The incorporation of $^{35}SO_4^{2-}$ into PAPS was rather better with the *O. danica* cell-free preparation than

[9] BANDURSKI, R. S. (1965) *Plant Biochemistry* (BONNER, J. and VARNER, J. E., eds.), p. 471. Academic Press, New York.
[10] HODSON, R. C. and SCHIFF, J. A. (1969) *Arch. Biochem. Biophys.* **132**, 151.
[11] DAVIES, W. H., MERCER, E. I. and GOODWIN, T. W. (1966) *Biochem. J.* **98**, 369.
[12] MERCER, E. I. and THOMAS, G. (1969) *Phytochemistry* **8**, 2281.
[13] ASAHI, T. (1964) *Biochim. Biophys. Acta* **82**, 58.
[14] SEGEL, I. H. and JOHNSON, M. J. (1963) *Arch. Biochem. Biophys.* **103**, 216.
[15] BANNERJEE, R. K. and ROY, A. B. (1966) *Mol. Pharmacol.* **2**, 56.

with that of *O. malhamensis*. Tris buffer, pH 8·5, proved rather better than phosphate buffer, pH 7·5, with the cell-free preparations of both algae; the reason for this difference is probably one of pH rather than the nature of the buffer itself since it has been shown that the pH optimum of APS kinase in yeast is 8·5–9·0.[16] The "boiled enzyme" and "no enzyme" controls (incubations 2 and 3, Table 1) failed to produce any $AP^{35}S$ or $PAP^{35}S$; the only radioactive material detected after incubation was residual $^{35}SO_4^{2-}$. The "boiled pyrophosphatase" and "no pyrophosphatase" controls (incubations 4 and 5, Table 1) supported an incorporation of $^{35}SO_4^{2-}$ into PAPS some 4–5 times lower than did the complete incubation mixture, showing not only the importance of pyrophosphatase in the PAPS-synthesizing system but also the likely presence of some pyrophosphatase in the algal cell-free preparations. Subsequent experimentation with the *O. danica* cell-free preparation showed that best incorporation of $^{35}SO_4^{2-}$ into PAPS was obtained when 50 μmol of ATP were present in a complete incubation mixture whose other components were present in the quantities given above. No $PAP^{35}S$ could be detected when 5 or 10 μmol of ATP were present and very little at the 75 μmol level; however when 30 μmol of ATP were present about 25% of the $PAP^{35}S$ found at the 50 μmol level was obtained. Similarly it was found that a 2 hr incubation period gave the greatest yield of $PAP^{35}S$. The quantity of $PAP^{35}S$ produced increased steadily with increasing incubation time up to 2 hr but then fell away to 85% of the 2 hr value after 3 hr presumably due to the presence of PAPS-degrading enzymes in the cell-free preparation. Such enzymes occur widely in both animal and plant tissues and include 3′-nucleotidases,[17,18] 5′-nucleotidases[17] and APS- and PAPS-sulphohydrases[19] which can result in poor apparent incorporation of $^{35}SO_4^{2-}$ into PAPS when crude enzyme preparations are used.

TABLE 1 INCORPORATION OF $^{35}SO_4^{2-}$ INTO 3′-PHOSPHOADENOSINE 5′-PHOSPHOSULPHATE BY CELL-FREE PREPARATIONS OF *Ochromonas danica* AND *O. malhamensis*

Incubation mixture	Radioactivity (dpm) in 3′-Phosphoadenosine 5′-phosphosulphate*			
	O danica		*O malhamensis*	
	Tris[a]†	Phosphate[b]	Tris[a]	Phosphate[b]
(1)	23275	12000	15600	10500
(2)	0	0	0	0
(3)	0	0	0	0
(4)	5600	3700	3200	1800
(5)	5000	1500	2500	1000

* In some experiments a radioactive zone co-chromatographing with authentic APS was observed Its radioactivity was 5–10% that of PAPS.

† Average of four experiments.

(1)—Complete system. 1 ml enzyme preparation, 50 μmol buffer; 50 μmol ATP; 25 μmol mercaptoethanol; 150 μCi $^{35}SO_4^{2-}$, 1 unit pyrophosphatase, (2)—Boiled enzyme preparation; (3)—Minus enzyme preparation, (4)—Boiled pyrophosphatase, (5)—Minus pyrophosphatase, (*a*)—Enzyme prepared and incubated in Tris buffer, final pH 8·5, (*b*)—Enzyme prepared and incubated in phosphate buffer, final pH 7 5

[16] ROBBINS, P W. and LIPMANN, F. (1958) *J Biol Chem.* **233,** 681
[17] ROBBINS, P W. and LIPMANN, F (1957) *J Biol Chem.* **229,** 837
[18] BRUNNGRABER, E G (1958) *J Biol Chem* **233,** 472.
[19] ABRAHAM, A and BACHHAWAT, B K (1964) *Indian J Biochem* **1,** 192

Effect of molybdate, chromate and tungstate on the incorporation of $^{35}SO_4^{2-}$ *into APS and* (b) *PAPS by a cell-free preparation of* Ochromonas danica

Bandurski *et al* [20] showed that ATP sulphurylase is relatively non-specific with respect to the anion which participates in reaction (1); all the group VI anions will substitute for SO_4^{2-}. However, when molybdate, chromate or tungstate are incubated with ATP and ATP sulphurylase, AMP and pyrophosphate result. It is assumed that ATP sulphurylase catalyses the formation of the adenyl-anion anhydride but that the latter is very short-lived and breaks down to yield AMP and the anion. These anions are therefore competitive inhibitors of ATP sulphurylase and will thus inhibit the incorporation of $^{35}SO_4^{2-}$ into APS and hence into PAPS

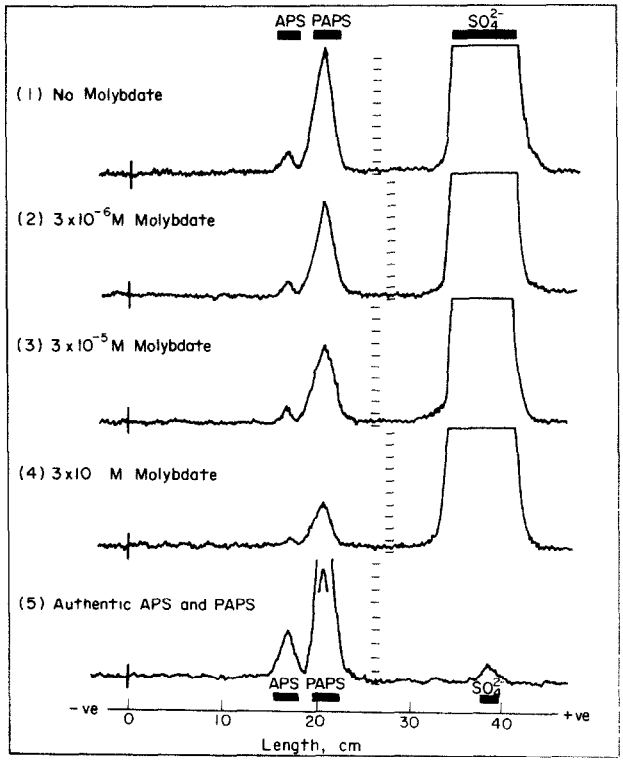

FIG 1 RADIOSCANS OF PAPER ELECTROPHORETOGRAMS SHOWING THE EFFECT OF MOLYBDATE ON THE INCORPORATION OF $^{35}SO_4^{2-}$ INTO APS AND PAPS BY A CELL-FREE PREPARATION OF *Ochromonas danica*
The *O danica* cell-free preparation (1 ml) was incubated anaerobically in darkness for 2 hr with 50 μmol Tris buffer, 50 μmol ATP, 25 μmol $MgCl_2$, 25 μmol mercaptoethanol 150 μCi $^{35}SO_4^{2-}$ 1 unit of pyrophosphatase and 0 μmol (1), 0 006 μmol (2), 0 06 μmol (3) and 0 6 μmol (4) of sodium molybdate (final vol 2 ml, final pH 8 5) The ^{35}S-labelled compounds produced were separated by paper electrophoresis An authentic sample of $AP^{35}S$ and $PAP^{35}S$ formed from $^{35}SO_4$ by a rat liver enzyme preparation was also run on the same paper electrophoretogram for comparison purposes (5)

The effect of molybdate, chromate and tungstate at three different concentrations (3×10^{-4}, 3×10^{-5} and 3×10^{-6} M), on the ability of the *O danica* cell-free preparation to incorporate $^{35}SO_4^{2-}$ into PAPS was examined Aliquots (1 ml) of the cell-free preparation were incubated under anaerobic conditions and in the absence of light for 2 hr at

[20] BANDURSKI R S, WILSON, L G and ASAHI, T (1956) *J Am Chem Soc* **78**, 6408

30° with 50 μmol ATP, 25 μmol $MgCl_2$, 25 μmol mercaptoethanol, 150 μCi $^{35}SO_4^{2-}$, 1 unit of pyrophosphatase, 50 μmol Tris buffer, pH 8·5, and each of the inhibitors at the concentrations given previously. The result of the molybdate experiment is shown in Fig. 1. Traces 1–4 depict radioscans of high voltage electrophoretograms of the incubation mixtures containing no molybdate, 3×10^{-6} M, 3×10^{-5} M and 3×10^{-4} M molybdate respectively. Trace 5 is a radioscan of authentic $AP^{35}S$ and $PAP^{35}S$ separated on the same electrophoresis run. It is apparent from the radioscans that molybdate inhibits APS and PAPS synthesis and that the degree of inhibition increases with increasing concentration of molybdate. The $AP^{35}S$ and $PAP^{35}S$ zones were eluted and radioassayed by liquid scintillation counting. It was found that 3×10^{-6} M molybdate brought about a 36·4% inhibition of $^{35}SO_4^{2-}$ incorporation into PAPS whilst 3×10^{-5} M and 3×10^{-4} M molybdate caused 50·7 and 67·3% inhibition respectively The same pattern of results was obtained with chromate and tungstate. The former anion brought about the following inhibitions: 3×10^{-6} M, 64·4%; 3×10^{-5} M, 75·4% and 3×10^{-4} M, 100% whilst the latter brought about 19·7%, 38·1% and 58·5% inhibition respectively at these concentrations.

The results of the experiments carried out in sections (a) and (b) are entirely consistent with the presence of a PAPS-synthesizing system in *O. danica* and *O malhamensis*. The fact that addition of pyrophosphatase to the cell-free preparations increases the incorporation of $^{35}SO_4^{2-}$ into PAPS whilst group VI anions, known inhibitors of ATP sulphurylase, decrease it strongly suggest that the enzymes concerned are ATP sulphurylase, APS kinase and pyrophosphatase.

Since these algae are capable of forming PAPS from sulphate it is likely that Haines' suggestion[6] that PAPS is the immediate source of the primary and secondary sulphates of the chlorosulpholipids is correct.

EXPERIMENTAL

Biological material Ochromonas danica Pringsheim 933/2 and *Ochromonas malhamensis* Pringsheim 933/1a cultures were obtained from the Culture Collection of Algae and Protozoa, The Botany School, Cambridge Both algae were grown in shake culture in the light at 25° on the chemically defined heterotrophic media of Aaronson and Baker [21]

Preparation of cell-free enzyme system Algal cells were harvested in the logarithmic phase of growth by centrifugation at 2000 *g* for 10 min They were then washed with either 0 1 M Tris-HCl, pH 7 0, containing 50 mM mercaptoethanol or 0 1 M phosphate buffer, pH 7 0, containing 50 mM mercaptoethanol Cells washed in the former soln were then resuspended in it (5 ml/g wet wt) and disrupted in a French pressure cell The cell crush was then centrifuged at 86000 *g* for 1 hr at 0° The resulting supernatant was dialysed for 3 hr at 4° against 0 05 M Tris buffer, pH 9 0, containing 50 mM mercaptoethanol Cells washed with the phosphate–mercaptoethanol buffer were put through a similar procedure save that they were disrupted in 0 1 M phosphate buffer, pH 7 0, containing 50 mM mercaptoethanol and their supernatant fraction dialysed against 0 05 M phosphate buffer, pH 8 0, containing 50 mM mercaptoethanol The dialysed supernatants were used as the cell-free enzyme system The protein content of the cell-free preparations was determined by the Biuret method[22] and was always in the range 25–35 mg/ml

Incubation of cell-free enzyme system Aliquots (1 0 ml) of the enzyme preparations were used in all incubations and were mixed with 1 0 ml of a soln containing all other reagents, the total vol of all incubations was therefore 2 ml In the experiments described in section (a) the enzyme preparations were incubated with 50 μmol Tris buffer, pH 9 0, or phosphate buffer, pH 8 0, 50 μmol ATP, 25 μmol $MgCl_2$, 25 μmol mercaptoethanol, 150 μCi $^{35}SO_4^{2-}$ and 1 unit of pyrophosphatase The $^{35}SO_4^{2-}$ was supplied by the Radiochemical Centre, Amersham, as an aq soln (pH 6–8, carrier free) Pyrophosphatase was obtained, as a crystalline suspension in 3 M $(NH_4)_2SO_4$, from Boehringer Co (London) Ltd The final pH of the incubation mixtures which utilized Tris as the buffering system was 8 5 whilst that for the incubation mixtures which utilized phosphate was 7 5 In the experiments described in section (b) the enzyme preparations were incubated with 50 μmol of Tris buffer, pH 9 0, 50 μmol ATP 25 μmol $MgCl_2$ 25 μmol mercaptoethanol 150 μCi $^{35}SO_4^{2-}$ 1 unit of pyrophosphatase

[21] AARONSON, A and BAKER, H (1959) *J Protozool* **6**, 282
[22] GORNALL, A G, BARDAWILL, C J and DAVID, M M (1949) *J Biol Chem* **172**, 751

and 0 6, 0 06 or 0 006 μmol of sodium molybdate, sodium chromate or sodium tungstate All incubations were carried out in Thunberg tubes under anaerobic conditions (atmosphere of argon or oxygen-free nitrogen) for 2 hr at 30° in the absence of light They were terminated by immersing the tubes in boiling H_2O for 3 min The protein precipitate which resulted from this heating was sedimented by centrifugation at 12000 *g* for 10 min and the supernatant removed for electrophoresis

Electrophoretic separation of APS and PAPS The procedure adopted was a modification of that used by Segel and Johnson[14] differing only in respect of the higher voltage (2250 V or 45 Vcm $^{-1}$) used The separation was carried out on Whatman 3MM paper using 0 2 M Tris buffer pH 8 1 and gave excellent separation of APS and PAPS from each other and from SO_4^{2-}, with mobilities, relative to SO_4^{2-}, close to the values quoted[14] Separation was complete in 40 min The paper was then dried at 110° and cut up into strips 3 5 cm wide which were scanned for radioactivity The radioactive zones were cut out and eluted 3× with 50% (*v*/*v*) aq EtOH containing 2% (*v*/*v*) 0 880 NH_3 The eluates from each zone were evaporated to dryness under reduced pressure at 25° and redissolved in a known quantity of 50% (*v*/*v*) aq EtOH Aliquots were then dispersed in *10 ml NE 250 liquid scintillation solution (Nuclear Enterprises Ltd Edinburgh) and assayed for radioactivity*

Preparation of PAP^{35}S using a rat liver enzyme system The method of Bannerjee and Roy[15] was used for the preparation of PAP^{35}S from $^{35}SO_4^{2-}$ and ATP its purification and its subsequent assay

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