

# Localization of a sulphate-activating system within *Euglena* mitochondria

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Intact mitochondria, obtained from *Euglena gracilis* Klebs var. *bacillaris* Cori mutant W<sub>10</sub>BSmL, which lacks plastids, and purified on Percoll density gradients, form adenosine 3'-phosphate 5'-phosphosulphate from sulphate. The optimal conditions include addition of 17 mM-Tricine/KOH, pH 7.6, 18 mM-MgCl<sub>2</sub>, 250 mM-sucrose, 5.66 mM-sodium ADP (or 0.94 mM-sodium ATP), 1 mM-K<sub>2</sub>SO<sub>4</sub>, carrier-free <sup>35</sup>SO<sub>4</sub><sup>2-</sup> (32.1 μCi) and 1.0 mg of mitochondrial protein in a total volume of 2.65 ml and incubation at 30 °C. Experiments with the inhibitor of adenylate kinase P<sup>1</sup>, P<sup>5</sup>-di(adenosine 5'-)pentaphosphate indicate that ATP is the preferred substrate for sulphate activation; ADP is utilized by conversion into ATP via adenylate kinase. ATP sulphurylase, adenylylsulphate kinase (APS kinase) and inorganic pyrophosphatase constitute the sulphate-activating system; ADP sulphurylase is undetectable. Fractionation of *Euglena* mitochondria with digitonin and centrifugation allowed the separation of outer-membrane vesicles and mitoplasts as judged by electron microscopy and selected enzymic markers. The detergent-labile association of the sulphate-activating system with the mitoplasts (similar to that of adenylate kinase), the fact that most of the adenosine 3'-phosphate 5'-phosphosulphate formed by intact mitochondria is found in the surrounding medium, and the ease with which nucleotide substrates reach the activating system in intact organelles, suggest that the enzymes of sulphate activation are located on the outer surface of the mitochondrial inner membrane.

## INTRODUCTION

Sulphate activation involves the sulphate adenylyl-transferase (ATP sulphurylase) (EC 2.7.7.4)-catalysed reaction of ATP and sulphate to form adenosine 5'-phosphosulphate. A second enzyme, adenylylsulphate kinase (APS kinase) (EC 2.7.1.25) catalyses the reaction of adenosine 5'-phosphosulphate and ATP to form adenosine 3'-phosphate 5'-phosphosulphate. Inorganic pyrophosphatase (EC 3.6.1.1) is usually required to help offset the unfavourable equilibrium of the first reaction by catalysing the hydrolysis of pyrophosphate, one of the products (De Meio, 1975; Schiff, 1983). Although the sulphate-activating enzymes have been studied in a variety of organisms, including *Euglena* (Wilson & Bandurski, 1956; Nissen & Benson, 1961; Abraham & Bachhawat, 1963; Davies *et al.*, 1966), they have only been localized in a cellular organelle in the case of spinach (*Spinacia oleracea*) chloroplasts (Schwenn & Trebst, 1976). Since evidence exists that APS sulphotransferase (adenylyl sulphate: thiol sulphotransferase), organic thio-sulphate reductase (ferrodoxin: sulphogluthione oxidoreductase) and *O*-acetylserine (thiol)-lyase (EC 4.2.99.8), enzymes of assimilatory sulphate reduction, are located in the mitochondria of *Euglena gracilis* var. *bacillaris* (Brunold & Schiff, 1976), it seemed likely that the enzymes of sulphate activation might also be located in this organelle. In the present paper we show that the sulphate-activating enzymes are present in highly purified mitochondria from *Euglena*, define the optimal conditions for sulphate activation and present data concerning the

location of the sulphate-activating enzymes within the mitochondria. Brief reports of this work have appeared previously (Saidha *et al.*, 1983, 1984).

## EXPERIMENTAL

### Growth of organism

Wild-type *Euglena gracilis* Klebs var. *bacillaris* Cori and two bleached mutants, W<sub>3</sub>BUL and W<sub>10</sub>BSmL, were grown at 26 °C in the dark on Hutner's pH 3.5 medium (Greenblatt & Schiff, 1959) with vitamin B<sub>12</sub> decreased to 50 ng/litre. The cultures were shaken on a rotary shaker (120 rev./min). Cultures from the late-exponential phase of growth (about 10<sup>6</sup> cells/ml after 5 days of growth) were used for isolation of crude mitochondria. All the media for cell growth, and the glassware, plasticware etc. used for isolation of mitochondria, were autoclaved at 103.5 kPa (15 lbf/in<sup>2</sup>) at 120 °C for 20 min and all transfers of cultures were made aseptically and under green safelights (Schiff, 1972). All solutions were sterilized either by autoclaving or by Millipore (0.45 μm pore size) filtration as appropriate.

Although wild-type cells and cells of mutant W<sub>3</sub>BUL yielded mitochondria that carried out sulphate activation, W<sub>10</sub>BSmL was used in all the experiments reported here, since this strain lacks organized proplastids that may contaminate the mitochondrial fraction (Osafune & Schiff, 1983).

Cells were routinely counted in a Coulter model A counter in 0.8% NaCl and 0.01% ethylmercurithiosali-

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cylate (thimerosal) after suitable dilution (Zeldin & Schiff, 1967).

### Preparation of mitochondria

The method used (Gomez-Silva *et al.*, 1985a), including modifications added in the present work, was as follows. The cells were harvested at 367 g for 5 min. All operations from cell harvest to isolation of mitochondria were performed at 4 °C. The combined cell pellets obtained from 6–8 litres of cell cultures (about 30–40 g wet wt.) were resuspended in about 100 ml of 50 mM-potassium phosphate buffer, pH 7.4, containing 0.3 M-sucrose (Buffer I) and centrifuged at 367 g for 5 min. The pellet was resuspended in 100 ml of Buffer I. To this suspension 300 mg of crude trypsin (Sigma, type II, containing chymotrypsin) suspended in 2–4 ml of Buffer I and passed through a sterile Millipore filter (0.45 µm pore size) was added. This mixture was incubated at 4 °C with gentle stirring for 45–60 min until microscopic examination indicated that spontaneous cell breakage had begun. At this point, 100 ml of Buffer I was added, and the suspension was centrifuged at 1000 g for 3 min. The cell pellet was gently resuspended in about 50 ml of 25 mM-Hepes/KOH buffer, pH 7.4, containing 0.25 M-sucrose and 0.005 M-EDTA (Buffer II). This suspension was passed through a sterilized [70% (v/v) ethanol followed by sterile water] Yeda press at 3.45 MPa (500 lbf/in<sup>2</sup>) of N<sub>2</sub>. The broken cell suspension was collected in 50 ml of Buffer II and was centrifuged at 1000 g for 5 min to remove whole cells and cell fragments. The supernatant fluid was then centrifuged at 10000 g for 10 min to obtain the crude mitochondrial pellets. These washed mitochondria were resuspended in a few millilitres of Buffer II and 0.5–1.0 ml was layered on top of a discontinuous Percoll density gradient prepared in 18 ml nitrocellulose tubes. This gradient was composed of layers (starting at the bottom of the tube) containing the following concentrations of Percoll in Buffer II: 60% (v/v), 3.0 ml; 30% (v/v), 3.5 ml; 20% (v/v), 3.5 ml; 10% (v/v), 3.0 ml; and 5% (v/v), 3.0 ml. The gradient was centrifuged at 29000 g for 30 min in a Beckman SW 27.1 rotor. Three fractions appeared at the interfaces between the 60% and 30% Percoll (Band A); between 30% and 20% Percoll (Band B); and between 20% and 10% Percoll (Band C). Two further fractions appeared in the 10% layer (Band D) and in the 5% layer (Band E). Each fraction was collected by suction, diluted with 20 ml of Buffer II and centrifuged at 10000 g for 10 min. The pellets were resuspended in Buffer II to a final concentration of about 10 mg of protein/ml. Alternatively, 1.0 ml gradient fractions (from bottom to top) were obtained by using a Büchler Polystaltic pump.

### Digitonin fractionation of mitochondria

The highly purified and intact mitochondria obtained by Percoll-gradient centrifugation in Band A were further fractionated into a low-speed pellet (mitoplasts), a high-speed pellet (outer membrane vesicles) and a high-speed supernatant fraction (intermembrane components solubilized from other fractions by digitonin treatment). The method of Day & Wiskish (1975) for digitonin fractionation of mitochondria was used with certain modifications. The mitochondrial suspension contained 9 mg of protein/ml of buffer II. To this suspension 0–2 mg of digitonin/9 mg of mitochondrial protein was added from a concentrated stock and this

mixture was kept on ice and stirred continuously for 30 min. The suspension was then diluted with 4 vol. of Buffer II and centrifuged at 30000 g for 30 min at 4 °C to obtain the low-speed pellet. The supernatant fluid was then centrifuged at 170000 g for 8 h to obtain the high-speed pellet. The supernatant fluid remaining on top of the high-speed pellet (170000 g supernatant fraction) was also recovered. Unfractionated mitochondria, not treated with digitonin, were used as controls for comparing various enzymic activities, protein and adenosine 3'-phosphate 5'-phospho[<sup>35</sup>S]sulphate formation from [<sup>35</sup>S]sulphate. The ratios of digitonin to mitochondrial protein used in the present work did not appear to affect the activities of any of the enzymes. However, higher ratios than those reported here were found to decrease succinate dehydrogenase activity and activate adenylate kinase activity.

### Assay for adenosine 3'-phosphate 5'-phospho[<sup>35</sup>S]sulphate formation from [<sup>35</sup>S]sulphate

After incubation of mitochondria or mitochondrial fractions in individual experiments, the suspension was chilled on ice for 10 min and then centrifuged for 10 min in a Microfuge (about 10000 g). Non-radioactive (carrier) adenosine 3'-phosphate 5'-phosphosulphate (20 µg/incubation) was added to the supernatant fluid. Acid-washed charcoal (15 mg/ml) was then added to adsorb the nucleotides (Fankhauser *et al.*, 1981). After thorough vortex-mixing the mixture was centrifuged at 5000 g for 10 min at 4 °C. The charcoal was resuspended in the supernatant fluid, and the mixture was again centrifuged. The supernatant fluid was discarded and the charcoal was washed with two 2 ml portions of cold distilled water adjusted to pH 7.0 with LiOH, by centrifugation at 5000 g for 10 min at 4 °C. The washes, containing various salts used in the reaction mixture and excess <sup>35</sup>SO<sub>4</sub><sup>2-</sup>, were discarded. The charcoal pellet was then extracted with three 2 ml portions of aq. 1% (v/v) NH<sub>3</sub> in 50% (v/v) ethanol, by using centrifugation at 5000 g for 10 min at 4 °C. The eluates were combined and were concentrated *in vacuo* at 30 °C to about 2 ml, frozen in liquid N<sub>2</sub> and freeze-dried. The dried powder was redissolved in 0.15–0.30 ml of distilled water adjusted to pH 7.0 with LiOH. A known volume of this sample, with additional carrier adenosine 3'-phosphate 5'-phosphosulphate (20 µg per portion spotted), was applied to Whatman 3MM filter paper (57 cm × 20 cm) and was subjected to electrophoresis (EC apparatus) in 0.1 M-sodium borate buffer, pH 8.0, at 1800 V for 70 min with ice/water cooling. After the paper had been dried, the adenosine 3'-phosphate 5'-phosphosulphate spot was located under 253.7 nm u.v. light, excised, cut into small pieces, placed in a scintillation vial with a mixture containing 0.4% 2,5-diphenyloxazole and 0.012% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene and the radioactivity was measured in a Beckman model LS-150 liquid-scintillation counter. An area of paper similar in size to each sample but away from the radioactive spots was treated similarly to provide a control for background radioactivity.

### Purification and identification of adenosine 3'-phosphate 5'-phosphosulphate

Adenosine 3'-phosphate 5'-phosphosulphate was purified by the methods of Tsang *et al.* (1976) and Fankhauser *et al.* (1981), with slight modifications. As a modification

to the method of Tsang *et al.* (1976), the use of 1.0 M-triethylammonium bicarbonate at pH 7.5 instead of 0.4 M-triethylammonium bicarbonate (pH 7.5) for elution of adenosine 3'-phosphate 5'-phosphosulphate was found to be more rapid, with a better resolution on the same type of Sephadex A-25 column. The methods of Frankhauser *et al.* (1981) used in identifying adenosine 5'-phosphoramidate by selective hydrolysis and paper electrophoresis [except that ethanol/NH<sub>3</sub> (see above) was used to elute adenosine 3'-phosphate 5'-phosphosulphate throughout the procedure] were used in the identification of adenosine 3'-phosphate 5'-phosphosulphate in the present work. Acid treatment of adenosine 3'-phosphate 5'-phosphosulphate at low pH (1.0) was done as described previously for adenosine 5'-phosphoramidate (Frankhauser *et al.*, 1981), and treatment with 3'-nucleotidase (EC 3.1.3.6) as previously described by Robbins & Lipmann (1957), and the products were purified by charcoal adsorption and paper electrophoresis (Frankhauser *et al.*, 1981) with 0.025 M-citrate buffer, pH 5.8 (Schmidt *et al.*, 1974). Non-radioactive spots were localized by using 253.7 nm u.v. light, and radioactive spots were localized by exposure of the electrophoretograms to Kodak no-screen X-ray film.

#### Assay of enzyme activities

ATP sulphurylase activity was determined by measuring the enzymic liberation of P<sub>i</sub> from ATP in the presence of molybdate and inorganic pyrophosphatase (Wilson & Bandurski, 1958). Condition for the assay of APS kinase were as previously described (Burnell & Whatley, 1975). Inorganic pyrophosphatase was assayed by the method of Shaw & Anderson (1972), but with 20 mM-Tricine, pH 7.6, as the buffer. P<sub>i</sub> formed was measured by a method described previously (Lebel *et al.*, 1978). ADP-sulphurylase (EC 2.7.7.4) activity was measured as phosphate uptake during the formation of ADP (Burnell & Anderson, 1973), but instead of the generation of adenosine 5'-phosphosulphate *in situ* with ATP, sulphate and sulphate adenyltransferase, synthetic adenosine 5'-phosphosulphate (2 mM or 2 μmol/ml) was used as the substrate. The formation of ADP was checked by paper electrophoresis in 0.025 M-citrate buffer, pH 5.8, on Whatman 3 MM filter paper (57 cm × 20 cm) at 1800 V for 1 h with ice/water cooling. Adenylate kinase (EC 2.7.4.3) was assayed by the electrophoretic method of Criss & Pradhan (1978), and inhibition of this activity was achieved by the addition of P<sup>1</sup>, P<sup>5</sup>-di(adenosine 5'-)pentaphosphate (Lienhard & Secemski, 1973). Alternatively, adenylate kinase was assayed spectrophotometrically at room temperature by coupling the formation of ATP to the reduction of NADP<sup>+</sup> with hexokinase and glucose-6-phosphate dehydrogenase (Schnaitman & Greenawalt, 1968). NADH:cytochrome *c* reductase was assayed as described by Sottocasa *et al.* (1967). Fumarase (EC 4.2.1.2) was assayed at 30 °C by using a method (Massey, 1955) based on the formation of fumarate, which is measured as an increase in absorbance at 250 nm. In order to minimize the loss of fumarase activity, the isolated mitochondria or mitochondrial fractions were resuspended in 1–2 ml of a stabilization buffer [15 mM-triethanolamine (pH 7.8)/5 mM-EDTA/10 mM-L-malic acid/20 mM-2-mercaptoethanol; H. Shibata & S. D. Schwartzbach, personal communication] before assay. One unit of fumarase produces a change in A<sub>250</sub> of 0.001/min. Succinate

dehydrogenase (EC 1.3.99.1) was assayed at 30 °C by the method of Davis & Merrett (1973). The reaction was started by the addition of phenazine methosulphate and the reduction of 2,6-dichloroindophenol was measured as a decrease in A<sub>600</sub>. A molar absorption coefficient of 21.9 × 10<sup>3</sup> litre · cm<sup>-1</sup> · mol<sup>-1</sup> (Nelson & Tolbert, 1970) was used to calculate the amount of 2,6-dichloroindophenol reduced. One unit of succinate dehydrogenase reduces 1 μmol of 2,6-dichloroindophenol/min. Protein was assayed by the method of Bradford (1976), with bovine serum albumin as the standard.

ADP obtained from Sigma contained small quantities of ATP as an impurity and was purified by using a column (1.5 cm × 10.0 cm) of Bio-Rad anion-exchange resin (AG1; X4; 200–400 mesh) as previously described (Rosing *et al.*, 1977). Isolated mitochondria or mitochondrial fractions were prepared for electron microscopy by addition of glutaraldehyde *in situ*, followed by further manipulations which have been described elsewhere (Gomez-Silva *et al.*, 1985b).

#### RESULTS AND DISCUSSION

Mutant W<sub>10</sub>BSmL of *Euglena gracilis* Klebs var. *bacillaris* Cori was used for preparation of mitochondria, since this mutant has been shown to lack plastids (particularly proplastids when grown in darkness) (Osafune & Schiff, 1983), which could contaminate the mitochondrial preparations. The crude mitochondrial fraction from W<sub>10</sub> was subjected to further separation on a Percoll step gradient (Fig. 1). The formation of adenosine 3'-phosphate 5'-phosphosulphate from sulphate was catalysed by all fractions to some extent, in the presence of either ADP or ATP, but activity was highest in fractions A and B, which contain purified intact mitochondria. Fraction A is enriched in contracted mitochondria and fraction B is enriched in mitochondria in the orthodox configuration (Gomez-Silva *et al.*, 1985a). Since fraction A gave the highest activity and yielded the most mitochondrial protein, this fraction was used in all subsequent work.

That adenosine 3'-phosphate 5'-phosphosulphate is indeed the product formed from sulphate in the presence of fraction-A mitochondria is demonstrated by the data in Fig. 2. The major <sup>35</sup>S-labelled product showed exact co-electrophoresis with the authentic material at pH 5.8. Acidification of a solution of the product leads to hydrolysis at room temperature; the non-radioactive

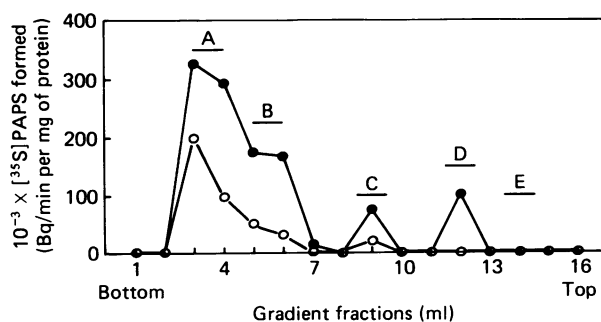


Fig. 1. Formation of adenosine 3'-phosphate 5'-phospho[<sup>35</sup>S]-sulphate ([<sup>35</sup>S]PAPS) from <sup>35</sup>SO<sub>4</sub><sup>2-</sup> by various mitochondrial fractions separated on a Percoll gradient

The complete system [containing either ADP (○) or ATP (●)] was the same as that shown in Table 2.

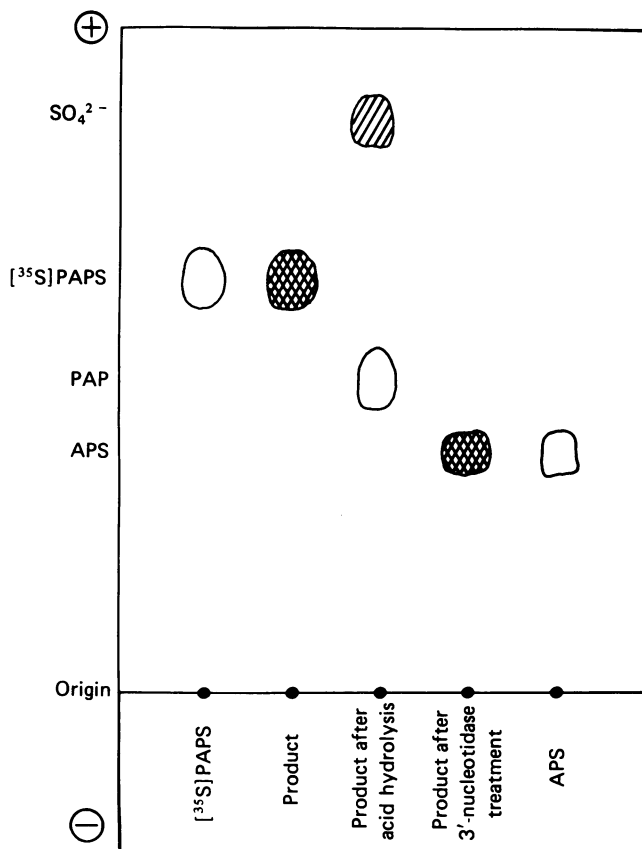


Fig. 2. Identification of the product formed from  $^{35}\text{SO}_4^{2-}$  by highly purified (Fraction A) mitochondria from *Euglena* as adenosine 3'-phosphate 5'-phospho $^{35}\text{S}$ ulphate ( $^{35}\text{S}$ ]PAPS) by paper electrophoresis of the compound and its hydrolysis products

The spots are coded to indicate whether they contain radioactivity and/or show quenching in u.v. light. ■, Radioactivity; □, u.v.; ▨ u.v. and radioactivity. Standards shown along the left margin include adenosine 3',5'-diphosphate (PAP), adenosine 5'-phosphosulphate (APS) as well as adenosine 3'-phosphate-5'-phosphosulphate and sulphate.

product shows quenching in u.v. light and shows co-electrophoresis with adenosine 3',5'-diphosphate, and the radioactive product shows co-electrophoresis with sulphate. On treatment with 3'-nucleotidase from rye grass (*Lolium perenne* Li.), all of the material moves as a radioactive spot that shows co-electrophoresis with adenosine 5'-phosphosulphate. These properties are all characteristic of adenosine 3'-phosphate 5'-phosphosulphate (Fankhauser *et al.*, 1981) and serve to confirm the identity of the product.

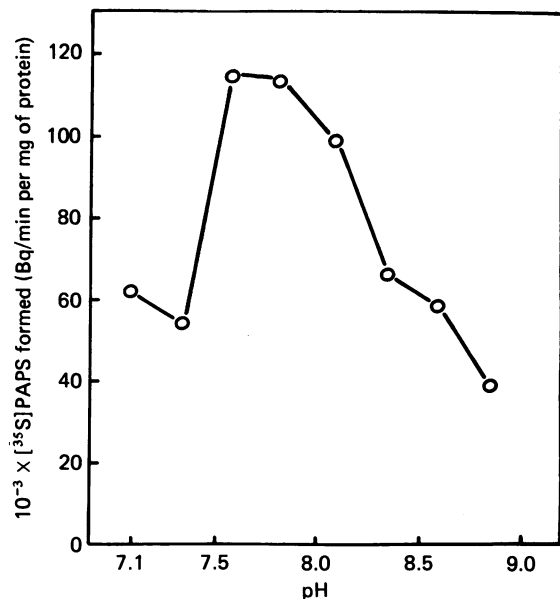
Although adenosine 3'-phosphate 5'-phosphosulphate is formed by highly purified intact mitochondria, nearly all of the compound accumulated in the presence of either ATP or ADP is found outside the organelles after incubation (Table 1). This indicates that the product is actively excreted from the organelles or that the sulphate-activating system is located external to the permeability barrier of the mitochondria (the mitochondrial inner membrane). When mitochondria were incubated for the usual time, re-isolated by centrifugation and re-incubated, formation of adenosine 3'-phosphate 5'-phosphosulphate and its appearance in the supernatant fraction during the second incubation were the same as in the first (results not shown), indicating that the sulphate-activating system is tightly bound within the mitochondria and is not released into the surrounding medium during incubation. Although adenosine 3'-phosphate 5'-phosphosulphate is the major product found inside and outside the mitochondria after incubation, small amounts of other radioactive spots are also found after paper electrophoresis of the supernatant fraction and the mitochondrial extract. One of these has the mobility of adenosine 5'-phosphosulphate; the others have not been identified.

A number of experiments were performed to optimize the conditions for the formation of adenosine 3'-phosphate 5'-phosphosulphate by the mitochondria (Figs. 3 and 4). The system shows a broad pH optimum with a peak in the region of pH 7.6–7.8 (Fig. 3). The broad optimum is probably the result of the participation of the several enzymes known to be required for sulphate activation (ATP sulphurylase, APS kinase and in-

Table 1. Adenosine 3'-phosphate 5'-phospho $^{35}\text{S}$ ulphate recovered from inside and outside the mitochondria after incubation of the organelles in the complete system

The complete assay mixture contained, in a total volume of 2.65 ml: Tricine/KOH, pH 7.6, 17 mM;  $\text{MgCl}_2$ , 18 mM; sucrose, 250 mM;  $\text{Na}^+$ -ADP, 5.66 mM, or  $\text{Na}^+$ -ATP, 0.94 mM; protein, 0.85 mg;  $\text{K}_2\text{SO}_4$ , 1 mM, and  $^{35}\text{SO}_4^{2-}$ , 32.0  $\mu\text{Ci}$ . The incubation mixture was shaken for 1 h at 30 °C. After incubation the mixture was separated by centrifugation into supernatant fluid ('outside') and mitochondrial pellet. The internal ('inside') adenosine 3'-phosphate 5'-phosphosulphate of samples 1 and 3 was extracted by subjecting the mitochondrial pellets (resuspended in 2.65 ml of buffer II) to two 10 s bursts of sonication (Branson model 200 sonifier with microtip, continuous power, at 0 °C) with an interval of 5 s between them, followed by centrifugation in a Microfuge at top speed; the supernatant fraction was retained for analysis. Samples 2 and 4 were treated similarly, except that the mitochondrial suspensions were made 1.0% (v/v) with respect to Triton X-100 and sonication was omitted.

Sample no.	Nucleotide added	$10^{-3} \times$ Adenosine 3'-phosphate 5'-phospho $^{35}\text{S}$ ulphate formed (Bq/min per mg of protein)	Distribution (%)	
			Inside	Outside
1	ADP (5.66 mM)	62.7	3.98	96.02
2	ADP (5.66 mM)	71.0	4.92	95.08
3	ATP (0.94 mM)	194.0	0.80	99.20
4	ATP (0.94 mM)	192.3	2.60	97.40



organic pyrophosphatase) (Robbins, 1962; Abraham & Bachhawat, 1963). Either ADP at 5.6 mM or ATP at 0.94 mM is optimal, although ATP yields somewhat higher activity.  $\text{MgCl}_2$  yields the highest activity at 18 mM, and a sucrose osmoticum of 250 mM is best. Under the conditions used, formation of adenosine 3'-phosphate 5'-phosphosulphate is a linear function of mitochondrial protein and the time of incubation (Fig. 4).

The minimal requirements for sulphate activation are indicated in Table 2. Addition of  $\text{MgCl}_2$  and ADP (or ATP) is indispensable for good activity, but deletion of sucrose osmoticum leads to somewhat higher activity, despite the lower activities found at concentrations

Fig. 3. Influence of pH on the formation of adenosine 3'-phosphate 5'-phospho[ $^{35}\text{S}$ ]sulphate ( $^{35}\text{S}$ ]PAPS) from  $^{35}\text{SO}_4^{2-}$  by fraction A mitochondria from *Euglena*

The complete system was the same as that in Table 2; the pH was adjusted by the addition of KOH.

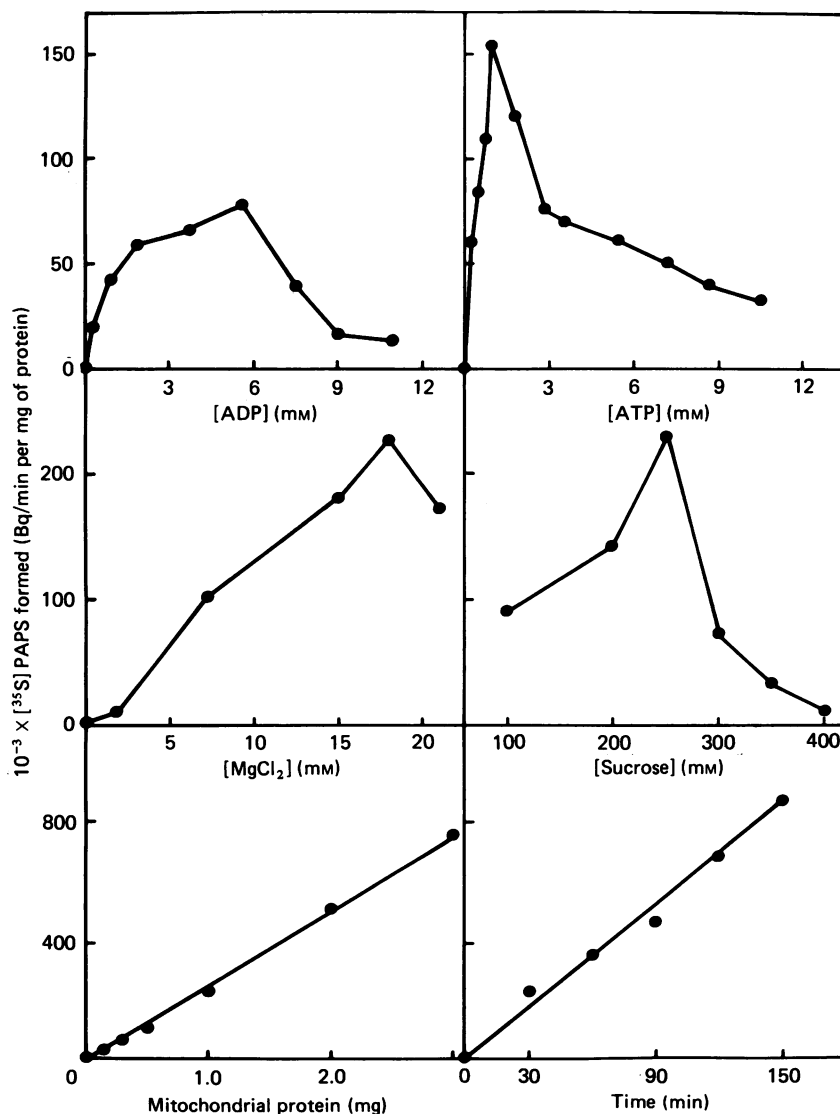


Fig. 4. Influence of various parameters on the formation of adenosine 3'-phosphate 5'-phospho[ $^{35}\text{S}$ ]sulphate ( $^{35}\text{S}$ ]PAPS) from  $^{35}\text{SO}_4^{2-}$  by fraction-A mitochondria from *Euglena*

The complete system was the same as shown in Table 2, but each parameter was varied as indicated in the Figure.

**Table 2. Minimal requirements for incorporation of  $^{35}\text{SO}_4^{2-}$  into adenosine 3'-phosphate 5'-phosphosulphate by *Euglena* mitochondria.**

The complete assay mixture contained, in a total volume of 2.65 ml: Tricine KOH, pH 7.6, 17 mM;  $\text{MgCl}_2$ , 18 mM; sucrose, 250 mM;  $\text{Na}^+$ -ADP, 5.66 mM; protein, 1 mg;  $\text{K}_2\text{SO}_4$ , 1.0 mM and  $^{35}\text{SO}_4^{2-}$ , 32.1  $\mu\text{Ci}$ . The incubation mixture was shaken for 1 h at 30 °C.

	$10^{-3} \times$ Adenosine 3'-phosphate 5'-phospho $^{35}\text{S}$ sulphate formed (Bq/min per mg of protein)	
	Expt. 1	Expt. 2
Complete system	105	123
– $\text{MgCl}_2$	5	—
– ADP	15	—
– Sucrose	126	—
Complete system (enzyme heated 100 °C, 5 min)	—	0

between the optimum and zero (Fig. 4). An osmotically intact organelle does not seem to be required for sulphate activation, probably because nucleotide di- or triphosphates are added and the participation of other mitochondrial functions such as oxidative phosphorylation is not required. Under iso-osmotic conditions, the sulphate-activating enzymes appear to be quite accessible to the substrates, but, as the osmoticum is decreased, the organelles undoubtedly swell and render the enzymes less accessible. At zero osmoticum, the organelles are probably lysed, making the enzymes highly accessible. As we will see, this explanation is not inconsistent with the localization studies to be described below.

Sulphate activation ordinarily utilizes ATP in both the first step (the formation of adenosine 5'-phosphosulphate catalysed by ATP sulphurylase) and the second step (the formation of adenosine 3'-phosphate 5'-phosphosulphate catalysed by APS kinase) (De Meio, 1975; Schiff, 1983). However, an ADP sulphurylase has been described that has only been assayed in the reverse direction (from

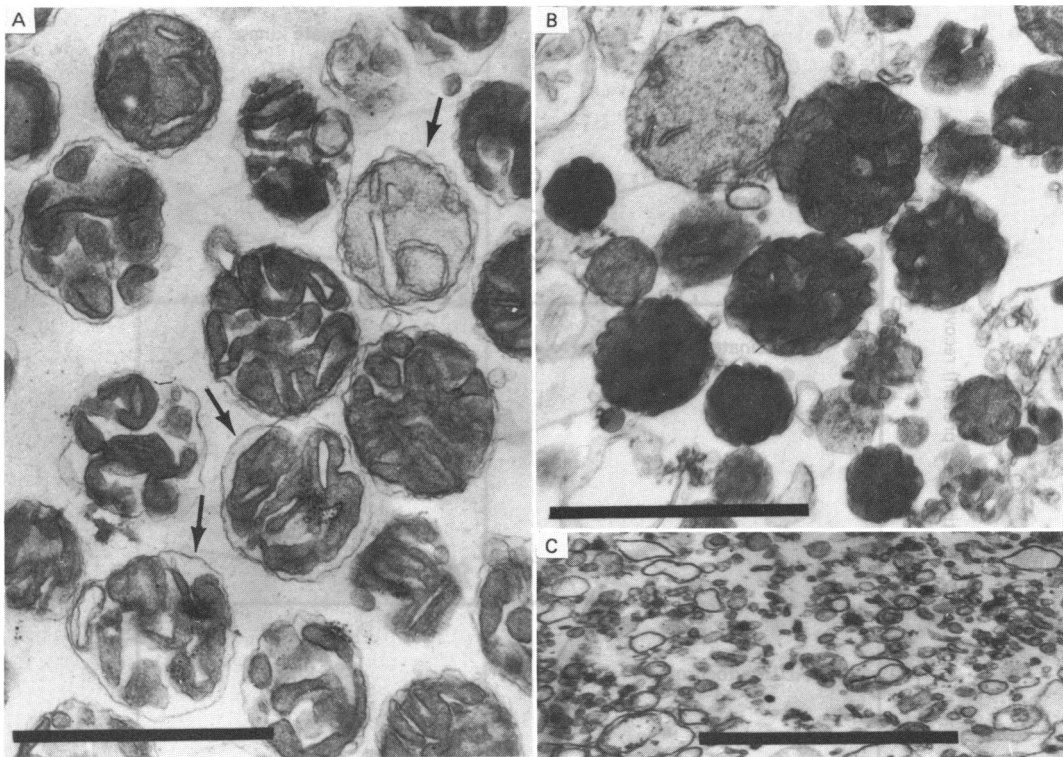
orthophosphate and adenosine 5'-phosphosulphate to ADP and sulphate). It was of interest, therefore, to determine how ADP and ATP were utilized by the mitochondrial sulphate-activating system. Since the conversion of two molecules of ADP into ATP plus AMP is readily accomplished under catalysis by adenylate kinase, an inhibitor of this enzyme [ $P^1, P^5$ -di(adenosine 5'-)pentaphosphate] was used in further experiments shown in Table 3. As the concentration of the inhibitor is raised, the formation of adenosine 3'-phosphate 5'-phosphosulphate from ADP and sulphate is progressively diminished, with 97% inhibition occurring at the highest concentration used. At the highest concentration of the inhibitor, the formation of adenosine 3'-phosphate 5'-phosphosulphate from sulphate and ATP is unaffected. Other data (Table 3) indicate that the highest concentration of the inhibitor effectively blocks ATP formation from ADP via adenylate kinase by 92%. Thus it is reasonable to conclude that ATP is the preferred substrate for sulphate activation via ATP sulphurylase and that ADP is active only through conversion into ATP via adenylate kinase. In agreement, ADP sulphurylase is undetectable (results not shown), although we can readily detect ATP sulphurylase, APS kinase and inorganic pyrophosphatase in these mitochondria (see below). The effective concentration of inhibitor required in these studies is considerably higher than has been used by others to inhibit highly purified rabbit muscle adenylate kinase (Lienhard & Secemski, 1973), but the data in the present experiments indicate that this concentration is necessary to inhibit the formation of ATP from ADP (Table 3). Perhaps binding of adenylate kinase to the mitochondrial membrane changes its reactivity with the inhibitor, or the adenylate kinase of *Euglena* mitochondria may have a lower affinity for the inhibitor than the purified muscle enzyme. Another possibility is that the inhibitor is enzymically degraded to some extent. We have noticed on electrophoretograms prepared from control incubation mixtures that the quenching spot of the inhibitor decreases somewhat during incubation, with the concomitant formation of other quenching spots.

To learn more about the location of the sulphate-activating system within the mitochondria, fractionation of the organelle with various concentrations of digitonin was undertaken. This fractionation yielded three com-

**Table 3. Inhibition of ATP and adenosine 3'-phosphate 5'-phospho $^{35}\text{S}$  sulphate formation in *Euglena* mitochondria by  $P^1, P^5$ -di(adenosine 5'-)pentaphosphate**

The composition of the reaction mixtures for the assay of the formation of adenosine 3'-phosphate 5'-phosphosulphate is given in the legend to Table 1. Assay of the formation of ATP from ADP (i.e. adenylate kinase activity) was done as described by Criss & Pradham (1978).

$[P^1, P^5\text{-Di(adenosine 5'-)pentaphosphate}]$ (M)	$10^{-3} \times$ Adenosine 3'-phosphate 5'-phospho $^{35}\text{S}$ sulphate formed with ADP as substrate (Bq/min per mg of protein)		$10^{-3} \times$ Adenosine 3'-phosphate 5'-phospho $^{35}\text{S}$ sulphate formed with ATP as substrate (Bq/min per mg of protein)		ATP formed from ADP ( $\mu\text{mol}/\text{min}$ per mg of protein)	
		Inhibition (%)		Inhibition (%)		Inhibition (%)
0	51.03	0	124.18	0	1.05	0
$3.7 \times 10^{-4}$	36.21	29	—	—	—	—
$3.7 \times 10^{-3}$	18.10	64	—	—	—	—
$3.7 \times 10^{-2}$	4.98	90	—	—	0.28	73
$9.4 \times 10^{-2}$	1.12	97	105.52	15	0.08	92



**Fig. 5.** Transmission electron micrographs of (A) fraction-A mitochondria from *Euglena*, (B) mitoplasts obtained by treatment of fraction-A mitochondria with digitonin (1 mg/mg of protein) followed by centrifugation at 30000 g, and (C) outer-membrane vesicles obtained by centrifugation of the supernatant fraction from B at 170000 g

The bar indicates 1.0  $\mu\text{m}$ . The arrows indicate the mitochondrial outer membranes.

**Table 4.** Distribution of the sulphate-activating system and related constituents within the mitochondria as determined by fractionation with digitonin (1 mg/9 mg of protein)

Adenylate kinase activity was assayed by the method of Schnaitman & Greenawalt (1968). All of the other constituents were assayed as described in the Experimental section. Abbreviation used: ND, non-detectable.

Expt. no.	Constituents from 100 $\mu\text{l}$ of unfractionated mitochondria	Amount recovered				Recovery (%) $\left[ \frac{(2)+(3)+(4)}{(1)} \times 100 \right]$
		Unfractionated mitochondria (1)	Mitoplasts (2)	Intermembrane components (3)	Outer membrane (4)	
1	Protein ( $\mu\text{g}$ )	470.0	280.0	80.0	60.0	89.4
	Succinate dehydrogenase ( $\mu\text{mol}/\text{min}$ )	3.7	3.7	ND	ND	100.0
	NADH:cytochrome <i>c</i> reductase [rotenone-insensitive] (pmol/min)	4.5	1.0	0.7	3.0	104.4
	Fumarase (pmol/min)	351.0	329.0	ND	ND	93.7
	Adenylate kinase (pmol/min)	86.6	64.5	12.0	8.8	98.4
	ATP sulphurylase (pmol/min)	41.4	26.5	9.3	6.3	101.6
	APS kinase (pmol/min)	5.4	4.6	0.5	ND	94.4
	Adenosine 3'-phosphate 5'-phospho[ $^{35}\text{S}$ ]sulphate formed from $^{35}\text{SO}_4^{2-}$ (Bq/min)	106800	84420	2520	5760	86.8
2	Protein ( $\mu\text{g}$ )	141.3	103.7	30.0	20.0	108.7
	Succinate dehydrogenase ( $\mu\text{mol}/\text{min}$ )	2.3	2.0	ND	ND	86.9
	NADH:cytochrome <i>c</i> reductase (pmol/min)	6.4	1.3	ND	4.5	90.6
	Inorganic pyrophosphatase (pmol/min)	8.0	6.7	0.3	ND	87.5

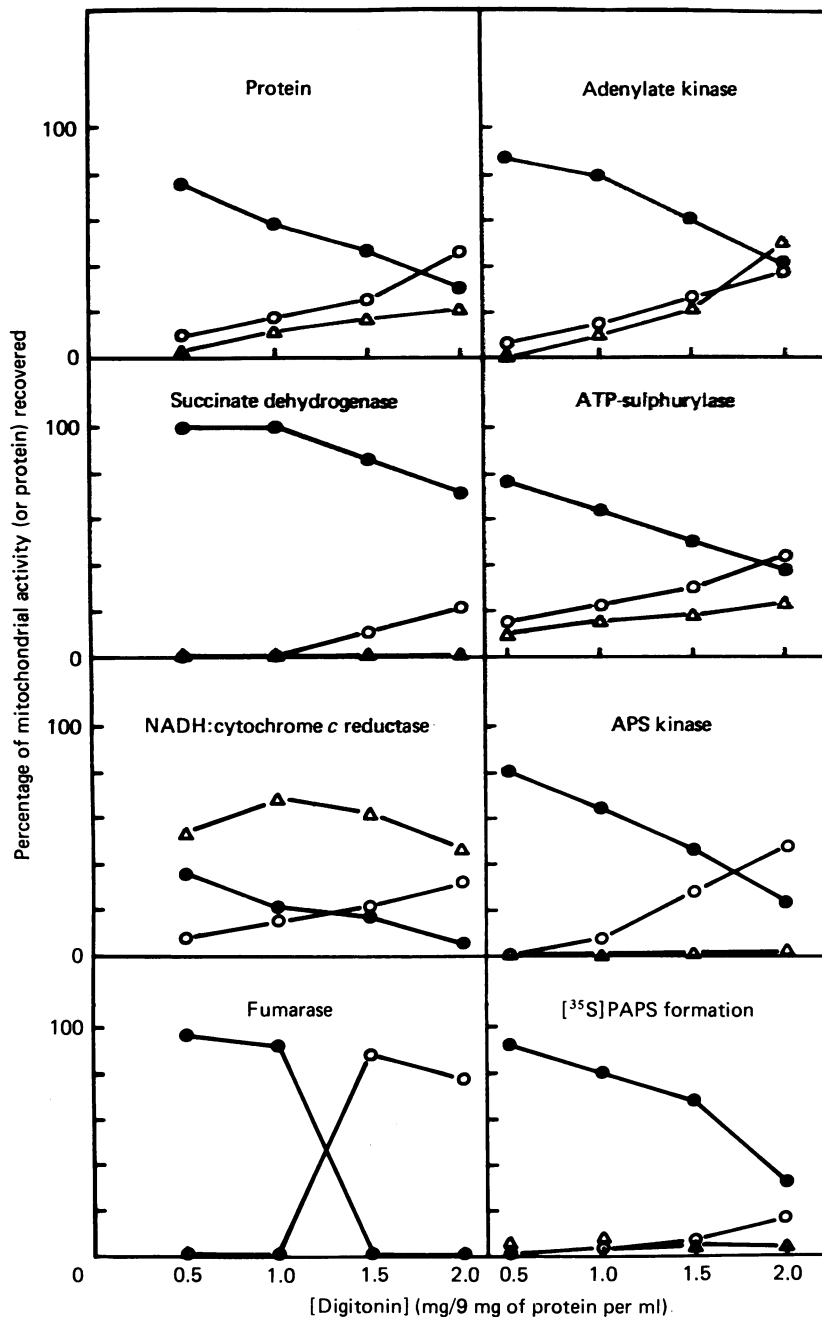


Fig. 6. Distribution in various centrifugal fractions of a number of constituents after treatment of fraction-A mitochondria from *Euglena* with various concentrations of digitonin

Key to symbols: ●, 30000 g pellet; △, 170000 g pellet; ○, 170000 g supernatant. Abbreviations used: APS, adenosine 5'-phosphosulphate, [<sup>35</sup>S]PAPS, adenosine 3'-phosphate 5'-phospho[<sup>35</sup>S]sulphate.

ponents in each case: a 30000 g pellet, a 170000 g pellet and a 170000 g supernatant fraction. Transmission electron micrographs of the two centrifugal pellets and the intact mitochondria from which they were prepared are compared in Fig. 5. The intact mitochondria are surrounded by a thin outer membrane with a thicker inner membrane surrounding the matrix. After fractionation with 1 mg of digitonin/9 mg of protein, the 170000 g pellet is seen to consist of membrane vesicles formed from the mitochondrial outer membrane. The 30000 g pellet contains the mitoplasts (mitochondria lacking the outer membranes); the only membrane around these structures

is now the mitochondrial inner membrane. These identifications are supported by assays for marker enzymes in the various fractions (Fig. 6). At all digitonin concentrations the rotenone-insensitive NADH:cytochrome *c* reductase, characteristic of mitochondrial outer membranes (Sottocasa *et al.*, 1967; Schnaitman *et al.*, 1967), is highly enriched in the 170000 g pellet, thought to contain outer-membrane vesicles. Fumarase, a soluble enzyme of the mitochondrial matrix is found in the 30000 g pellet (the mitoplast fraction) at low digitonin concentrations, but is rapidly solubilized at higher concentrations. Adenylate kinase, thought to be attached



to the outside of mitochondrial inner membranes (Arron *et al.*, 1976), is also found in the 30000 g pellet at low digitonin concentrations, but, as the concentration of the detergent is raised, the activity associated with the mitoplasts rapidly decreases and appears in the supernatant and outer-membrane fractions.

The enzymes of the sulphate-activating system behave essentially like adenylate kinase. They are associated with the mitoplasts at low digitonin concentrations, but their activity in this fraction decreases and rises in the supernatant and the outer-membrane fractions as the digitonin concentration is raised. Adenosine 3'-phosphate 5'-phosphosulphate formation from sulphate follows the same pattern as the individual activating enzymes. As may be seen in Table 4, the recoveries of the various enzyme activities are quite good at 1 mg of digitonin/9 mg of protein, and the components of the sulphate-activating system (ATP sulphurylase, APS kinase and inorganic pyrophosphatase), as well as the formation of adenosine 3'-phosphate 5'-phosphosulphate from sulphate, are highly enriched in the mitoplast fraction along with succinate dehydrogenase, fumarase and adenylate kinase, but are not enriched in the outer-membrane fraction, which contains most of the NADH:cytochrome *c* reductase activity. Taking together the data from Figs. 5 and 6 and from Table 4, the sulphate-activating system appears to be most closely associated with the adenylate kinase and is probably located along with that enzyme on the outside of the inner membranes of *Euglena* mitochondria. Such a location is also consistent with the findings that the sulphate-activating system has ready access to the ATP formed from ADP by adenylate kinase, that the adenosine 3'-phosphate 5'-phosphosulphate formed readily leaves the mitochondria during incubation and that the sulphate-activating enzymes have ready access to the ATP or ADP added externally. The outer membrane of the mitochondrion does not appear to hinder the passage of small molecules significantly; the inner membrane is thought to be the selective permeability barrier of the organelle (Vignais, 1976). In agreement, treatment of *Euglena* mitochondria with 40  $\mu$ M-carboxyatractyloside, a potent inhibitor of ATP or ADP transport across the inner membrane (Palmieri & Klingenberg, 1979), does not affect the formation of adenosine 3'-phosphate 5'-phosphosulphate from sulphate and ADP (results not shown).

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