4. α -(α -L-Aspartyl)-L-lysine and α -(β -L-aspartyl)-L-lysine cyclize to α -(L-aminosuccinyl)-L-lysine in 11N-hydrochloric acid at 80°, but this derivative of aminosuccinimide is much less stable than that formed from the corresponding ϵ -aspartyl-lysines.

5. ϵ -(Aminosuccinyl)-lysine is formed when bacitracin A is treated with 11N-hydrochloric acid at 80°. It is also formed during acid hydrolysis of the cell walls of certain strains of lactobacilli. The cell walls of other strains of lactobacilli do not yield this compound, although they contain lysine and aspartic acid residues.

One of us (D.L.S.) is indebted to the Medical Research Council for a scholarship. Infrared spectra were determined by Dr F. B. Strauss. The analysis was by Weiler and Strauss.

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The Production of an Esterase Inhibitor from Schradan in the Fat Body of the Desert Locust

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The oxidative conversion of the systemic insecticides schradan (bisdimethylaminophosphonous anhydride) and dimefox (bisdimethylaminofluorophosphine oxide) into powerful anticholinesterases has been studied in mammalian liver homogenates (Davison, 1954, 1955; O'Brien, 1956, 1957; Fenwick, Barron & Watson, 1957), and shown to be dependent on the presence of Mg^{2+} or Ca^{2+} ions, nicotinamide and diphosphopyridine nucleotide. The microsome fraction of liver cells could effect the conversion if fortified with reduced diphospho-

pyridine nucleotide. O'Brien & Spencer (1953, 1955) reported that many intact insect tissues could produce the same schradan metabolite as did liver, but all attempts to make an active tissue homogenate were unsuccessful. Cockroach-gut homogenates fortified with nicotinamide, diphosphopyridine nucleotide and Mg^{2+} ions failed to convert schradan (O'Brien, 1957) and dimefox (Fenwick *et al.* 1957) into such compounds, and it was suggested that a different mechanism might be involved.

An investigation of the conversion of schradan by the tissues of the desert locust was undertaken in order to determine whether there is a fundamental difference of mechanism between mammals and insects. The choice of insect was governed by its size, availability and economic importance.

MATERIALS AND METHODS

Schradan, diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and flavine mononucleotide (FMN) were obtained from L. Light and Co. Ltd.; dimefox from Fisons Pest Control Ltd.; flavinadenine dinucleotide (FAD) from Sigma Chemical Co.; DPNH (reduced DPN) was prepared by the reduction of DPN with dithionite (Colowick & Kaplan, 1955), and TPNH (reduced TPN) by the enzymic method of Nason & Evans (1953). *p*-Nitrophenyl acetate was synthesized by the method of Kaufmann (1909).

Fourth-instar nymphs of the desert locust (*Schistocerca gregaria* Forsk.) were provided at regular intervals by the Anti-Locust Research Centre, 1 Princes Gate, London, S.W. 7. The insect cage stood in a thermoregulated room, and was lit and heated internally by an electric-light bulb which was automatically switched on at 8 a.m. and off at midnight. The mean day temperature inside the cage was 35°, falling to 25° at night. A tray of water stood beneath the wire-gauze floor of the cage, and the humidity was further influenced by a jar of fresh or cold-stored green grass and a dish of drinking water in the cage. The grass diet was supplemented by a mixture of grass meal, bran, dried whole milk and dried autolysed yeast (The Distillers Co. Ltd.) in the proportions 2:2:2:1 (by vol.).

The tissues were removed from the decapitated insects and placed in an ice-cooled dry beaker. Air sacs and tracheae were generally not separated from the fat body after it was found that their presence did not affect schradan conversion. Testes were removed before the fat body, as otherwise they tended to become incorporated in it. The unwashed tissue was homogenized with a glass Potter-Elvehjem tube and pestle. Fat-body tissue is very soft, and five or six up-and-down movements of the pestle were sufficient to release the fat from the cells.

Esterase determination. The principle of the method of Huggins & Lapides (1947) was used, the rate of liberation of *p*-nitrophenol from *p*-nitrophenyl acetate being measured. The substrate could be kept for up to a month at -15° as a 50 mM-solution in dry methanol. For each series of estimations it was diluted (1/25) with water, and used within 1 hr. Esterase preparation (1 ml.) was added to 3 ml. of 0.01 M-phosphate buffer, pH 7.4 (Umbreit, Burris & Stauffer, 1949), in a 1 cm. diam. spectrophotometer tube. A blank contained water instead of esterase preparation. At zero time, 1 ml. of substrate solution was added to the first tube and its optical density at 400 m μ immediately measured with a Unicam SP 400 instrument. Subsequent tubes were treated likewise at intervals of 1 min., and the tubes allowed to stand at room temperature. At 15 min. a series of final readings was started, and the change of optical density of each tube was referred to a *p*-nitrophenol standard curve. The blank (spontaneous hydrolysis) value was subtracted from each. The activity, expressed as μ moles of substrate hydrolysed/hr./ml. of enzyme, was

directly proportional to enzyme concentration up to an optical-density change of 0.33.

Schradan conversion by intact tissue. Batches of fat-body tissue (30 mg. wet wt.) were incubated in air for 30 min. at 37° in 2 ml. of 'insect Ringer' solution (Thomsen, 1952), buffered with 10% (v/v) of 0.1 M-phosphate buffer, pH 7.4, in the presence of schradan at concentrations of 25, 50, 100 and 500 μ M. The tissue was then homogenized in the medium, water was added to give a total of 1 ml./3-6 mg. of tissue, and the suspension was centrifuged for 2 min. at 600 g. A volume (1 ml.) of supernatant was taken for esterase measurement after removal of the upper layer of fat. Converting power was expressed as the mean of the four values of $\log(100/b)/\text{mg. dry wt./concn. of schradan (M)}$ which were so obtained, where *b* is the percentage of the initial esterase which remained.

Dry weight and fat content were obtained by homogenizing a weighed sample of tissue in a small volume of water and shaking with ether. After a brief centrifuging, a portion of the ether extract was dried at room temperature and weighed. The aqueous suspension, freed of the ether layer, was dried at 110° and weighed.

Schradan conversion by homogenates. A fat-body homogenate in water was incubated for 3 hr. at 37° to reduce its Schradan-converting power. Schradan concentrations up to mM had no significant effect on its esterase activity. mM-Schradan was therefore usually used in subsequent conversion experiments, and schradan was omitted from controls. The components were placed in 6 in. $\times$ 1 in. boiling tubes shaken in a water bath at 37° and open to the air. A typical reaction mixture consisted of 0.2 ml. of fat-body suspension, 0.2 ml. of inhibitor or activator dissolved in 0.25 M-sucrose and 0.1 ml. of 5 mM-schradan in 0.1 M-phosphate buffer, pH 7.4. At the end of the incubation period (usually 30 min.) water was added to stop the reaction by dilution and cooling, and to give a convenient concentration of esterase, which was then estimated.

Centrifugal fractionation. A homogenate of 150-200 mg. wet wt. of fat body/ml. of 0.25 M-sucrose solution was centrifuged for 2 min. in a bench centrifuge (about 600 g), and the supernatant was decanted after removal of the upper layer of fat by suction. This did not reduce the schradan-converting activity of the suspension. The supernatant was then centrifuged at 0° for a further 30 min. at 18 000 g. The resulting clear supernatant was carefully pipetted off and replaced by an equal volume of sucrose solution in which the sedimented particles were resuspended.

RESULTS

Intact tissue

The intact gut and fat body of locusts of various ages of the fifth-instar and adult forms were tested for schradan conversion, with endogenous esterase as an indicator of inhibitor produced. The gut (slit longitudinally and cleaned of food) showed no activity with mM-schradan at 37°, pH 7.4, but fat body possessed definite but erratic activity. This was found to vary with the age of the insect, although males and females of the same age did not differ appreciably. A systematic investigation of the fat body of the fifth-instar and adult locusts

(fourth-instar and younger forms are too small to constitute a convenient source of material) showed that schradan-converting power began to decline sharply 2 days before the final moult, and rose slightly during the first 3 weeks of adult life (Table 1). It was noticed also that the esterase content declined during the fifth-instar and remained low in the adult. Fat body from fourth-sixth day fifth-instar locusts was therefore used in subsequent experiments.

Fat-body homogenates

A homogenate of fat body in 0.25M-sucrose was found to be surprisingly active in schradan conversion. mM-Schradan incubated for 30 min. at 37° with a 5% (wet wt.) suspension of fat body, buffered with phosphate buffer, pH 7.4, caused a marked inhibition of the endogenous esterase.

Rate of reaction. When time of incubation was plotted against log (100/*b*) (Aldridge, 1950), an S-shaped curve was obtained (Fig. 1), probably owing to the initial gradual increase of the inhibitor concentration and the final decline of activity of the homogenate. However, log (100/*b*) gave a better approximation to a straight line than percentage inhibition (i.e. 100−*b*) when plotted against time, and so was used as an estimate of the converting power of a fat-body preparation.

Buffers. Since O'Brien (1957) stated that schradan conversion by whole cockroach gut is inhibited by phosphate buffer, glycylglycine and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffers at pH 7.4 (Colowick & Kaplan, 1955) were substituted for phosphate, but with no advantage.

Metals. CaCl₂ and MgSO₄ both enhanced the converting power of fresh sucrose homogenates to about the same extent (e.g. in a typical experiment, mM-CaCl₂ soln. increased the inhibition of esterase from 25 to 40%). The optimum effect was achieved

with CaCl₂ at concentrations of 0.8 mM or higher. BaCl₂ and MnCl₂ had a small activating effect. ZnCl₂ (mM), CuSO₄ (0.1 mM), FeCl₂ (mM), CoCl₂, NaCl and Na₂SO₄ (all 10 mM) had none.

Cofactors. The presence of 10 mM-nicotinamide in the homogenizing medium did not improve converting power. The diphosphopyridine nucleotidase activity was measured and found to be less than 5% of that of rat liver (/g. fresh wt.), which probably accounts for the lack of effect with nicotinamide.

Fig. 1. Conversion of schradan by fat-body homogenate. Fat-body suspension, 60 mg./ml. in 0.25M-sucrose and 10 mM-MgSO₄, was incubated with mM-schradan, pH 7.4, at 37°. Samples were removed at intervals, and diluted with cold water and their esterase activities measured. ●, Percentage inhibition of esterase; ○, log (100/*b*), where *b*=percentage of initial esterase remaining.

Table 1. Variation with age of schradan-converting power of intact fat body

In each case the tissue from at least four locusts (equal numbers of males and females) was pooled. Details of procedure are given in the Materials and Methods section. The final moult generally occurred on the eighth day of the fifth instar.

Age (days)	Fat body/locust (mg. wet wt.)	Fat (%)	Dry wt. (%)	Esterase activity*	Schradan conversion†
Fifth instar					
4	50	28	23	0.49	1.6
5	56	24	23	0.36	1.6
6	71	32	19	0.31	1.3
7	75	33	14	0.21	0.05
8	50	27	11	0.23	0.06
Adult					
7	89	59	15	0.27	0.02
11	125	64	16	0.17	0.17
14	219	64	14	0.17	0.21
19	131	55	11	0.33	0.30

* μmoles of *p*-nitrophenyl acetate hydrolysed/15 min./mg. dry wt.
† Mean of four values of log (100/*b*)/m-concn. of schradan/mg. dry wt.; *b*=percentage of initial esterase remaining.

DPN and TPN (mm) were added to the homogenate in sucrose with or without nicotinamide, but had no effect.

Dialysis for 16 hr. at 2° against isotonic sucrose soln. resulted in complete loss of activity, which was not restored by DPN, TPN, DPNH, TPNH or by the clear supernatant obtained by centrifuging for 1 hr. at 18 000 g.

Fractionated homogenate. Both the supernatant and particulate fractions obtained by centrifuging contained enough esterase for a schradan-conversion test to be carried out. Neither was active alone, but when they were recombined full activity was restored.

Function of the supernatant. DPNH was prepared chemically and shown to fortify rat-liver microsomes in the conversion of schradan, but it would not replace the supernatant. Nor would a system generating DPNH, consisting of an extract of dried baker's yeast (containing alcohol dehydrogenase), ethanol and DPN.

A TPNH-producing system, comprising an extract of acetone-dried pig heart (containing isocitric dehydrogenase), DL-isocitrate (0.01 M) and TPN, was next added to a fat-body particle preparation, which could then convert schradan. The

efficiency of conversion depended on the amount of TPN added, and reached a maximum with about 2 mm-TPN. TPNH was prepared with the same pig-heart enzyme, which was then destroyed by boiling in alkaline solution. Schradan conversion by particles was shown to depend on TPNH concentration, reaching an optimum equal to the rate with supernatant, at about mm (Fig. 2).

When TPN was added to the fat-body supernatant, an increase in optical density at 340 m μ occurred which appeared to be limited by the concentrations of endogenous substrates, for when isocitrate, glucose 6-phosphate or malate was added, it increased further. Dialysis removed the TPN-reducing activity and addition of these substrates restored it (Fig. 3). There was no increase in optical density at 340 m μ when DPN was added to the supernatant.

No conversion was observed by particles in the presence of mm-hydrogen peroxide or 2 mm-DL-ascorbic acid.

Endogenous-reduced triphosphopyridine nucleotide. When the supernatant was replaced by the TPN-reducing system, TPN had to be added to an

Fig. 2. Effect of TPNH on schradan conversion by fat-body particles. Particle suspensions in 0.25 M-sucrose, equivalent to about 12% wet wt. of fat body, were incubated with TPNH and mm-schradan, pH 7.4, at 37° for 30 min., and the residual esterase activity was then determined. The two curves were obtained with two different fat-body preparations, the lower one (●) having stood overnight at 2° before use.

Fig. 3. Reduction of TPN by fat-body supernatant fraction equivalent to 20 mg. wet wt./ml., at pH 7.4, temp. 20°. TPN (mm) was added at zero time in the presence (○) and in the absence (●) of MgSO₄ (10 mm). The dialysed supernatant was inactive until glucose 6-phosphate (x), D-isocitrate (Δ) or L-malate (▲), all 5 mm, were added, at 2 min.

extent of about 2 mm in order to regain full activity, although the TPN-reducing power of the pig-heart isocitrate system was much greater than that of the supernatant. Therefore an attempt was made to estimate the amount of endogenous TPNH. Optical density at 340 mμ indicated a maximum possible concentration of 0.24 mm in the supernatant from a 200 mg./ml. homogenate. TPN was added to a supernatant preparation buffered with 1 % NaHCO₃ saturated with CO₂, pH 7. When the optical density at 340 mμ had risen to a maximum, excess of lithium pyruvate was added, and the optical density declined as the reaction

$\text{malate}^{2-} + \text{TPN}^+ \rightarrow \text{pyruvate}^- + \text{CO}_2 + \text{TPNH}$ was reversed. Similarly, when pyruvate was added to the supernatant without TPN, a small decline in optical density occurred (Fig. 4), and a calculation based on this change showed an approximate concentration of 45 μM-TPNH, or about 18 μM in a typical reaction mixture. Thus there seems to be a discrepancy factor of more than 10 between the endogenous TPNH and the amount that had to be added to replace it. Possibly the higher efficiency of the natural coenzyme can be explained in terms of binding and orientation.

Function of calcium and magnesium. CaCl₂ (mm) and MgSO₄ (10 mm) (see Fig. 3) both caused a small increase in the rate of reduction of TPN by the supernatant. In an experiment to test the effect of these metal ions on the particle fraction, the particle concentration was reduced, in the presence of a constant concentration of added TPNH, until it became rate-limiting. Under these conditions, neither CaCl₂ nor MgSO₄ had any effect on the rate of schradan conversion.

Inhibitors of conversion. The effects of inhibitors (brought to pH 7.4) were estimated by the per-

centage reduction of log (100/b) they caused. The results with whole suspensions are shown in Table 2, and with TPNH-particle preparations in Table 3. The inhibition by *p*-chloromercuribenzoate was reversed by mM-glutathione. Adenosine triphosphate (2 mm) did not restore the activity after treatment with 2:4-dinitrophenol.

Nature of the particle-bound enzyme. In view of the inhibitory effects of chelating agents and mepacrine (quinacrine, atabrine) a number of unsuccessful attempts were made to demonstrate

Table 2. *Inhibitors of schradan conversion in fat-body homogenates*

Inhibitors were pre-incubated for 5 min. at 37°, pH 7.4, with the fat-body preparation (centrifuged 2 min. at 600 g in 0.25 M-sucrose + 10 mM-MgSO₄), before addition of mM-schradan. The remaining esterase activity was determined after a further 30 min. at 37°.

Inhibitor	Concn. of inhibitor (mm)	Inhibition of schradan conversion*
Anaerobiosis	—	100
8-Hydroxyquinoline	0.25	29
	1.0	83
αα'-Dipyridyl	0.5	44
	1.0	74
Diethyldithiocarbamate	0.25	39
	1.0	74
Ethylenediaminetetra-acetate (EDTA)	1.0	22
	5.0	64
Potassium cyanide	5.0	0
Pyrophosphate	1.0	0
Citrate	1.0	0
Mepacrine	0.05	31
	0.25	73
<i>p</i> -Chloromercuribenzoate	0.25	53
	0.5	100
2:4-Dinitrophenol	0.05	15
	0.5	50
Antimycin A	2 μg./ml.	0

* Estimated by percentage decrease of log (100/b).

Table 3. *Inhibitors of schradan conversion by the fat-body particulate fraction*

The particles were fortified with enzymically prepared TPNH (0.5 mm final concn.), and incubated with inhibitor and schradan (mm), pH 7.4, at 37° for 30 min. before measuring residual esterase activity.

Inhibitor	Concn. of inhibitor (mm)	Inhibition of schradan conversion*
αα'-Dipyridyl	1	97
Mepacrine	0.25	75
<i>p</i> -Chloromercuribenzoate	0.25	100
2:4-Dinitrophenol	0.5	61

* Estimated by percentage decrease in log (100/b).

Fig. 4. Oxidation of TPNH in the supernatant fraction. ●, TPN (mm) was added at zero time to a fat-body supernatant buffered with NaHCO₃-CO₂, pH 7; ○, no TPN added. Arrows indicate the points at which pyruvate (10 mm) was added.

the participation of a heavy metal, and of a flavine prosthetic group. For example:

(i) A fat-body homogenate, buffered with tris buffer, pH 7.4, was treated with $\alpha\alpha'$ -dipyridyl followed by FeSO_4 at twice the concentration. No restoration of activity occurred.

(ii) A particle suspension was treated with ethylenediaminetetra-acetate (EDTA) and then dialysed for 3 hr. at 2° against isotonic sucrose. All activity (in combination with supernatant fraction) was lost and none was restored by adding Fe^{2+} , Fe^{3+} or Cu^{2+} ions. It was subsequently found that mere dialysis of a particle suspension against sucrose soln. for 3 hr. inactivated it (the presence of pieces of dialysis tube in the control suspension had no effect). This was not due to removal of cofactors, since washing the particles once in sucrose soln. and re-sedimenting and resuspending them did not reduce their activity. A brief 45 min. dialysis of particles against 25 mM-EDTA caused only a very small decline in activity. Therefore the technique of dialysis was abandoned.

(iii) A homogenate was treated with dipyridyl sufficient to inhibit almost completely its schradan conversion. The particles were then separated by centrifuging and resuspended in fresh sucrose soln., in the hope that the metal, together with the excess of dipyridyl, would have been removed. However, when the particles were tested, in combination with untreated supernatant, their activity was found to be normal. Evidently the dipyridyl is weakly bound to the enzyme molecule and its inhibition is readily reversible. 8-Hydroxyquinoline inhibition was also reversed by washing.

(iv) FAD (0.4 mM) had no effect on the activity of a fat-body homogenate. The effect of mepacrine (0.25 mM) was not reduced by the previous addition of FAD or FMN (0.25 mM).

(v) Homogenates were incubated at various acid pH values before restoring to 7.4 and measuring schradan conversion. Pre-incubation for 6 min. at 37° and pH 4.4 was needed to cause a substantial reduction of activity, but it was not restored by Fe^{2+} ions (0.5 mM) or FAD (0.1 mM) or both.

Non-enzymic activation

A reaction mixture consisting of ascorbic acid, EDTA and ferrous (or ferric) iron, which accomplishes certain aromatic hydroxylations, has been described by Udenfriend, Clarke, Axelrod & Brodie (1954). This system also produced an esterase inhibitor when incubated with schradan, and controls showed that ascorbic acid was not essential.

Convenient concentrations were: FeSO_4 (5 mM), EDTA (5 mM), schradan (20 mM). EDTA solution was neutralized with NaOH, and the system was buffered with phosphate buffer, pH 7, and shaken

in air. The product of this reaction was applied at 1/20 final dilution to an esterase preparation to assess its inhibitory action. The amount of EDTA added was just sufficient to prevent precipitation of the iron by the phosphate buffer.

The following facts were observed:

(i) Fe^{2+} ions could not be replaced by Fe^{3+} , Cu^{2+} , Co^{2+} or MoO_4^{2-} ions, nor had ferrocyanide any action on schradan.

(ii) Production of inhibitor stopped within 3 min. at 37° , 8 min. at 22° (Fig. 5) or 16 min. at 2° .

(iii) Much less inhibitor was formed if the reaction occurred in an evacuated Thunberg tube.

(iv) If FeSO_4 was pre-incubated for 5 min. with EDTA in air before adding schradan, no inhibitor was formed, but if the pre-incubation was anaerobic, inhibitor formation was normal. When FeSO_4 was tipped into EDTA solution from the side arm of a Warburg flask, a rapid uptake of O_2 occurred, to the extent of 1 equivalent of O_2 /atom of Fe (Fig. 5). If the molecular ratio of EDTA to Fe^{2+} ions was reduced to less than one, consumption of O_2 was limited to 1 equivalent/molecule of EDTA.

(v) In the presence of FeSO_4 , unchelated and unbuffered, some inhibitor was produced from schradan, and pre-incubation of the FeSO_4 in air had no effect. However, the amount of inhibitor detectable again quickly reached a steady level, possibly due to the setting up of an equilibrium, the inhibitor being unstable at the low pH involved.

(vi) Reducing agents were added in the hope of promoting the reaction, but metabisulphite pre-

Fig. 5. Model system. Curve A: units of inhibitor produced (b = percentage of initial esterase remaining after incubating with inhibitor for 15 min. at 37°). EDTA (5 mM), schradan (20 mM), FeSO_4 (5 mM) were added at zero time. pH 7, temp. 22° . Curve B: rate of oxygen uptake. EDTA (10 mM), FeSO_4 (10 mM) were added at zero time, pH 7, temp. 20° .

vented, and thiosulphate reduced, the formation of inhibitor. Ascorbic acid (40 mM), at pH 5.7 and 37°, increased the inhibitor production, but did not prolong it beyond 3 min. When ascorbic acid was tipped, in a Warburg flask, into a Fe^{2+} -ion-EDTA mixture in which uptake of O_2 had ceased, a further rapid uptake occurred, equivalent to complete oxidation of the ascorbic acid to dehydroascorbic acid.

A Co^{2+} ion-2 histidine chelate, which combines reversibly with molecular O_2 (see Martell & Calvin, 1952), was ineffective in producing an esterase inhibitor from schradan.

Dimefox

A few experiments were carried out with dimefox in order to compare its behaviour with that of schradan. It was also converted into an anti-esterase by the particles of the fat body fortified with TPNH, and the converting power of eighth-day fat body was considerably lower than that of fifth-day fifth-instar. However, a given concentration of dimefox caused the same degree of esterase inhibition in intact or homogenized fat body as did approx. 50 times the molar concentration of schradan, although in rat liver equal concentrations of the two compounds produce about the same effect (Fenwick *et al.* 1957).

Experiments in vivo

The LD_{50} values for schradan and dimefox administered by intrathoracic injection of solutions in 'insect Ringer' (0.01 ml./g. body wt.) were approx. 1800 and 45 $\mu\text{g./g.}$ respectively, with young-adult locusts.

There was no appreciable difference in toxicity of dimefox between young adults and fifth-day fifth-instar nymphs. With schradan, the nymphs were probably slightly less susceptible than adults. An injection of 2000 $\mu\text{g./g.}$ caused the death of 70% young adults and 30% of fifth-day fifth-instar nymphs.

DISCUSSION

It has been shown that in locust fat body, as in rat liver, the production of an esterase inhibitor from schradan and dimefox depends upon the presence of the particulate and soluble fractions of the cell contents, requires oxygen, and is activated by Ca^{2+} or Mg^{2+} ions. The soluble supernatant can be replaced by TPNH (as distinct from DPNH in the rat), and contains at least three enzymes capable of catalysing the reduction of TPN, namely glucose 6-phosphate and isocitric dehydrogenases and malic enzyme. The function of Ca^{2+} and Mg^{2+} ions is probably to activate these enzymes.

This strange need for a reducing agent in an

oxidative process has been discussed in a review article by Brodie (1956), in connexion with the metabolism of various drugs. It was suggested that a peroxide-like compound, formed during the oxidation of TPNH, might then oxidize the drug. O'Brien (1957) demonstrated the formation of hydrogen peroxide on the addition of DPNH to mouse-liver homogenates, but could not correlate the rate of its formation with that of schradan conversion. Furthermore, hydrogen peroxide would not replace DPNH in fortifying a microsome suspension, although if catalase was added as well, conversion of schradan did occur.

Mason, Fowles & Peterson (1955) proposed that the Cu^+ ions of phenolase are oxidized to Cu^{2+} ions during the process of transfer of oxygen to the substrate. A reducing system would be needed to regenerate the active form of the enzyme, and Velick (1956) suggested that this might also be the function of TPNH in Brodie's (1956) drug-metabolizing systems, if the reduced form of a metallo-enzyme were involved.

It was therefore interesting to observe that the conversion of schradan by the intracellular particles of locust fat body, supplemented with TPNH, was prevented by $\alpha\alpha'$ -dipyridyl. It was hoped that chelating agents would provide a means of removing the supposed metal from the enzyme, and that its activity would then be restored by adding the appropriate metal ion, as has been done with a number of metallo-enzymes. However, the inhibition proved to be reversible by washing the particles, so presumably the metal is not removed, but merely blocked, by the chelating agents used. A similar phenomenon occurred in the inhibition of copper-containing uricase by potassium cyanide, which was reversed by dialysis (Mahler, Hübscher & Baum, 1955). Nor was the commonly used technique of dialysis against cyanide solution of value, since (a) dialysis inactivated the particle suspension, conceivably owing to a lowering of pH at the dialysis membrane (Gordon, 1957), and (b) cyanide did not inhibit the conversion of schradan. The lack of inhibition by cyanide, pyrophosphate or citrate cannot be used as an indication of the nature of the enzyme system, since the permeability of the particle membrane to these materials is unknown.

Although no direct evidence for the participation of a metal in the natural system could be obtained, it has been shown that Fe^{2+} ions, particularly in the form of a chelated complex with EDTA, in equimolecular proportions, can catalyse the oxidative formation of an esterase inhibitor from schradan. The reaction ceases as the complex is auto-oxidized (presumably to the ferric form), and Fe^{3+} ions among others are inactive, which is of particular interest in view of the need for a reducing agent in

animal systems. The addition of ascorbic acid enhanced inhibitor production, and it was itself oxidized, but ascorbic acid could not replace TPNH in the natural system. The spontaneous uptake of oxygen by a Fe^{3+} ion-EDTA complex may have some practical interest in dealing with an enzyme whose activity is measured manometrically (see, for example, Schepartz, 1953). If Fe^{2+} ion is added to an enzyme that has been inhibited by EDTA, a brief uptake of oxygen will occur which should not be confused with the restoration of enzyme activity.

Mepacrine inhibits enzymes with FMN and FAD prosthetic groups (Haas, 1944; Mackler, Mahler & Green, 1954), and was shown by Haas (1944) to compete with FMN for the apoenzyme of TPN cytochrome *c* reductase. The inhibition was irreversible, but could be prevented by the previous addition of small amounts of prosthetic group. The effect of mepacrine on schradan conversion was not changed by the presence of FAD or FMN, but again such negative results must be assessed with caution in view of the particle-bound nature of the system.

The fact that inhibition of conversion by *p*-chloromercuribenzoate can be reversed by glutathione (cf. Mahler, 1954) may indicate that the vital -SH groups are concerned with orientating the substrate on the enzyme surface rather than binding prosthetic groups.

It seems probable that further information about the nature of this oxidizing system must await its solubilization and purification, but in the meantime it is possible to surmise that the mechanism is of the type discussed by Mason *et al.* (1955), with the possible participation of a flavin nucleotide as an intermediate hydrogen carrier:

(Spencer & O'Brien, 1957). Or alternatively, without a change of valency of the metal ion:

The large difference in toxicity between schradan and dimefox may be connected with the much greater overall efficiency of dimefox conversion with esterase-inhibition in fat-body preparations *in vitro*, if this implies a similar high efficiency in other tissues, e.g. the nerve cord. It has not been determined whether the higher efficiency is due to faster conversion, slower destruction of the product as compared with rat liver, or a higher susceptibility of the esterase. It is not likely that conversion in the fat body contributes materially to the lethal effect *in vivo*, since considerable variations in fat-body activity are not related to changes in toxicity.

SUMMARY

1. The capacity of the fat body of the desert locust to convert schradan into an esterase inhibitor is high in young fifth-instar nymphs, but falls sharply before the final moult.
2. The conversion requires oxygen, reduced triphosphopyridine nucleotide and a particle-bound enzyme system.

representing the net reaction:

where AH_2 is an oxidizable substrate such as glucose 6-phosphate, E is the enzyme protein, M is a metal ion and Sch.O is the esterase inhibitor, probably the methylol derivative of schradan

3. It is inhibited by chelating agents (reversibly), and by mepacrine, *p*-chloromercuribenzoate and 2:4-dinitrophenol.

4. No requirement for a metal or flavine prosthetic group could be shown with the particle-bound system.

5. A Fe^{2+} ion-ethylenediaminetetra-acetic acid complex can catalyse the oxidative formation of an inhibitor from schradan.

6. A mechanism for the simultaneous oxidation of schradan and reduced triphosphopyridine nucleotide is suggested.

7. The requirements for the conversion of dimefox to an anti-esterase are the same as those for schradan, but the rate of esterase inhibition is much higher. Dimefox is also considerably more toxic than schradan to the locust, but LD_{50} does not vary with fat-body converting activity.

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Cytochromes and some Respiratory Enzymes in Mitochondria from the Spadix of *Arum maculatum*

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The discovery by James & Beevers (1950) that the rapid respiration of the *Arum* spadix is unaffected by cyanide stimulated interest in the mitochondria of this tissue. Mitochondria isolated from the spadix of *Arum maculatum* have been shown to be similar to those obtained from many other plant and animal tissues in that they are able to oxidize intermediates of the citric acid cycle (Hackett & Simon, 1954; James & Elliott, 1955) and contain cytochromes *a*, *b* and *c* (Bendall & Hill, 1956). However, these oxidations are inhibited to only a small extent by cyanide (James & Elliott, 1955) and are exceptionally rapid. It has also been shown (Bendall & Hill, 1956) that in addition to the normal cytochrome system *Arum* mitochondria contain a relatively large amount of cytochrome *b*, which can be oxidized by air in the presence of

cyanide. It was suggested that cytochrome *b*, accounts for a significant part of the cyanide-stable respiration of the mitochondria.

Simon (1957) has recently measured the rates of oxidation of succinate and cytochrome *c* at different stages of development of the spadix, and determined the effect of cyanide and antimycin A on these enzyme systems. He has shown that the succinic-oxidation system rapidly increases in activity as the spadix grows and matures, and that in the mature spadix cytochrome *c* oxidase and the succinic system are about equally active, despite the comparatively small effect of cyanide on the succinic system.

Further studies on the respiratory enzymes and cytochromes in *Arum* mitochondria are described in the present paper. The effects of cyanide and