An Endogenous Inhibitor of Microsomal Mixed-Function Oxidases in Homogenates of the Southern Armyworm (*Prodenia eridania*)

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1. A potent inhibitor of microsomal mixed-function oxidation has been isolated from the gut contents of larvae of the southern armyworm (*Prodenia eridania*).
2. Progressive inhibition is associated with a soluble proteinase with a molecular weight of approx. 26000.
3. Although the proteolytic activity of the inhibitor can be reduced by phenylmethanesulphonyl fluoride and other reagents, these materials are only partially effective in protecting the microsomal enzymes.
4. Inhibitory activity is decreased in the presence of bovine serum albumin.

It is now generally accepted that insects metabolize insecticides and other foreign compounds by means of microsomal enzymes, which appear to be biochemically similar to those found in mammalian liver (Smith, 1967; Terriere, 1968). In investigations of the microsomal enzymes of insect species, however, a major problem arises from the relatively small size of the organism and the consequently small amounts of tissue available. To obviate this problem it has become common practice to employ homogenates or centrifugal fractions obtained from whole insects. Enzyme activity in these 'whole preparations' is usually low and short-lived compared with the activity in similar fractions of mammalian liver, and it is highly questionable whether such preparations provide a realistic measure of the metabolic activity of the insect under investigation.

The microsomal enzymes responsible for drug and insecticide metabolism are not equally distributed throughout the insect, but are localized in the tissues of specific organs. Thus maximal epoxidase activity in the larvae of the southern armyworm (*Prodenia eridania*) is found in the tissues of the mid-gut (Krieger & Wilkinson, 1969). In contrast, the oxidation of *p*-nitrotoluene (Hook, Jordan & Smith, 1968) and desulphuration of parathion (Nakatsugawa & Dahm, 1962, 1967) take place primarily in the fat-body of the locust and cockroach respectively, although in the latter case activity is also found in other tissues. In microsomal fractions prepared from whole homogenates, therefore, the presence of large amounts of material derived from inactive tissues effectively dilutes that from the active tissue and consequently decreases the specific activity observed.

More important than the tissue dilution factor,

however, are the potential effects on enzyme activity of inhibitory materials released during the homogenization process (Terriere, 1968; Chakraborty, Sissons & Smith, 1967). Such natural inhibitors have been noted by several workers studying the microsomal enzymes of insects and clearly have considerable significance in the results of such investigations. Homogenates of whole houseflies are found to contain heat-stable diffusible materials that are inhibitory towards the activity *in vitro* of microsomal preparations from both insects and mammals (Matthews & Hodgson, 1964). Because the inhibitory substances are more concentrated in the head and thorax, housefly abdomens have been found to provide a more active and stable source of microsomal enzymes (Tsukamoto & Casida, 1967b; Chakraborty *et al.* 1967). In homogenates of locust and cockroach fat-body, materials sedimenting in heavier fractions have been found to be inhibitory towards both *p*-nitrotoluene oxidation (Hook *et al.* 1968; Chakraborty *et al.* 1967) and parathion desulphuration (Nakatsugawa & Dahm, 1965). Work in this laboratory has shown that, although the microsomal fraction prepared from homogenates of whole *Prodenia* larvae exhibit no mixed-function oxidase activity, extremely active microsomal preparations can be obtained from larval gut tissue (Krieger & Wilkinson, 1969). Subsequent work has indicated the presence in *Prodenia* gut contents of a potent endogenous inhibitor of microsomal mixed-function oxidation. It is therefore clear that several inhibitory materials are present in whole homogenates of different insect species and that these may seriously impede basic investigations on the nature of the microsomal enzymes in insects. This paper describes the isolation and characterization of an

inhibitory factor present in *Prodenia* gut contents. The epoxidation of aldrin, the hydroxylation of dihydroisodrin (R. I. Krieger, unpublished work) and the demethylation of *p*-chloro-*N*-methylaniline have been employed as indices of microsomal enzyme activity. A preliminary report of this work was presented at the National Meeting of the Entomological Society of America, Dallas, Texas, 2-5 December 1968.

MATERIALS

Insects. Eggs of the southern armyworm (*Prodenia eridania* Cramer) were initially obtained from the Niagara Chemical Division, FMC Corporation, Middleport, N.Y., U.S.A., and larvae were reared continuously under greenhouse conditions as reported by Krieger & Wilkinson (1969).

Chemicals. Analytical grade samples of aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo-exo-5,8-dimethanonaphthalene) and its 6,7-epoxide dieldrin were kindly supplied by the Shell Development Co., Modesto, Calif., U.S.A., as was a sample of monohydroxy-dihydroisodrin. Dihydroisodrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-endo-5,8-dimethanonaphthalene) was prepared by the catalytic reduction of isodrin over Raney nickel (Lidov, 1955). Paraaxon (*OO*-diethyl *O*-4-nitrophenyl phosphate) was synthesized by Mr James Nolan, of the Division of Biological Sciences, Cornell University. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP⁺, NADPH, *p*-chloro-*N*-methylaniline, phenylmethanesulphonyl fluoride, vitamin-free casein, trypsin, soya-bean trypsin inhibitor, serum albumin, ovalbumin and cytochrome *c* were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Di-isopropyl phosphorofluoridate was purchased from Aldrich Chemical Co., Cedar Knolls, N.J., U.S.A. All other chemicals and solvents employed were of analytical-reagent grade.

METHODS

Source and preparation of microsomes. The guts of sixth-instar *Prodenia* larvae were removed and the dark-green gut contents cleared by means of a rolling motion of the finger. The gut tissue was thoroughly rinsed in ice-cold 1.15% (w/v) KCl and homogenized in cold 1.15% KCl with a hand-operated TenBroeck tissue grinder. The concentration of the crude homogenate was five guts/ml. Gut tissue microsomal fractions were obtained by centrifugation for 1 h at 105 000g of the supernatant from an earlier 15 min spin at 12 000g. All operations were carried out at 0-4°C and centrifugation was accomplished with an International Equipment Co. (IEC) B-60 ultracentrifuge with an A-211 angle-head rotor. The microsomal pellet was resuspended in an appropriate volume of 1.15% KCl such that the final microsomal protein concentration was approx. 2-4 mg/ml. Protein concentration was determined by a modified biuret method (Fincham, 1954), with bovine serum albumin as standard.

Preparation and partial purification of the gut-contents inhibitor. The gut contents of sixth-instar larvae were

dispersed in water. The resulting mixture was centrifuged at 105 000g for 2 h in an A-211 angle-head rotor in an IEC B-60 ultracentrifuge. The particulate material was discarded and the soluble fraction freeze-dried to yield a dark-green residue (fraction I), which could be stored for extended periods at 4-8°C without loss of inhibitory activity.

Solutions containing 100 mg of fraction I/ml were applied to a column (50 cm × 2 cm) of Sephadex G-50 gel and were eluted at 3-4°C with water at a flow rate of 30 ml/h. The inhibitory fraction eluted with the void volume and on freeze-drying gave a yellow material (fraction II), which could be stored at 4°C for a limited time and was used in most of the characterization studies. For molecular-weight estimations a column (30 cm × 2.5 cm) of Sephadex G-75 gel was employed. The column was equilibrated and eluted at 3-4°C with mM-KCl-7 mM-tris-HCl buffer, pH 7.8 (at room temperature), and samples (1 ml) containing approx. 5 mg of inhibitor fraction II were chromatographed at a flow rate of 25 ml/h. Consecutive fractions (2 ml) of the column eluate were collected and assayed for protein at 280 nm in a Unicam SP.800 spectrophotometer equipped with a scale-expansion accessory. Each fraction was assayed for inhibitory activity towards the gut microsomal epoxidase. Molecular-weight standards of Blue Dextran, serum albumin, ovalbumin, soya-bean trypsin inhibitor and cytochrome *c* were chromatographed on the same column under identical conditions and column fractions were monitored spectrophotometrically at 280 nm.

Assay of microsomal enzyme activity. Incubations were carried out aerobically in 25 ml flasks shaken in a water bath at 30°C. A standard 5 ml incubation mixture contained: tris-HCl buffer, pH 7.8 (50 mM); glucose 6-phosphate (2.4 mM); glucose 6-phosphate dehydrogenase (1.6 units); NADP⁺ (51 μM); KCl (27 mM); aldrin (100 μg) or dihydroisodrin (100 μg) in ethanol (25 μl). After 10 min reactions were stopped by addition of 4 ml of acetone, and after extraction with light petroleum (b.p. 40-70°C) dieldrin or monohydroxydihydroisodrin was determined by g.l.c. by methods reported by Krieger & Wilkinson (1969). The 2 ml *N*-demethylase assay mixtures (Kupfer & Bruggeman, 1966) contained identical concentrations of incubation components and *p*-chloro-*N*-methylaniline (500 μg). The 20 min incubations at 30°C were terminated by addition of 2 ml of 0.75 M-H₂SO₄ containing 58 mg of *p*-dimethylaminobenzaldehyde. The mixture was centrifuged for 15 min at 15 000g and the *E*₄₄₅ was read against appropriate zero-time and reagent blanks. The reactions were normally initiated by addition of crude tissue homogenates or microsomal fractions (1-2 mg of protein).

Assay of proteolytic enzyme activity. Proteolytic activity of the gut-contents inhibitor was measured spectrophotometrically by the caseinolysis method of Kunitz (1947). The 3 ml incubation mixtures containing 22 mM-sodium-potassium phosphate buffer, pH 7.8, vitamin-free casein (0.5%, w/v) and appropriate amounts of the inhibitor were shaken in a water bath at 30°C for 20 min. The reaction was initiated with substrate and terminated by addition of 2 ml of trichloroacetic acid (5%, w/v). After centrifugation at 12 000g for 12 min the *E*₂₈₀ of the supernatant was measured against appropriate zero-time and reagent blanks.

In experiments to determine the effect on caseinolysis of several chemicals and selected anions and cations the candidate materials were preincubated for various times with about 1mg of fraction II before addition of the substrate.

RESULTS

Preliminary investigations established the complete absence of epoxidase activity from homogenates or microsomal fractions of whole *Prodenia* larvae (Krieger & Wilkinson, 1969). It was, however, established that extremely active microsomal enzyme preparations could be obtained from tissues of the larval gut. These results strongly suggested that some inhibitory material was being released during homogenization of the whole larvae, and recombination of homogenates of different fractions of the larvae with the active gut preparation clearly demonstrated that potent inhibitory activity was associated with the larval gut contents. The absence of inhibitory activity from homogenates of the red kidney bean (*Phaseolus vulgaris*) on which the larvae were reared indicated that inhibition was truly a property of the larval gut contents and was not an artifact associated with some component of the ingested food material.

Properties of fractions of the gut-contents inhibitor. The soluble nature of the inhibitor is shown by the fact that after centrifugation of an aqueous suspension of the crude gut contents for 2h at 105 000g inhibitory activity is retained in the supernatant. Only a trace of inhibitory activity is associated with the 105 000g sediment, which consists largely of partially digested food particles.

Freeze-drying of aqueous solutions of the inhibitor had no effect on inhibitory activity and this procedure allowed the accumulation of large amounts of fraction I. No loss of activity of freeze-dried fraction I occurred on storage at 4–8°C for periods of up to 1 year. Although freeze-dried fraction II was considerably less stable, it could be stored at 4–8°C for at least 1 week with little loss of inhibitory activity. The use of freeze-dried fractions of the inhibitor was also extremely useful in providing a measure of inhibitory activity based on dry weight of the solid material.

Aqueous solutions of fraction I were remarkably heat-stable, and no loss in inhibitory activity was observed after solutions were kept at 100°C for up to 15min. Activity was, however, destroyed when solutions of fraction I were evaporated to dryness at 100°C or when the solid material was burnt at 600°C. Aqueous solutions of fraction II were, in contrast, heat-labile and all inhibitory activity was lost in less than 1min when heated at 100°C. At 30°C, however, the inhibitor was stable for several hours and even after 24h at this temperature

Table 1. *Purification of gut-contents inhibitor*

Inhibitory activity was measured toward aldrin epoxidation by using the standard incubation medium (5ml) with about 1mg of gut microsomal protein. Addition of the microsomal suspension initiated the 10min reaction. I_{50} values were determined by plotting percentage inhibition as a function of log (dry wt. of inhibitor added); the weight of fraction III was estimated from the E_{280} .

Inhibitor	I_{50} (mg/5ml of incubation mixture)
Crude mixture	2.18
Fraction I	0.97
Fraction II (Sephadex G-50)	0.48
Fraction III (Sephadex G-100)	0.03

solutions still retained 70% of their original inhibitory activity.

The inhibitory material was non-diffusible and no loss of activity was observed after dialysis against water at 4°C for 36h.

Partial purification of the gut-contents inhibitor. The inhibitory potency of various fractions of the inhibitor towards microsomal epoxidation is shown in Table 1 in which I_{50} values (50% inhibition) are based on the dry weight of inhibitor/5ml of standard incubation mixture. Removal of the particulate material of the crude gut contents resulted in a twofold purification (fraction I), and passage through Sephadex G-50 gel resulted in a further doubling of inhibitory activity on a dry-weight basis (fraction II). Maximum purification was achieved by passage of the inhibitor through Sephadex G-100 gel (fraction III). The I_{50} value of fraction III (0.03mg/5ml of incubation mixture) represents an approximately 70-fold purification, although in this case the weight of the inhibitor was estimated from its E_{280} . Solutions of fraction III were colourless, in contrast with the yellow and dark-green colours associated with fractions II and I respectively.

Marked inhibitory activity was also exhibited towards the epoxidase enzymes associated with mouse liver microsomal fractions and crude homogenates of house-cricket (*Acheta domesticus*) Malpighian tubules (G. M. Benke & C. F. Wilkinson, unpublished work). In addition to its effect on epoxidation, the material was inhibitory towards other types of mixed-function oxidation in microsomal fractions from *Prodenia* gut tissues and mammalian liver, including the *N*-demethylation of *N*-methyl-*p*-chloroaniline and the hydroxylation of dihydroisodrin.

Effect of preincubation of components of the reaction mixture with gut-contents inhibitor. Preliminary experiments indicated that the inhibition of aldrin

Table 2. *Preincubation of various components of the reaction mixture with gut-contents inhibitor*

During the 10 min preincubation period the flasks containing 2.5 ml of tris-HCl buffer and the indicated components were shaken in a water bath at 30°C. After 10 min the incubation medium was completed and the reaction initiated by substrate addition. The complete incubation medium contained (final concns. in 5 ml): tris-HCl, pH 7.8 (50 μ M); glucose 6-phosphate (2.4 mM); KCl (27 mM); NADP⁺ (51 μ M); glucose 6-phosphate dehydrogenase (1.6 units); microsomal protein (1.5 mg); aldrin (100 μ g) added in ethanol (25 μ l). Aqueous solutions of inhibitor containing about 0.2 mg dry wt. of fraction II were used.

Preincubation medium	Relative epoxidase activity
Complete, not preincubated	1.00
Complete (no inhibitor)	1.00
Complete + inhibitor, not preincubated	0.70
Complete + inhibitor - microsomes*	0.70
Complete + inhibitor	0.15
Microsomal suspension + inhibitor	0.15
Glucose 6-phosphate + inhibitor	0.70
NADP ⁺ + inhibitor	0.70
Aldrin + inhibitor*	0.70

*Reaction was initiated by addition of microsomal suspension.

epoxidation was progressive. This factor was investigated in a number of preincubation experiments, the results of which are shown in Table 2.

The components of the reaction mixture were preincubated with 0.5 mg of fraction II for 10 min at 30°C. The reaction mixture was then completed by addition of aldrin, microsomal suspension or other components omitted during the preincubation period, and dieldrin production was determined over a 10 min incubation period. The results in Table 2 show that maximum inhibition (85%) was obtained after preincubation of the inhibitor with the complete incubation mixture or with the microsomal suspension. This amounts to about a three-fold increase over the 30% inhibition obtained with no preincubation and demonstrates the progressive nature of the inhibition. Preincubation of the inhibitor with components of the NADPH-generating system in the absence of microsomes indicated no inhibition of this system and suggests that inhibition results directly from some interaction with the microsomal suspension. That the inhibitor was not interfering with the NADPH-generating system was further substantiated by the fact that the degree of inhibition observed was not affected by doubling the amounts of NADP⁺, glucose 6-phosphate and glucose 6-phosphate dehydrogenase in the reaction mixture. Nicotinamide (Schenkman, Ball & Estabrook, 1967) and pyrophosphate (Gil-

Fig. 1. Effect of bovine serum albumin (BSA) on epoxidation by: ○, gut microsomal preparation; ●, the same with gut-contents inhibitor fraction II present. The reaction was initiated by adding microsomal suspension and epoxidation activity was determined as described in the Methods section.

lette, Grieb & Sasame, 1963), which are often added to microsomal incubations to prevent enzymic degradation of NADP⁺ and NADPH, had no effect on the inhibition at concentrations of 1 mM.

Effect of bovine serum albumin. In view of the reported ability of bovine serum albumin to stimulate the enzymic activity of housefly microsomes (Tsukamoto & Casida, 1967a; Ray, 1967) it was decided to investigate the effect of this material on the inhibition of microsomal epoxidation by the inhibitor. Fig. 1 shows that bovine serum albumin at concentrations up to 80 μ g/5 ml of incubation mixture produced up to a sevenfold enhancement of microsomal epoxidase activity in the presence of inhibitor. A 1.5-fold increase in dieldrin formation was also observed to result from the addition of bovine serum albumin to controls in the absence of the inhibitor.

Kinetics of inhibition. A double-reciprocal plot (Lineweaver & Burk, 1934) clearly indicates that the apparent K_m (20 μ M) for aldrin epoxidation was unchanged when fraction II inhibited the reaction by 45% (Fig. 2). This value should be interpreted with some caution as a result of the aqueous insolubility of the substrate and the observed dependence of K_m on the concentration of microsomal protein employed (Lewis, Wilkinson & Ray, 1967; Krieger & Wilkinson, 1969).

Proteolytic nature of the gut-contents inhibitor. The physical characteristics of the inhibitor, together

Fig. 2. Lineweaver-Burk plot for dieldrin formation in the presence (●) and absence (○) of gut-contents inhibitor fraction II. Incubation mixture and conditions were as described in the Methods section. [S], Aldrin added ($\mu\text{mol}/5\text{ml}$ of incubation mixture); v , dieldrin produced ($\mu\text{mol}/\text{min}$).

with the progressive nature of its inhibition of microsomal epoxidation, suggested that the inhibitor might itself possess enzymic properties enabling it to degrade some essential component of the microsomal system. That bovine serum albumin exhibited a marked protective effect on aldrin epoxidase in the presence of the inhibitor further indicated that inhibition might be associated with proteolytic activity.

Preliminary experiments with the insoluble-hide-powder proteolysis assay method of Hess, Ciaccio & Nelson (1957) established that the inhibitor did indeed possess significant proteolytic activity; this was supported by subsequent characterization studies in which the hydrolysis of casein was employed as a more suitable measure of proteolytic activity.

Throughout each of the purification procedures employed in this investigation inhibitory activity of the inhibitor was found to be associated with proteolytic enzyme activity. This is illustrated in Fig. 3, which clearly shows the co-chromatographic relationship between caseinolysis and inhibitory activity in fractions of the inhibitor (fraction II) eluted from a Sephadex G-100 gel column. A similar co-chromatographic relationship between these two types of activity was also obtained on Sephadex G-75 gel eluted under similar conditions.

Estimation of the molecular weight of gut-contents inhibitor. Molecular-weight estimations (Andrews, 1964) were carried out with both Sephadex G-75 and G-100gels. In each case the elution volume of

Fig. 3. Sephadex G-100 gel filtration of gut-contents inhibitor fraction II. Samples (1ml) were applied to a column ($60\text{cm} \times 2.5\text{cm}$) of Sephadex G-100 equilibrated and eluted with mm-tris-HCl buffer, $\text{pH } 7.8$, containing 7mm-KCl at $2-4^\circ\text{C}$. Fractions (3ml) were collected with a Gilson fraction collector with a 280nm -absorption monitor; the flow rate was 30ml/h . Caseinolytic activity and epoxidase inhibitory activity were subsequently measured as described in the Methods section. ----, Protein; ----, inhibitory activity; ----, caseinolytic activity; —, Blue Dextran.

Fig. 4. Sephadex G-75 gel filtration of gut-contents inhibitor fraction II. Samples (1ml) were applied to a column ($30\text{cm} \times 2.5\text{cm}$) of Sephadex G-75 gel equilibrated and eluted with mm-tris-HCl buffer, $\text{pH } 7.8$, containing 7mm-KCl at $2-4^\circ\text{C}$. Fractions (2ml) were collected; the flow rate was 25ml/h . Blue Dextran, cytochrome c , ovalbumin, soya-bean trypsin inhibitor and bovine serum albumin standards were chromatographed in several combinations with the same operating parameters.

the inhibitory activity from inhibitor fraction II was determined and compared directly with a calibration curve obtained with several standard proteins of known molecular weight chromatographed under identical conditions. The standard materials employed were bovine serum albumin, ovalbumin, soya-bean trypsin inhibitor, cytochrome c and Blue Dextran. As indicated in Fig. 4 the inhibitory activity is associated with a fraction with a molecular weight of approx. 26000.

Fig. 5. Effect of incubation temperature on caseinolysis by gut-contents inhibitor fraction II. Standard incubation mixtures (3 ml) were shaken in a water bath at the indicated temperature for 10 min; caseinolysis was determined as described in the Methods section.

Proteolytic properties of the gut-contents inhibitor. Preliminary experiments indicated that caseinolytic activity increased linearly with the dry weight of inhibitor (fraction II) added over the range 0.5–4.0 mg/3 ml of incubation mixture. It was also established that the reaction was linear with time to about 40 min. Assays were therefore effected over a 20 min incubation period; 1 mg of fraction II gave rise to an E_{280} of 0.40.

Optimum pH. A steady increase in caseinolytic activity was associated with increasing pH, but no clearly defined optimum was observed. All incubations were measured against zero-time and reagent blanks and appropriate corrections were made for small amounts of non-enzymic alkaline hydrolysis. At about pH 7 the caseinolytic activity of the inhibitor was essentially the same whether measured in phosphate or borate-phosphate-acetate buffer.

Under storage conditions at 0°C solutions of the inhibitor appeared to be remarkably stable to extremes of pH. Thus, after adjustment to pH 7.8, solutions that had been stored at pH values in the range 2–10 for up to 24 h showed no decrease in either caseinolytic or inhibitory activity.

Effect of temperature on caseinolysis. Under the assay conditions employed caseinolytic activity of the inhibitor (fraction II) increased markedly with increasing temperature. The temperature profile (Fig. 5) indicates a maximum at about 45°C, but significant activity is still retained at temperatures

as high as 85°C. No activity was observed in incubations carried out at 0 or 100°C. In other experiments on the effect of temperature, portions of the enzyme solution were preincubated at different temperatures for 10 min and assayed for caseinolytic activity after cooling to 30°C. Only those solutions heated to temperatures above 45°C showed any loss of caseinolytic activity.

Effect of selected materials on caseinolysis. Proteolytic enzymes can be broadly classified as serine, thiol or metal proteinases (Hartley, 1960). A strong indication of the class to which a proteinase belongs may therefore be obtained by studying its sensitivity to reagents that are specific for those functional groups. Therefore an attempt was made to characterize further the proteinase associated with the inhibitor by its behaviour towards some of these reagents, and also to investigate the possibility of stabilizing microsomal enzyme activity.

The thiol-blocking reagent *p*-chloromercuribenzoic acid and the thiols cysteine, GSH, 2-mercaptoethanol, dithiothreitol and 2,3-dimercaptopropan-1-ol were each preincubated with fraction II for 15 min at concentrations from 1 μ M to 1 mM. Under these conditions no inhibition of caseinolysis was observed, showing that no thiol group accessible to *p*-chloromercuribenzoate was necessary for activity.

Preincubation of the inhibitor with citrate or EDTA at concentrations greater than 1 mM resulted in up to a three-fold stimulation of caseinolytic activity that was not affected by prior dialysis of either the substrate or the inhibitor. 2,2'-Bipyridyl had no observable effect.

Di-isopropyl phosphorofluoridate (Saunders & Stacey, 1948) and phenylmethanesulphonyl fluoride (Fahrney & Gold, 1963) are well-known 'serine' reagents, and both materials proved inhibitory towards the caseinolytic activity of the inhibitor after preincubation for 60 min at 30°C. Under these conditions, inhibition by mM-phenylmethanesulphonyl fluoride was 91%, considerably greater than the 14% inhibition observed with mM-diisopropyl phosphorofluoridate. A third 'serine' reagent, paraoxon (*OO*-diethyl *O*-4-nitrophenyl phosphate), and soya-bean trypsin inhibitor had no observable effect on caseinolysis under similar experimental conditions. Inhibition of caseinolysis was also observed with phenylthiourea (I_{50} 1 mM), although thiourea and urea showed no effect.

The effects of several anions and cations on the caseinolytic activity of the inhibitor were also determined at concentrations up to 10 mM or saturation. Each of the ions were added in aqueous solution and preincubated for 5 min with fraction II before addition of substrate. Appropriate reagent blanks were included for each ion tested.

Mg^{2+} proved to be a potent activator of caseinolytic

Table 3. *Effect of phenylmethanesulphonyl fluoride on activity of gut-contents inhibitor*

Complete incubation medium contained (final concns. in 5ml): tris-HCl buffer, pH 7.8 (50 μ M); glucose 6-phosphate (2.4 mM); KCl (27 mM); NADP⁺ (51 μ M); glucose 6-phosphate dehydrogenase (1.6 units); microsomal suspension (1mg of protein) and aldrin (100 μ g), dihydroisodrin (100 μ g) or *p*-chloro-*N*-methylaniline (500 μ g). After a 60min preincubation period at 30°C the reactions were initiated by microsomal enzyme addition and activity was determined as described in the Methods section. Abbreviations: PMSF, phenylmethanesulphonyl fluoride.

Incubation medium	Activity (%)		
	Epoxi- dation	Hydroxyl- ation	Demethyl- ation
Complete	100	100	100
Complete + fraction II	14	24	42
Complete + mM-PMSF			
+ fraction II	57	85	70
Complete + mM-PMSF	48	86	94

sis at concentrations greater than 1 mM, and Ca²⁺ also stimulated activity to a smaller extent. Mn²⁺ had no effect and inhibition was obtained with Cu²⁺, Ag²⁺, Zn²⁺, Hg²⁺, Fe³⁺, Sn²⁺ and Co²⁺, in decreasing order of effectiveness, 0.1 mM-Cu²⁺ resulting in approx. 65% inhibition.

No marked effects on caseinolysis were observed in the presence of F⁻, NO₃⁻, SO₄²⁻ or CN⁻ at concentrations up to 1 mM.

Stabilization of microsomal enzyme activity. The inhibition of caseinolysis by specific 'serine' reagents such as phenylmethanesulphonyl fluoride suggests that such materials can protect microsomal enzymes from the action of the inhibitor. Table 3 shows that at 30°C a 50min preincubation of the inhibitor with mM-phenylmethanesulphonyl fluoride decreases inhibition of microsomal epoxidation, hydroxylation and *N*-demethylation. The extent to which reversal of inhibition occurs appears to vary with the type of microsomal activity under investigation; it is largest with hydroxylation (61%) and smallest with *N*-demethylation (28%). Unfortunately, at the high concentration required for significant reversal of inhibition, phenylmethanesulphonyl fluoride itself inhibits microsomal enzyme activity. Thus, although *N*-demethylation and hydroxylation are inhibited by only 6 and 14% respectively in the presence of mM-phenylmethanesulphonyl fluoride, the 52% decrease in epoxidation activity clearly indicates that this compound has only limited use as a protective agent.

The cations found to inhibit caseinolytic activity of the inhibitor similarly inhibit the microsomal enzymes and consequently cannot be considered

as potential stabilizing agents for the latter in insect preparations.

DISCUSSION

The absence of mixed-function oxidase activity from microsomal fractions prepared from whole *Prodenia* larvae is undoubtedly due to the release of an inhibitor during the homogenization process. The inhibitor is present in the gut contents of the larva and is a soluble non-diffusible protein with a molecular weight of approx. 26000. In the crude form it is heat-stable, but it becomes considerably more labile on purification. The material is clearly different from the heat-stable diffusible inhibitory factor found in houseflies (Matthews & Hodgson, 1964) and the inhibitors from locust (Chakraborty *et al.* 1967) and cockroach (Nakatsugawa & Dahm, 1965) fat-bodies, both of which are associated with a particulate fraction sedimenting at 10000g.

Inhibitory activity of the gut-contents inhibitor closely parallels its proteolytic activity, and the progressive nature of the inhibition suggests that it results from a direct attack on the microsomal protein. Preincubation experiments indicate that inhibition does not result from the depletion of essential cofactors in the incubation medium. Although inhibition is of a general nature, different types of microsomal activity appear to vary in their susceptibility to the inhibitor. Thus under identical incubation conditions a concentration of the inhibitor that results in 86% inhibition of epoxidation causes only a 42% decrease in *N*-demethylation (Table 3), suggesting that the inhibitor may exhibit some selectivity in its ability to destroy binding sites of different substrates. Mammalian liver and insect microsomal fractions are both sensitive to the inhibitor.

Characterization of the inhibitor suggests that it is probably a serine proteinase not dissimilar to trypsin, and it is possible that the enzyme is a naturally occurring larval digestive proteinase. Although detailed characterization studies have not been made on other species, similar inhibitory activity has been found in the gut contents of all lepidopterous larvae examined in this laboratory. Further, the existence of significant proteolytic enzyme activity has been demonstrated in 10% (w/v) homogenates of several other insect species including housefly (*Musca domestica*), flour beetle (*Tenebrio molitor*) and house cricket (*Acheta domesticus*) (Table 4). It therefore appears that the importance of proteolytic enzymes released during homogenization of whole insects may have broad significance and should always be considered in initial investigations with new insect species. The importance of such inhibitory materials can be readily determined by measuring the degree of

Table 4. *Proteolytic activity of whole insect homogenates*

Homogenates (10%, w/v, in 1.15% KCl) of whole insects were prepared in a Waring Blendor. After filtration through nylon organdy the homogenates were assayed for proteolytic activity in 20mm-sodium-potassium phosphate buffer, pH 7.8, at 30°C. The reaction was initiated with addition of vitamin-free casein and was terminated after 10 min by addition of trichloroacetic acid (5%, w/v). E_{280} of the soluble fractions were measured against zero-time blanks. Caseinolytic activity was ranked according to the following scheme: + + +, > 0.100; + +, 0.050–0.100; +, 0.010–0.050 extinction units.

Insect	Caseinolytic activity
Southern armyworm (<i>Prodenia</i>)	+ +
Housefly (<i>Musca</i>)	+ + +
Cockroach (<i>Periplaneta</i>)	+
Cricket (<i>Acheta</i>)	+ +
Flour beetle (<i>Tenebrio</i>)	+ + +

inhibition of mammalian liver microsomal preparations by whole insect homogenates or by screening the homogenates for proteolytic enzyme activity by the caseinolysis assay method.

The presence of endogenous microsomal enzyme inhibitors in homogenates of whole insects clearly imposes severe limitation on the use of such preparations *in vitro* in investigation of comparative detoxication. With most of the larger species of insects presently being used the problem can usually be avoided by tissue fractionation and differential centrifugation of homogenates. When the inhibitor is a soluble material, as is the gut-contents inhibitor, the preparation of a microsomal pellet allows removal of the inhibitor in the 100000g supernatant and substantially increases the stability of the enzyme preparation obtained. Because the extent of inhibition increases with both time and temperature, further enhancement of activity is obtained by ensuring that all operations are carried out rapidly at low temperatures. Unfortunately, a great many insects of economic importance and biochemical interest are so small that dissection on a preparative scale is not technically feasible, and the use of whole homogenates is necessary. One possibility for making the results of such investigation more meaningful is by including in the homogenization medium materials that deactivate the endogenous inhibitory materials.

At high concentrations specific 'serine' reagents such as di-isopropyl phosphorofluoridate and phenylmethanesulphonyl fluoride effectively blocked the caseinolytic activity of the inhibitor and were also partially successful in protecting microsomal enzyme activity in the presence of the

inhibitor. However, these compounds are themselves inhibitory towards microsomal enzyme activity, and for this reason are not suitable protective agents.

The incorporation of phenylthiourea into homogenization media to inhibit the browning reaction of tyrosinase enabled Williamson & Schechter (1968) to prepare active microsomal preparations from several species of lepidopterous larvae. Phenylthiourea is an effective inhibitor of caseinolysis by the gut-contents inhibitor from *Prodenia* (I_{50} 1mM) and moreover has no marked effect on microsomal enzyme activity at fairly high concentration. However, its inclusion in the homogenization medium [50% saturation in 1.15% (w/v) potassium chloride] did not enable the preparation of active microsomal fractions from whole *Prodenia* larvae.

Bovine serum albumin has been reported to stimulate epoxidation (Ray, 1967) and carbamate metabolism (Tsukamoto & Casida, 1967a) in housefly microsomes, and as a result it has been suggested that the albumin is able to bind the inhibitors released during homogenization. Bovine serum albumin exhibited a marked protective effect on microsomal activity in the presence of the inhibitor, probably by providing an alternative protein substrate for it. It is likely that the activity of albumin in stabilizing housefly microsomes occurs at least partially by a similar mechanism, although in this case the concentration of inhibitory material in the head and thorax suggests the presence of inhibitors other than those associated with the gut.

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