Enzymic Sulphation of *p*-Nitrophenol and Steroids by Larval Gut Tissues of the Southern Armyworm (*Prodenia eridania* Cramer)

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1. An enzyme system that catalyses the sulphation of *p*-nitrophenol, cholesterol, α -ecdysone, β -sitosterol, dehydroepiandrosterone, oestrone and four other steroids of plant and insect origin was obtained from the soluble fraction of southern-armyworm gut tissues. 2. The enzyme system required ATP and inorganic sulphate, and activity was slightly enhanced in the presence of GSH. 3. The properties of this enzyme system with respect to pH, temperature, substrate and protein concentrations and various cofactors and reagents were studied. At -23°C the enzyme preparation could be stored for 2 weeks without drastic loss of activity. At the end of storage for 1 month the loss of activity was approx. 21%. 4. The possible involvement of this enzyme system in insect endocrine control is discussed.

Although sulphate conjugates of phenol were first isolated and identified in mammals in the latter part of the last century, the mechanism of this reaction was not fully established until the discovery of the enzymic activation of sulphate and the isolation and identification of active sulphate, 3'-phosphoadenylyl sulphate (adenosine 3'-phosphate 5'-sulphatophosphate) some 15–20 years ago (Bernstein & McGilvery, 1952; DeMeio *et al.*, 1953; Segal, 1955; DeMeio *et al.*, 1955; Hilz & Lipmann, 1955; Robbins & Lipmann, 1956*a,b*, 1957). Studies on sulphate transfer from 3'-phosphoadenylyl sulphate to various acceptors including mammalian hormonal steroids and L-tyrosine methyl ester have been actively pursued in the last few years.

In mammals much of the recent emphasis on sulphoconjugation has been centred around the purification and characterization of the individual enzymes within the complex-forming family of sulphotransferases (Nose & Lipmann, 1958; Banerjee & Roy, 1966, 1968; Hidaka *et al.*, 1969; Mattock & Jones, 1970; McEvoy & Carroll, 1971). It now appears that in mammalian tissues there are at least four different sulphotransferases (Dodgson & Rose, 1970; Roy, 1970), which according to acceptor specificity are designated as phenol sulphotransferase (3'-phosphoadenylyl sulphate-phenol sulphotransferase, EC 2.8.2.1), 3β -hydroxy steroid sulphotransferase (3'-phosphoadenylyl sulphate- 3β -hydroxy steroid sulphotransferase, EC 2.8.2.2), oestrone sulphotransferase (3'-phosphoadenylyl sulphate-oestrone sulphotransferase, EC 2.8.2.4) and L-tyrosine methyl ester sulphotransferase. Of the enzymes that have been partially purified, phenol sulphotransferase is reported to have been purified more than 2000-fold

from male rat liver (McEvoy & Carroll, 1971). This purified enzyme did not catalyse the sulphation of dehydroepiandrosterone, butan-1-ol, L-tyrosine methyl ester, 1-naphthylamine or 5-hydroxytryptamine (serotonin).

Although sulphate conjugates have been detected *in vivo* in many insect species after treatment with various phenolic compounds (Smith, 1955, 1964, 1968; Binning *et al.*, 1967; Yang & Wilkinson, 1971), no information is presently available about the mechanism of sulphoconjugation in insects or the nature of the enzymes involved. Further, little if any consideration has been given to establishing the potential physiological role of this reaction.

In a previous study (Yang & Wilkinson, 1971) *p*-nitrophenyl sulphate was isolated from the faeces of sixth-instar larvae of the southern armyworm (*Prodenia eridania*) treated orally with *p*-nitro[$^{14}\text{C}_2$]-phenol and was established to be the major excreted metabolite. The present investigation describes the isolation and characterization from this species of an enzyme system that catalyses the sulphation of *p*-nitrophenol and various steroids of insect, mammalian and plant origin.

Materials and Methods

Experimental animals

The southern-armyworm culture was maintained from eggs kindly supplied by the Niagara Chemical Division, FMC Corporation, Middleport, N.Y., U.S.A., and the rearing conditions were those previously reported by Krieger & Wilkinson (1969). Early-sixth-instar larvae were used throughout this study.

Preparation of sulphotransferases

The guts of 50 southern-armyworm larvae were removed posteriorly after excision of the tip of the abdomen. After the removal of the attached Malpighian tubules, the guts were cut open longitudinally and the food contents were removed along with the peritrophic membrane. The gut tissues were then rinsed three times in 0.15M-KCl, blotted and homogenized in 4 vol. of 0.15M-KCl with a TenBroeck tissue grinder. The crude homogenate was centrifuged at 10000g for 15 min and the resulting supernatant further centrifuged at 100000g for 1 h in an International Equipment Co. B-60 ultracentrifuge. All operations were carried out at 0–4°C. The high-speed supernatant constituted the enzyme source. It contained approx. 6mg of protein/ml and was stored at –23°C.

Steroids and other chemicals

Campesterol (24 α -methyl-5-cholesten-3 β -ol), cholesteryl sulphate, 22,25-bisdeoxyecdysone (2 β ,3 β ,14 α -trihydroxy-5 β -cholest-7-en-6-one), dihydrobrassicasterol (24 β -methyl-5-cholesten-3 β -ol), α -ecdysone (2 β ,3 β ,14 α ,22R,25-pentahydroxy-5 β -cholest-7-en-6-one), β -sitosterol (24 β -ethyl-5-cholesten-3 β -ol) and stigmaterol (5,22-stigmastadien-3 β -ol) were kindly provided by Dr. W. E. Robbins, Insect Physiology Laboratory, Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md., U.S.A. ATP (disodium salt), cholesterol (5-cholesten-3 β -ol), dehydroepiandrosterone (3 β -hydroxyandrost-5-en-17-one), dehydroepiandrosterone 3-sulphate, oestrone [3-hydroxy-1,3,5,(10)-oestratrien-17-one], oestrone 3-sulphate, GSH, *p*-nitrophenol and *p*-nitrophenyl sulphate (potassium salt) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. All other chemicals and reagents were of analytical-reagent grade.

Preparation of buffers, cofactors and substrates

Sodium citrate buffers (0.5M) were prepared by adjusting 0.5M-sodium citrate to the required pH values with 0.5M-citric acid, and sodium phosphate buffers (0.5M) were prepared similarly by using 0.5M-Na₂HPO₄ and 0.5M-NaH₂PO₄. Tris-HCl buffers (0.5M) were prepared by adjusting known volumes of 1M-tris to the required pH values with conc. HCl (approx. 12M) and then diluting to twice of the original volume with water.

ATP and GSH were both prepared as 0.1M solutions with water and were adjusted to pH 6.6 with 1M-NaOH. *p*-Nitrophenol was prepared as an aqueous 5mM solution with the aid of 1 drop of ethanol/25ml. All the above solutions were kept in the freezer in 5ml fractions. The steroids were

prepared as 12.5mM solutions in 1,4-dioxan (spectral grade) and the solutions were kept in the freezer.

Assay of enzyme activity

Sulphotransferase activity was measured by one of two methods, depending on the substrate used. For the sulphation of *p*-nitrophenol, method A, a modification of the method described by Hilz & Lipmann (1955), was employed. The incubation mixture contained, in a total volume of 0.5ml, 50 μ mol of tris-HCl, pH 7.5, 5 μ mol each of ATP, K₂SO₄, MgCl₂ and GSH, 0.25 μ mol of *p*-nitrophenol and enzyme preparation (in 0.15M-KCl) containing approx. 0.3mg of protein. After 1h incubation at 38°C the reaction was terminated by the addition of 2ml of ethanol. The protein was removed by centrifugation and 0.5ml of the resulting supernatant was added to 5ml of 0.1M-NaOH. The extinction of the solution was measured at 400nm and the amount of *p*-nitrophenyl sulphate formed was determined from the decrease of the extinction relative to the non-enzymic controls.

For comparing the sulphation of steroids with that of *p*-nitrophenol, method B, a modification of the Methylene Blue method described by Banerjee & Roy (1967), was employed. The incubation mixture and other conditions were the same as those described above except that sodium phosphate buffer, pH 7.5, was used instead of tris-HCl and the enzyme was prepared in 0.05M-sodium phosphate buffer, pH 7.5. The steroids (0.125 μ mol) were added to the incubation mixture as solutions in 1,4-dioxan. The enzymic reaction was terminated by the addition of 2.5ml of ethanol, and, after centrifugal precipitation of the denatured protein, 2.5ml of the supernatant was evaporated to dryness in a boiling-water bath. The subsequent procedure was identical with that described by Banerjee & Roy (1967) except that the extinction of the chloroform extract of the Methylene Blue-sulphate complex was measured at 600nm. The sulphation of dehydroepiandrosterone, oestrone and *p*-nitrophenol was determined from the respective standard curves obtained with corresponding sulphate esters, and the sulphation of campesterol, cholesterol, 22,25-bisdeoxyecdysone, dihydrobrassicasterol, α -ecdysone, β -sitosterol and stigmaterol was estimated from the standard curve obtained with cholesteryl sulphate.

Spectrophotometric measurements were made with a Bausch and Lomb Spectronic 20 spectrophotometer, although the results were confirmed by using a Norelco Unicam SP.800 spectrophotometer equipped with a scale-expansion accessory. Unless otherwise stated, sulphotransferase activity is expressed in terms of nmol of sulphate ester formed/h per mg of protein. All experiments were repeated at least twice and the data represent mean values.

Protein determinations were effected by the modified Lowry method described by Chaykin (1966).

Results

Distribution of sulphotransferase activity in various tissues and in the subcellular fractions of the gut tissues

The sulphation of *p*-nitrophenol by various tissue preparations of southern-armyworm larvae was studied in the following manner. Crude tissue homogenates were prepared from fat-body, gut, haemolymph and Malpighian tubules of ten southern-armyworm larvae in ice-cold 0.15 M-KCl. The protein concentration of each preparation was adjusted to within the range 3–7 mg/ml, and 0.1 ml of each preparation was assayed for sulphotransferase activity (method A). The results indicate that whereas haemolymph showed no activity, gut, Malpighian tubules and fat-body formed 23.1, 13.0 and 2.7 nmol of *p*-nitrophenyl sulphate/h per mg of protein respectively.

Since the gut homogenate appeared to possess the highest sulphotransferase activity and since the gut constituted the most conveniently isolated enzyme source, a study was conducted to establish the subcellular localization of sulphotransferase activity in this organ. The results given in Table 1 indicate that the sulphotransferase activity was present exclusively in the soluble fraction of the cell.

Effects of cofactors and other reagents

The effects of various reagents and cofactors on the sulphation of *p*-nitrophenol by southern-armyworm larval gut enzymes were evaluated by using the assay conditions of method A. By comparison with the complete incubation system, in which 164.5 nmol of *p*-nitrophenyl sulphate/h per mg of protein was formed, the absence of GSH, MgCl₂, K₂SO₄ and ATP resulted in approx. 11, 87, 97 and 100% decreases in

activity respectively. The slight but consistent activity observed in the absence of added K₂SO₄ is possibly due to the presence of trace amounts of inorganic sulphate derived from either the tissues or the reagents employed.

Effects of substrate concentration and protein concentration

The effect of substrate concentration on sulphotransferase activity was studied at nine different concentrations of *p*-nitrophenol between 0.04 and 1 mM. Enzyme activity increased progressively with increasing concentration of *p*-nitrophenol and approached a maximal limiting value at approx. 0.3–0.4 mM. The concentration of *p*-nitrophenol employed as a routine in this investigation was 0.5 mM.

The effects of the amount of added enzyme preparation on the sulphation of *p*-nitrophenol was tested at eight different protein concentrations between 0.05 and 1.2 mg of protein/assay. Enzyme activity was a linear function of protein concentration up to approx. 0.7 mg/assay.

Effects of pH and temperature

Sodium citrate (pH 2.6–5.9), sodium phosphate (pH 6.0–8.0) and tris-HCl (pH 7.5–9.0) buffers were employed to study the effect of pH on the rate of sulphation of *p*-nitrophenol. Fig. 1 shows that maximum activity was obtained at pH 7.5 and that the enzyme system was considerably less active at pH values lower than 6 and higher than 8.5. The results also suggest that, of the three buffers employed, tris-HCl is the best medium for the sulphotransferases.

The effect of temperature on sulphotransferase activity was investigated up to 45°C. Under the reaction conditions employed the rate of sulphation

Table 1. *Sulphotransferase activity in subcellular fractions of southern-armyworm larval gut homogenates*

The appropriate subcellular fractions were prepared from a homogenate of the gut tissues of 50 southern-armyworm larvae by differential centrifugation. Centrifugal conditions employed were as follows: cell debris+mitochondria (pellet at 10000g for 15 min), mitochondrial supernatant (supernatant at 10000g for 15 min), microsomal fraction (pellet at 100000g for 60 min) and soluble fraction (supernatant at 100000g for 60 min). Before incubation the protein concentration of each fraction was adjusted to within the range 3–6 mg/ml; 0.1 ml of each fraction was assayed for the sulphation of *p*-nitrophenol under the assay conditions (method A) described in the text.

Fraction	<i>p</i> -Nitrophenyl sulphate formed (nmol/h per mg of protein)
Crude homogenate	18.6
Cell debris+mitochondria	0
Mitochondrial supernatant	104.4
Microsomal fraction	0
Soluble fraction	191.0

Fig. 1. Effect of pH on the sulphation of *p*-nitrophenol by southern-armyworm larva sulphotransferases

●, Sodium citrate buffer; ▲, sodium phosphate buffer; □, tris-HCl buffer. Specific activity is expressed as nmol of *p*-nitrophenyl sulphate formed/h per mg of protein and assay conditions (method A) are as described in the text.

Fig. 2. Time-course study on the sulphation of *p*-nitrophenol by southern-armyworm larva sulphotransferases

●, Reaction initiated by the addition of enzyme preparation; ○, reaction initiated by the addition of substrate. Specific activity is expressed as nmol of *p*-nitrophenyl sulphate formed/mg of protein and assay conditions (method A) are as described in the text.

of *p*-nitrophenol increased markedly between 24° and 38°C, but declined at higher temperatures.

Time-course study

Initial experiments on the time-course of the reaction with *p*-nitrophenol were carried out in incubations in which the reaction was initiated by addition of the enzyme preparation. Under these conditions a short lag period was consistently observed at the initial portion of the curve (Fig. 2). This phenomenon might be the result of the limited availability of 3'-phosphoadenylyl sulphate, which is presumably synthesized from ATP and inorganic sulphate by ATP sulphurylase (ATP-sulphate adenylyltransferase, EC 2.7.7.4) and adenylyl sulphate phosphokinase (ATP-adenylyl sulphate 3'-phosphotransferase, EC 2.7.1.25). This speculation is supported by the fact that a smooth curve was observed when, in later experiments, substrate instead of enzyme was employed to initiate the reaction (Fig. 2).

Effect of storage

Since the results of previous experiments indicated that the southern-armyworm larval gut sulphotransferases were rather stable, a study was conducted to compare the activity of a fresh preparation with that of preparations stored at -23°C for various times. Fig. 3 represents the mean results of three separate experiments and shows a 21% loss of activity when the enzyme preparation was stored for 32 days; about half of this loss occurred during the first week.

Fig. 3. Effect of storage on the sulphation of *p*-nitrophenol by southern-armyworm larva sulphotransferases

The enzyme preparation was a 100000g supernatant of a gut tissue homogenate of 50 southern-armyworm larvae in 0.15M-KCl. After preparation the enzyme was divided into 1 ml portions, which were stored at -23°C. At appropriate time-intervals these were assayed under conditions (method A) described in the text.

Reactivity of southern-armyworm larval gut sulphotransferases

The activity of southern-armyworm larval gut sulphotransferases towards various steroids of insect, mammalian and plant origin was evaluated and compared with that towards *p*-nitrophenol. Although, as shown in Table 2, the addition of 10 μl of 1,4-dioxan inhibited the rate of sulphation of *p*-nitrophenol by approx. 10%, this was found to be a more suitable solvent than propane-1,2-diol, *NN*-dimethyl-

Table 2. *Sulphotransferase activity towards various steroids relative to that towards p-nitrophenol*

p-Nitrophenol (0.25 μ mol) was added to the incubation mixture as 50 μ l of an aqueous 5 mM solution as described in the text. The steroids were prepared as 12.5 mM solutions in 1,4-dioxan, and a 10 μ l portion of the appropriate solution was employed for enzyme assay. The assay conditions (method B) are as described in the text. The values in parentheses indicate the activity relative to that (100%) towards *p*-nitrophenol.

Substrate	Sulphate ester formed (nmol/h per mg of protein)
<i>p</i> -Nitrophenol	158.8 (100%)
<i>p</i> -Nitrophenol+1,4-dioxan (10 μ l)	142.6 (89.8%)
Dehydroepiandrosterone	60.3 (38.0%)
Oestrone	34.5 (21.7%)
Cholesterol	4.3 (2.7%)
α -Ecdysone	6.6 (4.2%)
22,25-Bisdeoxyecdysone	11.3 (7.1%)
β -Sitosterol	10.9 (6.9%)
Stigmasterol	1.5 (0.9%)
Campesterol	4.3 (2.7%)
Dihydrobrassicasterol	5.7 (3.6%)

formamide, dimethyl sulphoxide or methanol on the basis of such factors as substrate solubility, enzyme inhibition and interference with the extraction of the Methylene Blue-sulphate complex (R. S. H. Yang, unpublished work). The results (Table 2) indicate that the southern-armyworm larva sulphotransferases were active in the sulphation of various steroids, including certain well-known mammalian and insect hormones and common plant steroids. The activity was especially high with dehydroepiandrosterone and oestrone, which are often used as substrates in studies involving mammalian steroid sulphotransferases.

Discussion

The results of this investigation indicate that a sulphotransferase system is present in the gut tissues of southern-armyworm larvae and is active in the sulphation of *p*-nitrophenol and various steroids of insect, mammalian and plant origin. With respect to the sulphation of *p*-nitrophenol, the crude insect enzyme system is more active than some of the mammalian preparations in various states of purification (Table 3), although the validity of such comparisons is always open to some question in view of differences in tissue, methods of enzyme preparation and assay. Nevertheless, comparative studies in this laboratory on the sulphation of *p*-nitrophenol by rat liver and southern-armyworm gut high-speed supernatants (100000g) have revealed that the insect system is about three times as active as that from rat liver (R. S. H. Yang, unpublished work). Preliminary studies have shown that an active insect sulphotransferase system is also present in gut and

Malpighian-tubule preparations of the Madagascar cockroach (*Gromphadorhina portentosa*) (R. S. H. Yang, unpublished work).

On the basis of the subcellular distribution of the enzyme system (Table 1) and its requirement for ATP and inorganic sulphate, the insect enzyme system appears to be similar to that studied in mammals. It seems reasonable to assume that the insect system, like that in mammals, utilizes 'active sulphate', namely 3'-phosphoadenylyl sulphate, as the sulphate donor in the sulphation of various acceptors. This has not yet been established, however, and if this is so it is not known whether the biosynthesis of 3'-phosphoadenylyl sulphate in insects is preceded by the initial synthesis of adenylyl sulphate from ATP and inorganic sulphate. Although GSH was found to enhance the sulphation of *p*-nitrophenol by southern-armyworm larval gut sulphotransferases, the mechanism involved in this activation remains to be investigated.

In addition to its activity on *p*-nitrophenol, the southern-armyworm gut preparation was active in the sulphation of a wide variety of steroids, including the mammalian hormones dehydroepiandrosterone and oestrone and the insect moulting hormones α -ecdysone and 22,25-bisdeoxyecdysone (Table 2). Although some of these steroid sulphates can be considered the result of biochemical transformations for the purpose of detoxication, others may have important physiological implications. Sulphate esters of cholesterol, campesterol and β -sitosterol have been isolated and identified from the meconium of tobacco-hornworm (*Manduca sexta*) pupae (Hutchins & Kaplanis, 1969), and these steroids are known to be precursors in the biosynthesis of α -ecdysone in this species (Robbins *et al.*, 1971; Thompson *et al.*,

Table 3. *Activity of phenol sulphotransferase in various animal species*

All data were converted into nmol of *p*-nitrophenyl sulphate formed/min per mg of protein. The values in parentheses represent the degrees of purification.

Enzyme source	Specific activity (nmol/min per mg of protein)	Reference
Rabbit liver*	1.4	Nose & Lipmann (1958)
Lamb liver*	0.9	Nose & Lipmann (1958)
Rat liver*	0.7	Nose & Lipmann (1958)
Guinea-pig liver (17×)	2.8	Banerjee & Roy (1966)
(68×)	10.0	Banerjee & Roy (1968)
Rat liver†	0.3	McEvoy & Carroll (1971)
Rat liver (2179×)	645.0	McEvoy & Carroll (1971)
Southern-armyworm gut†	2.6	Present work

* Partially purified, but degree of purification not known.

† High-speed (100000g) supernatant. The specific activity of the southern-armyworm larval gut preparation was converted from that given in Table 2.

1972). Further, there is strong evidence that houseflies (*Musca domestica*) convert 22,25-bisdeoxyecdysone into sulphate and glucoside conjugates, and treatment of diapausing tobacco-hornworm pupae with this same material results in the formation of similar conjugates of both α -ecdysone and 20-hydroxyecdysone (Thompson *et al.*, 1972). By analogy with the suggested role of sulphotoconjugation in the metabolism of mammalian steroid hormones (Dodgson & Rose, 1970; Roy, 1970), it appears that the existence of sulphotransferases in insects can no longer be adequately explained solely in terms of their importance in detoxication or excretion or both. At the present time little or no information is available about the existence of arylsulphatases in insects, although this type of enzyme appears to be widely distributed in the animal kingdom (Dodgson & Rose, 1970; Nicholls & Roy, 1971; Roy, 1971). If arylsulphatases are indeed present in insects, it is possible that sulphotransferases and arylsulphatases might work in concert to form a metabolic mechanism for the regulation of insect steroids in general and more specifically for the activation and deactivation of insect-moulting hormones. A periodic rise and fall in the titre of the moulting hormone at certain stages of insect development has been observed in at least two different species (Burdette, 1962; Shaaya & Karlson, 1965a,b, Kaplanis *et al.*, 1966), but whether this is due to differential rates of biosynthesis and degradation or to the metabolic activation and deactivation of a constant hormone titre remains to be elucidated.

The sulphotransferase activity of the southern-armyworm preparation towards the insect and plant steroids investigated is relatively low compared with that for dehydroepiandrosterone and oestrone (Table 2). This might be explained in part in terms of

inadequate solubility, as both the insect and plant steroids were observed to be relatively insoluble in the incubation system employed. On the other hand, since the enzyme preparation was relatively crude, it is possible that different sulphotransferases with different acceptor specificities might be present in insects.

Despite the fact that the class Insecta represents the largest group of organisms in the animal kingdom, relatively little is known about insect biochemistry. The present study is the first report on sulphotoconjugation in insects at the enzymic level. The results of this investigation raise questions on the comparative aspects of the biosynthesis of 3'-phosphoadenylyl sulphate and the possible physiological role of sulphotoconjugation in insects.

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