Mechanism of Inhibition of Microsomal Mixed-Function Oxidation by the Gut-Contents Inhibitor of the Southern Armyworm (*Prodenia eridania*)

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A potent inhibitor of microsomal mixed-function oxidation reactions in insects had previously been isolated and partially purified from the gut contents of *Prodenia eridania* and shown to be associated with proteinase activity. Incubation of rat liver microsomal fraction with low concentrations of this inhibitor led to solubilization of NADPH-cytochrome *c* reductase, which was paralleled by the inactivation of reduction of cytochrome *P*-450 by NADPH and by the inhibition of NADPH-linked benzo[3,4]pyrene hydroxylation and aminopyrine demethylation. There was little or no effect on cytochromes *b*₅ and *P*-450, nor was the capacity of the latter catalyst to combine with exogenous substrates decreased. Contrary to the findings with NADPH, preincubation of microsomal fraction with the inhibitor did not cause a significant decrease in the rate of cytochrome *P*-450 reduction by NADH, supporting the assumption that different catalysts are involved in the electron transfer from NADH and NADPH to cytochrome *P*-450. The findings indicate the importance of taking the possible presence of endogenous inhibitors into consideration when evaluating low or absent mixed-function oxidation activities found in insect systems *in vitro*.

Cytochrome *P*-450 is the terminal oxidase involved in the microsomal mixed-function oxidation of a great number of exogenous lipid-soluble compounds, including various drugs and insecticides (see Conney, 1967, for review). A primary step in the mixed-function oxidation process involves the binding of the substrate to the oxidized form of cytochrome *P*-450, which most often is associated with the formation of the so-called type I spectral change (characterized by a peak at about 385 nm and a trough at about 420 nm) in the difference spectrum of the microsomal fraction (Schenkman, 1970). The cytochrome *P*-450-substrate complex is subsequently reduced and the available evidence suggests that NADPH-cytochrome *c* reductase is involved in the electron transfer from NADPH to the oxidized complex (Ernster & Orrenius, 1965; Orrenius, Berg & Ernster, 1969). Moreover, the reduction of the cytochrome *P*-450-substrate complex has been proposed to be the rate-limiting step in the overall mixed-function oxidation process (Gigon, Gram & Gillette, 1969).

Previous studies have revealed that the microsomal electron-transport components involved in

nicotinamide nucleotide oxidation respond differently to treatment of the microsomal fraction with proteolytic agents (Omura, Siekevitz & Palade, 1967; Ito & Sato, 1969; Orrenius *et al.* 1969). Treatment of liver microsomal fraction with trypsin has, for example, been shown to solubilize NADPH-cytochrome *c* reductase and cytochrome *b*₅, leaving the major portion of NADH-cytochrome *b*₅ reductase and cytochrome *P*-450 in the membrane-bound state. Thus it has been proposed that the former two enzymes may have a superficial localization in the microsomal membrane (Ito & Sato, 1969).

Studies on mixed-function oxidases in insects have sometimes been impeded by the presence in insect homogenates of various endogenous inhibitors (Terriere, 1968; Chakraborty, Sissons & Smith, 1967). Thus a potent inhibitor of microsomal mixed-function oxidation has been shown to be present in the gut contents of the southern armyworm (*Prodenia eridania*) (Krieger & Wilkinson, 1969). Further studies on the nature of this inhibitor revealed that inhibition was associated with a soluble proteinase with a molecular weight

of approx. 26 000 (Krieger & Wilkinson, 1970). The present study was designed to investigate the mechanism by which the gut-contents inhibitor present in *Prodenia* inhibits microsomal mixed-function oxidation and the results are discussed with special regard to the possible organization of the NADH- and NADPH-linked electron-transport systems in the microsomal membrane.

MATERIALS AND METHODS

The gut-contents inhibitor was isolated from the gut contents of sixth-instar *Prodenia* larvae and partially purified to produce 'Fraction II' (Krieger & Wilkinson, 1970). The inhibitor was stored in the freeze-dried condition.

Male Sprague-Dawley rats (200 g) were starved overnight and killed by decapitation. Liver microsomal fractions were isolated as described by Ernster, Siekevitz & Palade (1962). The microsomal fractions were washed once with 0.15 M-tris-HCl buffer, pH 8.0, and re-centrifuged at 105 000 g for 40 min. The pellets were resuspended in 0.25 M-sucrose at a protein concentration of 10 mg/ml.

Submitochondrial particles from ox heart were isolated as described by Löw & Vallin (1963).

The routine procedure for preincubation of microsomal fractions with the inhibitor involved incubation of liver microsomal fraction (4 mg of microsomal protein/ml), 0.1 M-tris-HCl buffer, pH 7.5, or 0.1 M-potassium phosphate buffer, pH 7.5, and various concentrations of gut-contents inhibitor, in a final volume of 1 ml, for 10 min at 30°C. At the end of the preincubation period the enzyme assays were immediately started. Incubations of microsomal fractions in the absence of inhibitor served as controls.

Protein was measured as described by Lowry, Rosebrough, Farr & Randall (1951).

NADPH-cytochrome *c* reductase activity was measured as described by Dallner (1963).

The concentration of cytochrome *b₅*, NADH- and NADPH-cytochrome *P*-450 reductase activities, and hexobarbital-induced type I spectral change, were measured as previously described (Orrenius *et al.* 1969) except that an Aminco-Chance split-beam/dual-wave-length recording spectrophotometer was employed for the recordings and the temperature was 30°C.

Oxidative demethylation of aminopyrine was measured as reported by Orrenius *et al.* (1969). The rate of hydroxylation of benzo[3,4]pyrene was assayed as described by Gnospelius, Thor & Orrenius (1969), except that NADP⁺ and the NADPH-generating system were omitted and NADPH was added, to a final concentration of 1.5 mM.

All chemicals employed were standard commercial products.

RESULTS

As shown in Fig. 1, incubation of rat liver microsomal fraction for 10 min at 30°C in the presence of various concentrations of gut-contents inhibitor resulted in decreased rates of hydroxylation of benzo[3,4]pyrene and oxidative demethylation of aminopyrine. The benzo[3,4]pyrene hydroxylation reaction appeared to be somewhat more sensitive to the action of the inhibitor than was aminopyrine demethylation.

The inhibition of the overall hydroxylation process by gut-contents inhibitor could be correlated with a solubilization of NADPH-cytochrome

Fig. 1. (a) Inactivation of benzo[3,4]pyrene hydroxylation by preincubation of liver microsomal fraction with gut-contents inhibitor at various concentrations. (b) Inactivation of oxidative demethylation of aminopyrine by preincubation of liver microsomal fraction with the inhibitor at various concentrations. Experimental details are given in the text.

Fig. 2. Release of protein and NADPH-cytochrome *c* reductase activity by preincubation of liver microsomal fraction with gut-contents inhibitor. Microsomal fractions were preincubated in the absence and presence of various concentrations of gut-contents inhibitor, as described in the Materials and Methods section, and re-centrifuged at 105000*g* for 40 min. Protein and NADPH-cytochrome *c* reductase activity were assayed in the pellet and supernatant. ●, Protein in the supernatant; ■, NADPH-cytochrome *c* reductase activity in the supernatant; ○, protein in the pellet; □, NADPH-cytochrome *c* reductase activity in the pellet.

Fig. 3. Inactivation of NADPH-cytochrome *P*-450 reductase activity by preincubation of liver microsomal fraction with gut-contents inhibitor. NADPH-cytochrome *P*-450 reduction was measured in the presence of 0.83 mM-hexobarbital. The following concentrations of inhibitor (µg/4 mg of protein per ml) were used during preincubation of the microsomal fraction: 0, 10, 50, 100, as marked on the appropriate curve. The arrows indicate the rapid addition of 5 µM-NADPH to start the reaction. The amount of microsomal protein added to the cuvette was 2 mg. — indicates the subsequent chemical reduction of cytochrome *P*-450 by a few milligrams of Na₂S₂O₄.

c reductase from the microsomal fraction, as revealed by the results presented in Fig. 2. It is noteworthy that extensive solubilization of the flavoenzyme occurred already at low concentrations of the inhibitor, which caused the release of only a very small amount of the total microsomal protein. Parallel to the solubilization of NADPH-cytochrome *c* reductase by the action of gut-contents inhibitor, there was an inactivation of NADPH-cytochrome *P*-450 reductase, measured in the presence of hexobarbital (Fig. 3). On the other hand, at the concentrations of the inhibitor employed, cytochrome *P*-450 could still be chemically reduced and there was no evidence for its conversion into the enzymically inactive cytochrome *P*-420 form.

The lack of significant effect of pretreatment of the microsomal fraction with gut-contents inhibitor on the magnitude of the hexobarbital-produced type I spectral change (Fig. 4) provides further evidence that cytochrome *P*-450 as such was not affected by the inhibitor and that the inhibitory action on mixed-function oxidation was due to the effect on the reductase portion of the enzyme system. This assumption is further supported by the relatively small effect of the inhibitor (added at concentrations found to cause maximal inhibition of NADPH-cytochrome *P*-450 reductase and NADPH-linked substrate hydroxylation) on cytochrome *P*-450 reduction by NADH (Fig. 5), which is most probably mediated by NADH-cytochrome *b*₅ reductase and possibly also involves cytochrome *b*₅. Only negligible amounts of cytochrome *b*₅ were released from the microsomal fraction owing to the effect of gut-contents inhibitor added at a concentration that caused maximal inactivation of NADPH-linked substrate

Fig. 4. Effect on hexobarbital-induced type I spectral change by preincubation of liver microsomal fraction with gut-contents inhibitor. The following concentrations of inhibitor (µg/4 mg of protein per ml) were used during preincubation of the microsomal fraction: 0, —; 50 µg, ----; 200 µg, - - - - . Each cuvette contained 4 mg of microsomal protein.

Fig. 5. NADH-cytochrome *P*-450 reductase activity in liver microsomal fraction preincubated with gut-contents inhibitor. The reaction was measured in the presence of 0.83 mM-hexobarbital. The following concentrations of inhibitor ($\mu\text{g}/4\text{ mg}$ of protein per ml) were used during preincubation of the microsomal fraction: 0, 10, 50, 100, as marked on the appropriate curve. The arrows indicate the rapid addition of $5\mu\text{M}$ -NADH to start the reaction. The amount of microsomal protein added to the cuvette was 4 mg. Subsequent addition of a few milligrams of $\text{Na}_2\text{S}_2\text{O}_4$ chemically reduced cytochrome *P*-450 to the same extent in all the four samples.

hydroxylation. However, incubation of the microsomal fraction at 30°C in the absence of added inhibitor caused a progressive release of part of the cytochrome *b*₅ content present in the microsomal fraction.

DISCUSSION

Several studies have revealed the presence of inhibitors of microsomal mixed-function oxidases in insect homogenates (Terriere, 1968) and in caterpillar preparations in particular (Krieger & Wilkinson, 1969; Kahn, 1969; Kuhr, 1970). Thus a potent inhibitor has been isolated and partially purified from the gut contents of *P. eridania* and shown to be a soluble proteinase (Krieger & Wilkinson, 1970). The present study was undertaken to investigate the mechanism by which gut-contents inhibitor inhibits mixed-function oxidation reactions in microsomal fractions. The results indicate that inhibition is due to solubilization of NADPH-cytochrome *c* reductase associated with the parallel inactivation of NADPH-linked cytochrome *P*-450 reduction. On the other hand, cytochrome *P*-450 as such, and NADH-linked electron transport, were virtually unaffected by pretreatment of the microsomal fraction with the inhibitor at concentrations that caused strong inhibition of the NADPH-linked functions. The fact that only a minor fraction of the microsomal protein (<5%) was released together with 90–100% of NADPH-cytochrome *c* reductase activity provides further support for the assumption that this enzyme has a superficial localization in the microsomal membrane and is readily attacked by proteolytic enzymes.

The effects of gut-contents inhibitor on the

microsomal electron-transport components are thus very similar to those previously reported for trypsin (Orrenius *et al.* 1969). There is no evidence for the assumption that there is a major difference in action between the two proteolytic agents, although the effects of preincubation of the microsomal fraction at low concentrations of the gut-contents inhibitor seem to be more restricted to the solubilization of NADPH-cytochrome *c* reductase activity, whereas trypsin (even at low concentrations) has been found also to cause a partial solubilization of cytochrome *b*₅ and a decreased capacity of cytochrome *P*-450 for substrate binding (Orrenius *et al.* 1969).

In conclusion, the present study has revealed that the gut-contents inhibitor present in *Prodenia* interferes with mixed-function oxidation by causing a release of NADPH-cytochrome *c* reductase from the microsomal membrane. Since gut-contents inhibitor seems to be very potent in this respect, it is obvious that, when studying mixed-function oxidation reactions in insects, one should undertake every possible effort to avoid contamination of the microsomal fraction with this (and other) inhibitors during the homogenization and cell-fractionation procedures.

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REFERENCES

- Chakraborty, J., Sissons, C. H. & Smith, J. N. (1967). *Biochem. J.* **102**, 492.
- Conney, A. H. (1967). *Pharmac. Rev.* **19**, 317.
- Dallner, G. (1963). *Acta path. microbiol. scand.* (Suppl.), p. 166.
- Ernster, L. & Orrenius, S. (1965). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **24**, 1190.
- Ernster, L., Siekevitz, P. & Palade, G. E. (1962). *J. Cell Biol.* **15**, 541.
- Gigon, P. L., Gram, T. E. & Gillette, J. R. (1969). *Molec. Pharmac.* **5**, 109.
- Gnosspeilus, Y., Thor, H. & Orrenius, S. (1969). *Chem.-biol. Interactions*, **1**, 125.
- Ito, A. & Sato, R. (1969). *J. Cell Biol.* **40**, 179.
- Kahn, M. A. Q. (1969). *J. econ. Ent.* **62**, 723.
- Krieger, R. I. & Wilkinson, C. F. (1969). *Biochem. Pharmac.* **18**, 1403.
- Krieger, R. I. & Wilkinson, C. F. (1970). *Biochem. J.* **116**, 781.
- Kuhr, R. J. (1970). *J. agric. Fd Chem.* **18**, 1023.
- Löw, H. & Vallin, I. (1963). *Biochim. biophys. Acta*, **69**, 361.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Omura, T., Siekevitz, P. & Palade, G. E. (1967). *J. biol. Chem.* **242**, 2389.
- Orrenius, S., Berg, A. & Ernster, L. (1969). *Eur. J. Biochem.* **11**, 193.
- Schenkman, J. B. (1970). *Biochemistry, Easton*, **9**, 2081.
- Terriere, L. C. (1968). *A. Rev. Ent.* **13**, 75.