3'-Phosphoadenosine-5'-phosphosulphate synthesis and involvement in sulphotransferase reactions in the insect, *Spodoptera littoralis*

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1. Synthesis of 3'-phosphoadenosine-5'-phosphosulphate from ATP and $^{35}\text{SO}_4^{2-}$ was demonstrated in homogenates of gut, Malpighian tubules and fat body of *Spodoptera littoralis*. 2. The enzyme system was most active in the gut tissue, and was primarily located in the cytosol fraction of the cell. Gut cytosol preparations were used as a source of the 3'-phosphoadenosine-5'-phosphosulphate generating system for more detailed studies. 3. Maximum synthesis required an incubation mixture containing Tris/HCl buffer (pH 7.5), ATP (20 mM), MgCl_2 (13.0 mM) and K_2SO_4 (3 mM). 4. The specific activity of the 3'-phosphoadenosine-5'-phosphosulphate synthesizing activity in gut cytosol increased during development of the sixth instar larva, reaching a peak at day 4. A sudden fall in specific activity was observed in the prepupal stage. 5. 3'-Phosphoadenosine-5'-phosphosulphate formation is the rate limiting process in the overall sulphation of *p*-nitrophenol in the gut cytosol preparations from *S. littoralis*. 6. It is concluded that the properties of the sulphate-activating system in this insect are similar to those reported for vertebrates.

In insects there is evidence that sulphoconjugation plays a major role in the detoxication and excretion of xenobiotics (Smith, 1968; Yang & Wilkinson, 1971). Sulphation is also involved in the regulation of the circulating pools of metabolites. For example, dopamine sulphate and *N*-acetyldopamine sulphate have been isolated in relatively large quantities from the cockroach, *Periplaneta americana*. These can serve as sources of dopamine and *N*-acetyldopamine which are incorporated into the cuticle during sclerotization (Bodnaryk & Brunet, 1974; Bodnaryk *et al.*, 1974; Brunet, 1980).

Sulphoconjugation is also apparently involved in the metabolism of juvenile hormones (Slade & Wilkinson, 1974; De Kort & Granger, 1981), sterols (Hutchins & Kaplanis, 1968; Yang & Wilkinson, 1972, 1973) and ecdysteroids (insect moulting hormones) (Koolman *et al.*, 1973; Russell & Price, 1977). It has been suggested that such polar ecdysteroid conjugates may serve as storage forms of the hormone (Willig *et al.*, 1971). Variation in the sulphotransferase and sulphohydrolase enzyme activities during development of *S. eridania* led to speculation as to their possible role in controlling the free ecdysteroid titre (Yang *et al.*, 1973).

Although sulphation is important, there is a paucity of information about the mechanism, par-

Abbreviations used: PAdoPS, 3'-phosphoadenosine-5'-phosphosulphate; AdoPS, adenosine 5'-phosphosulphate.

ticularly as insect sulphotransferase activity has only been measured using ATP, SO_4^{2-} and Mg^{2+} as cofactors (Yang & Wilkinson, 1972; Yang *et al.*, 1973). In vertebrates, it is generally accepted that the principal sulphate donor in sulphotransferase reactions is PAdoPS ('PAPS'; 3-phosphoadenylyl sulphate) which is synthesized by a two-step reaction requiring the enzymes sulphate adenylyltransferase (EC 2.7.7.4) and adenylylsulphate kinase (EC 2.7.1.25). Sulphate adenylyltransferase catalyses the formation of AdoPS ('APS', adenylylsulphate) which is itself utilized by adenylylsulphate kinase to form PAdoPS (Dodgson & Rose, 1970; Roy & Trudinger, 1970; De Meio, 1975).

In the present study, we have demonstrated a PAdoPS-generating system in an insect which appears to be the rate limiting step in the sulphation of *p*-nitrophenol, at least *in vitro*. The properties of the sulphate-activating system, including tissue and subcellular distribution and developmental pattern, are reported for the sixth instar larva of *S. littoralis*.

Materials and methods

Chemicals

ATP (sodium salt) and bovine serum albumin

were obtained from P-L Biochemicals, *p*-nitrophenol was from Sigma, carrier-free $^{35}\text{SO}_4^{2-}$ was from The Radiochemical Centre, Amersham, and Aquasol was from New England Nuclear. [^{35}S]AdoPS was a gift from Dr. G. F. White, Department of Biochemistry, University College, Cardiff, U.K. 3-Phosphoadenosine-5'-phospho[^{35}S]-sulphate was prepared by the method of Bailey-Wood *et al.* (1970). PEI-cellulose (LS 254) t.l.c. plates were obtained from Schleicher and Schüll, Dassel, Germany. All other chemicals were obtained from BDH and were 'Analar' grade.

Growth of insects

S. littoralis Boisd larvae were reared on an artificial agar-based diet prepared as described by McKinley (1970). The insects were maintained at a constant temperature of 25°C and a relative humidity of 65–70% while a 16h light period alternated with an 8h dark period. Under these conditions the sixth instar lasted 6 days and the whole life cycle was completed in 42 days.

Thin-layer chromatography and assay of radioactive metabolites

PAdoPS, AdoPS inorganic SO_4^{2-} and *p*-nitrophenyl sulphate from enzymic reaction mixtures were separated by t.l.c. Aqueous aliquots (10 μl) of the reaction mixtures, which had been deproteinized by immersion in boiling water for 15s and the precipitate removed by centrifugation, were chromatographed on PEI-cellulose plates impregnated with a fluorescent dye (254 nm) with 0.4M- NH_4HCO_3 for development. Radioactivity was located with a Panax radiochromatogram scanner, whereas non-radioactive compounds were located by viewing under u.v. light (254 nm). Typical R_F values are given in Fig. 1. The radioactive cellulose was then removed from the plastic support and transferred to a glass scintillation vial to which 1 ml of water and 8 ml of Aquasol scintillation fluid were added. The radioactivity was then assayed by using an Inter technique SL 4000 liquid-scintillation spectrometer. Counting efficiencies (approx. 65%) were routinely determined with dioctyl[^{35}S]sulphide as an internal standard.

Preparation of tissue homogenates

Fat bodies, guts (free of peritrophic membrane) and Malpighian tubules were dissected from 2–3-day old larvae (10) of the sixth instar, and together with the remaining carcasses, were washed with 0.15M-KCl. The tissues were homogenized with a Potter-Elvehjem homogenizer in 0.15M-KCl to give 20% (w/v) homogenates which were used immediately for assay of PAdoPS formation. Haemo-

lymph was drained from the larvae before dissection and was used directly for assay.

Subcellular fractionation

Since gut contained greater PAdoPS synthesizing activity than any of the other tissues examined (Table 1), this tissue was used for subsequent study. The guts of sixth instar larvae (50) were excised and chilled in insect Ringer solution (Bodenstein, 1946) and were cleaned by removing the peritrophic membrane together with the gut contents and were then rinsed in 0.15M-KCl. The tissue was homogenized as described earlier and the homogenate was then centrifuged at 750g for 10 min. The pellet was rehomogenized in 0.15M-KCl and centrifuged at 750g for 10 min. This procedure was repeated once more to give the nuclear pellet which was resuspended in 0.15M-KCl. A sample of the combined 750g supernatants was kept for assay and the rest was centrifuged at 17300g for 20 min to give a pellet which was resuspended in 0.15M-KCl to form the combined mitochondrial and lysosomal fraction. The preceding centrifugation procedures were carried out using a Sorvall RC2-B centrifuge and an SS-34 rotor. Finally, centrifugation of the 17700g supernatant at 100000g for 60 min in a T865-1 rotor of a Sorvall OTD-65 ultracentrifuge yielded the 'microsomal' and cytosol fractions. The subcellular fractionation was based on a procedure used for the gut tissue of the insect, *Calliphora vicina*, where the subcellular fractions were characterized by assay of appropriate marker enzymes (R. E. Isaac, K. K. Phua & H. H. Rees, unpublished work). Protein content of each fraction was estimated using the method of Gornall *et al.* (1949). For the routine preparation of gut cytosol, a single centrifugation of the 20% (w/v) homogenate at 100000g for 60 min was sufficient. Subcellular preparations (8–9 mg of protein/ml) could be stored in 0.1 ml volumes of 0.15M-KCl at –20°C for up to 6 days without loss of PAdoPS synthesizing or phenol sulphotransferase activities.

Crude rat liver sulphotransferase was prepared as described by Jenner & Rose (1973).

Enzyme reactions

Synthesis of PAdoPS for characterization and studies on tissue distribution of its formation. [^{35}S]PAdoPS was generated in an incubation mixture containing, at a final concentration, 10mM-ATP, adjusted to pH 7.5 with NaOH, 10mM-MgCl₂, 25mM-Tris/HCl buffer (pH 7.5) and 1.2 μCi of $^{35}\text{SO}_4^{2-}$. Reactions were initiated by the addition of 50 μl of insect tissue homogenate to make a final incubation volume of 110 μl and were carried out at 37°C. After 1h, reactions were terminated as described earlier by heating to 100°C for 15s.

Routine assay of PAdoPS synthesis. Standard reaction mixtures contained: 20 mM-ATP adjusted to pH 7.5 with NaOH, 13.0 mM-MgCl₂, 40 mM-Tris/HCl buffer (pH 7.5) and 3 mM-K₂SO₄ (all final concentrations) and 10 μ Ci of ³⁵SO₄²⁻. The reactions were initiated by the addition of 90 μ l (0.4 mg of protein) of the gut cytosol preparation to make a final incubation volume of 150 μ l. Incubation was carried out at 37°C for 1 h and the reactions were stopped by immersing the incubation tubes in boiling water for 15 s. PAdoPS was stable for up to 1 min at 100°C. Coagulated protein was sedimented by centrifugation at 500g for 6 min and 10 μ l of the resulting supernatant was used for t.l.c. Control determinations were made by adding enzyme to the reaction mixture immediately before boiling. After chromatography, the percentage incorporation of ³⁵S into PAdoPS was determined by scintillation counting. The amount of PAdoPS formed was calculated from the known specific activity of the ³⁵SO₄²⁻ as described by Mattock & Jones (1970). Under these reaction conditions, PAdoPS synthesis was linear with time for up to 1 h and with protein concentration up to 6 mg/ml.

Phenolsulphotransferase. This enzyme (EC 2.8.2.1) was assayed using *p*-nitrophenol as the acceptor. After incubation of the standard reaction mixture for PAdoPS synthesis with gut cytosol, 50 μ l of the deproteinized solution was used directly as the source of PAdoPS. A typical reaction mixture contained: 40 mM-Tris/HCl buffer (pH 7.5), 30 mM-EDTA, 20 mM-dithiothreitol and 0.5 mM-*p*-nitrophenol as final concentrations. The PAdoPS solution (50 μ l; 15 nmol) and 50 μ l of the cytosol preparation (from either insect gut or rat liver) containing sulphotransferase activity were added to make a final incubation volume of 200 μ l. After incubation at 37°C for 1 h, the reaction was terminated by the addition of 0.1 M-NaOH (1 ml). The absorbance of the solution at 400 nm, ϵ_{400} = 18200 (Dodgson & Spencer, 1953), was read against blanks prepared by replacing the *p*-nitrophenol in the incubation mixture with water. Controls were made by adding the cytosolic enzyme preparation immediately before the addition of alkali. The amount of *p*-nitrophenyl sulphate formed was calculated from the difference in the absorbance of the test and control incubations. Synthesis of PAdoPS during the incubation was prevented by the presence of EDTA (Brunngraber, 1958).

In other experiments phenolsulphotransferase activity was measured using ATP/Mg²⁺ and SO₄²⁻ as cofactors, thus relying on the synthesis of PAdoPS *in situ*. The reaction was carried out by adding *p*-nitrophenol to the standard incubation mixture for PAdoPS synthesis (concentrations in 200 μ l final volume: 15 mM-ATP, 10 mM-Mg²⁺, 2.3 mM-K₂SO₄ and 0.5 mM-*p*-nitrophenol) prior to the

addition of enzyme. Reactions were stopped by the addition of NaOH (1 ml), and the *p*-nitrophenyl-sulphate was estimated by measuring the decrease in the absorbance of *p*-nitrophenol at 400 nm.

Results

Identification of AdoPS and PAdoPS

To demonstrate the involvement of AdoPS and PAdoPS in sulphotransferase reactions it was first necessary to show that these nucleotides could be synthesized from ATP and inorganic sulphate in the absence of exogenous sulphate acceptor.

Incubation of ³⁵SO₄ with *S. littoralis* gut homogenate in the presence of ATP and Mg²⁺ resulted in the formation of two ³⁵S-labelled compounds which co-chromatographed with authentic AdoPS and PAdoPS on PEI-cellulose t.l.c. (Fig. 1a). There was an absolute requirement for ATP and Mg²⁺ in the formation of both compounds. An aliquot of this reaction mixture was incubated in the presence of EDTA with *p*-nitrophenol and a rat liver cytosol preparation, rich in sulphotransferase activity. Essentially all the radioactivity corresponding to PAdoPS, but not AdoPS, disappeared and was replaced by a ³⁵S-labelled component which co-chromatographed with *p*-nitrophenyl sulphate (Fig. 1b).

AdoPS formation varied little with increase in incubation time (results not shown) and was a minor product of the reaction except in the early part of the incubation period when the amount of PAdoPS

Fig. 1. Thin-layer radiochromatograms of ³⁵SO₄²⁻ metabolites in gut homogenates from *S. littoralis*. Incubations were performed in the absence (a) or in the presence (b) of *p*-nitrophenol and rat liver cytosol sulphotransferase. For experimental details see the text. Radioactive component I co-chromatographed with PAdoPS (R_F = 0.23), II with AdoPS (R_F = 0.34), III with SO₄²⁻ (R_F = 0.61) and IV with *p*-nitrophenyl sulphate (R_F = 0.43). O is the origin and F is the solvent front.

formed was low. These observations are to be expected if, as in other biological systems (De Meio, 1975), the formation of *AdoPS* is not favoured thermodynamically and will only proceed appreciably if the products of the reaction are continually removed.

Tissue and subcellular distribution of PAdoPS-synthesizing activity

The ability of the insect tissues to synthesize *PAdoPS* was investigated by incubating fat body, gut, Malpighian tubule and carcass homogenates with $^{35}\text{SO}_4^{2-}$, ATP and Mg^{2+} . Greatest incorporation of $^{35}\text{SO}_4^{2-}$ into *PAdoPS* was achieved by the gut (Table 1). Fat body tissue and Malpighian tubules could also synthesize *PAdoPS* to a lesser extent, but not haemolymph and carcass.

Using the routine assay for *PAdoPS* synthesis, a quantitative subcellular distribution pattern was obtained (Table 2). *PAdoPS*-synthesizing activity was mainly located in the cytosol fraction (100 000 g supernatant). Some activity in the other subcellular fractions might reflect contamination by the soluble enzymes, and in the case of the nuclear fraction by the presence of unbroken cells. Since *PAdoPS* synthesis predominated in the gut cytosolic fraction, this source of enzymic activity was used for all subsequent experiments.

Table 1. *Synthesis of [^{35}S]PAdoPS from ATP/Mg $^{2+}$ and $^{35}\text{SO}_4$ by various tissues of *S. littoralis**

For details see the text. —, *PAdoPS* synthesis not detected.

Tissue	Wet wt./ insect (mg)	[^{35}S]PAdoPS synthesis (% of the total synthesis in tissues from one insect)
Fat-body	28	9
Gut	42	67
Malpighian tubules	10	24
Carcass	250	—
Haemolymph	130	—

Conditions for maximum PAdoPS synthesis

Effect of pH. Three buffers (sodium citrate, pH 5.5–6.5; Tris/HCl, pH 6.5–9.0; NaHCO_3 , pH 9.0–10.00) were used to measure *PAdoPS* synthesis over a broad pH range (Fig. 2a). Maximum *PAdoPS* formation was observed at pH 7.5 with Tris/HCl as buffer.

Effect of substrate concentration. At a constant final concentration of 1 mM- K_2SO_4 the amount of *PAdoPS* formed varied with changes in both ATP and Mg^{2+} concentrations (Figs. 2b and 2c). *PAdoPS* synthesis was greatest at final concentrations of 20 mM-ATP and of 10–15 mM- Mg^{2+} . The formation of *PAdoPS* was also dependent upon the final sulphate concentration and was maximal at 3 mM- K_2SO_4 (Fig. 2d).

PAdoPS synthesis during the development of the sixth instar larva

The rate of formation of *PAdoPS* per mg of cytosol protein was determined at daily intervals and was shown to increase 2.5 fold during development from day 0 to day 4 (Fig. 3a). A sudden fall in the synthesis between day 5 and day 6 coincided with the onset of pupation. There was little fluctuation in the protein concentration of the gut cytosol between day 2 and day 5 (Fig. 3b). This developmental pattern was completely reproducible in another experiment.

Effect of peritrophic membrane homogenate on the PAdoPS-synthesizing activity of the gut cytosol

The peritrophic membrane was routinely removed from the lumen of the gut prior to homogenization and preparation of the gut cytosol fraction. The effect of this tissue on *PAdoPS* synthesis in the gut preparation was investigated as follows. A homogenate was prepared from the peritrophic membranes of 20 larvae in 0.15 M-KCl (0.5 ml). When an aliquot (45 μl) of this preparation was added to the standard incubation mixture, *PAdoPS* production was reduced by 34%. This apparent inhibition was

Table 2. *PAdoPS synthesis by subcellular fractions of *S. littoralis* gut tissue*

For details see the text. Absolute values are with respect to the complete fractions isolated from 50 larvae. Percentage recoveries were calculated from summing the protein content or *PAdoPS* synthesizing activity of the individual fractions and dividing this by the sum of the protein content or activity of the nuclear fraction plus 750 g supernatant (129 mg and 2259 nmol/h respectively).

Subcellular fraction	Percentage distribution				Recovery (%)
	Nuclear	Mitochondrial + lysosomal	Microsomal	Cytosol	
Protein	52	10	6	32	107
<i>PAdoPS</i> synthesis	7	5	5	83	108

Fig. 2. Effect of pH and substrate concentrations on PAdoPS synthesis by the gut cytosol from *S. littoralis*. The effect of pH (a) was determined with 40 mM-sodium citrate buffer (□), 40 mM-Tris/HCl buffer (○) or sodium bicarbonate buffer (△). Other panels show the effect of (b) ATP concentration in the presence of 13 mM-MgCl₂ and 1 mM-K₂SO₄, (c) Mg²⁺ concentration in the presence of 20 mM-ATP and 1 mM-K₂SO₄, and (d) SO₄²⁻ concentration in the presence of 13 mM-MgCl₂ and 20 mM-ATP. For experimental details see the text. In all experiments maximum synthesis of PAdoPS was between 100 and 120 nmol/h per mg of protein.

prevented by heating the peritrophic membrane homogenate to 100°C for 1 min.

Phenol sulphotransferase activity in the gut cytosol of *S. littoralis*

The reaction rate for the sulphation of *p*-nitrophenol starting from ATP/Mg²⁺ and inorganic sulphate was compared with the rate of sulphation when PAdoPS was already present in the incubation mixture. Results showed (Fig. 4a) that, following a lag period of 4 min, sulphation was linear for an incubation period of 1 h in the absence of preformed PAdoPS and that in this time-period 84 nmol of *p*-nitrophenyl sulphate/mg of protein were produced. The lag probably reflected the low initial level of endogenous PAdoPS. Phenolsulphotransferase activity was also measured in the presence of preformed PAdoPS as described in the Methods section. Transfer of sulphate from PAdoPS to *p*-nitrophenol was complete after 10 min, producing 90 nmol of *p*-nitrophenyl sulphate per mg of protein

(Fig. 4b); this indicated that all the PAdoPS had been utilized in the reaction. After this period no more *p*-nitrophenyl sulphate was formed since the presence of EDTA inhibited any synthesis *in situ* of the sulphate donor, PAdoPS. These results suggest that the rate-limiting step in the sulphation of *p*-nitrophenol from ATP/Mg²⁺ and inorganic sulphate is the formation of PAdoPS.

Discussion

In the present study we have shown that, as in other biological systems, the metabolism *in vitro* of inorganic sulphate in *S. littoralis* yields the activated sulphate nucleotides, AdoPS and PAdoPS. This insect species can readily synthesize both AdoPS and PAdoPS in a number of tissues, of which the gut is the most active. AdoPS is most probably an intermediate in the synthesis of PAdoPS from ATP and inorganic sulphate. Optimum conditions for the overall synthesis of PAdoPS were similar to those

determined for sulphate activation in vertebrates (Hall & Straatsma, 1966; Koizumi *et al.*, 1969; Scully *et al.*, 1970). In the latter studies the highest levels of *PAdoPS* synthesis were obtained with

10–13 mM- Mg^{2+} , 30–45 mM-ATP and a pH of 7.5 or 8.0. In this insect species the cytosolic location of *PAdoPS* synthesis was in common with the sub-cellular distribution in vertebrates (Koizumi *et al.*, 1969). In liver tissue the cytosol fraction also contains a number of enzymes which degrade *PAdoPS* and *AdoPS* (Dodgson & Rose, 1975), but their activity can be minimized by the presence of at least 0.1 mM-ATP (Denner *et al.*, 1973). In the present study it is unlikely that analogous enzyme activities have affected the observed rate of *PAdoPS* synthesis because of the high ATP concentration in the incubation medium.

PAdoPS synthesis in the gut cytosol fraction, expressed per mg of cytosol protein, varied during development of the sixth instar larva, reaching a peak at day 4. This twofold increase in specific activity coincides with increase in larval weight (R. E. Isaac, K. K. Phua & H. H. Rees, unpublished work) and therefore high biosynthetic activity. A sudden drop in activity was observed at the onset of the pre-pupal stage.

The peritrophic membrane protects the gut epidermis from ingested material and gut microflora. When a homogenate of this membrane was added to gut cytosol there was a decrease in *PAdoPS* synthesis. This decrease was not observed when the homogenate was heated at 100°C, thus suggesting that either proteolytic or nucleotide-degrading enzymes were responsible. Removal of the peritrophic membrane prior to homogenization of the gut was important not only in removing gut contents but also in preventing any inhibition of *PAdoPS* synthesis by the membrane homogenate. Our observations that sulphation of *p*-nitrophenol in the gut of *S. littoralis* was dependent upon the rate of *PAdoPS* synthesis suggests that caution should be exercised in interpretation of results of sulpho-

Fig. 3. *PAdoPS* synthesis (a) and protein concentration (b) in the gut cytosol fraction during development of the sixth instar larva of *S. littoralis*

For experimental details see the text. Pupation occurs at day 6.

Fig. 4. Sulphation of *p*-nitrophenol by the gut cytosol from *S. littoralis*

Experiments were performed using (a) ATP/ Mg^{2+} and K_2SO_4 as cofactors and (b) preformed *PAdoPS* as cofactor. For experimental details see the text.

transferase assays using ATP and inorganic sulphate as substrate. Yang *et al.* (1973) using such an assay showed that the phenolsulphotransferase activity in the closely related species, *S. eridania*, increases during the sixth instar, reaching a high level at day 5 of development. These results might be more accurately interpreted as reflecting the increase in the activity of the PAdoPS generating system.

In mammalian liver, it is recognized that the PAdoPS pool is very small (Mulder & Scholtens, 1978; Wong & Yeo, 1979), probably as a result of rapid sulphation of endogenous substrates (Spencer, 1960) and nucleotide degradation (Dodgson & Rose, 1975). Similarly, the presence of PAdoPS as a substrate for specific insect sulphotransferases would not only depend upon rates of synthesis and degradation but also upon competition with other sulphotoconjugating reactions.

Recently, Enderle & Koolman (1981) have demonstrated an ecdysteroid sulphotransferase in the fat body of the blowfly, *Calliphora vicina*, but it is not known whether the enzyme is specific for ecdysteroids. Future work in this area should result in a better understanding of the role of sulphotoconjugation in insect metabolism and development.

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