lignin with those of other workers on spruce lignin show the eucalypt lignin to have striking resemblance in chemical composition and properties to spruce lignin and suggests that, despite the presence of syringyl groups, the basic structure of the two lignins is fundamentally the same.

SUMMARY

1. Crude methanol lignin from immature and mature *Eucalyptus regnans* has been separated by countercurrent distribution into lignin and degraded lignin.

2. Chromatographic analysis of the separated lignin indicates the presence of a number of substances of differing dissociation constants. This is in accord with Brauns's conception of lignin as a group of amorphous substances.

3. The lignin of immature *E. regnans* is shown to possess the empirical formula based on a C_9 unit of $C_9H_{6.68}O_{1.30}(OH)_{1.05}(OH)_{0.35}(O \cdot CH_3)_{1.45}$, where the aliphatic hydroxyl-group content is represented by 1.05 and the phenolic hydroxyl-group content by 0.35. The lignin of mature *E. regnans* does not differ significantly from this.

4. An approximately equal number of syringyl and guaiacyl units are present in the lignin of *E. regnans*.

5. Potentiometric determination indicates that one in three of the C_9 units is phenolic and ionized in dimethylformamide, whereas spectrophotometric determination shows one in five to be ionized in methanolic 0.1N-potassium hydroxide.

6. The spectrophotometric data suggest that of the phenolic groups ionized in the 0.1N-potassium hydroxide 60% are syringyl groups.

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The Spontaneous and Induced Recovery of Fly-Brain Cholinesterase after Inhibition by Organophosphates

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The widely held belief is that organophosphates exert their insecticidal action through the inhibition of cholinesterase (Spencer & O'Brien, 1957; Fukuto, 1957). Mengle & Casida (1958), with 17 organophosphates in amounts corresponding to the

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LD₅₀, showed that cholinesterase *in vivo* was usually largely inhibited within a few hours, but always recovered in the survivors to 90% or more of normal within 1 day. These recoveries are greater than those found in the mammal *in vivo* (Davison, 1955). There appeared to be no connexion between the nature of the alkoxy substituents and

rate of recovery, as there is in mammals (Davison, 1955).

The validity of the data of Mengle & Casida (1958) was brought into doubt by the findings of Van Asperen (1958a), who made the point that workers measuring cholinesterase inhibition often obtained erroneous results. This was based upon the assumption that excess of inhibitor present in the tissue, but not bound to the enzyme, came in contact, especially during homogenization, with uninhibited enzyme. To avoid this effect Van Asperen homogenized whole flies directly in acetylcholine solution after 'knock down' (i.e. severe paralysis) with dimethyl-2:2-dichlorovinyl phosphate vapours. In this way there was no further inhibition of the enzyme because uninhibited enzyme was 'protected' by the acetylcholine from inhibition.

In the present study similar protection techniques were used and the results were compared with those obtained by the standard method of Mengle & Casida (1958). The comparison shows no difference between results obtained in the two methods and indicates that the rapid inhibition *in vivo* and recovery of cholinesterase activity is a real phenomenon.

In the mammal, spontaneous recovery *in vitro* of inhibited cholinesterase is well known (Hobbiger, 1951; Aldridge, 1953; Aldridge & Davison, 1953). Yet in the housefly Van Asperen (1958b) reported no spontaneous recovery *in vitro* after inhibition with dimethyl-2:2-dichlorovinyl phosphate. In the present work this compound and four additional organophosphate compounds were used to inhibit fly-head cholinesterase *in vitro* and with all five there was no spontaneous recovery. This failure to recover *in vitro* could be due to rapid 'ageing' of the inhibited cholinesterase to an irreversible form, as was reported to occur in mammals (Hobbiger, 1956; Davies & Green, 1956). This possibility has been examined in the present work by the use of potent cholinesterase activators *in vitro* and *in vivo*.

The paradoxical difference between effects *in vivo* and *in vitro* is examined below, and a possible explanation is proposed.

MATERIALS AND METHODS

Adult female houseflies, *Musca domestica* L. (CSMA-1948 strain), were used the day after emergence.

The organophosphates used were: malathion, *S*-1:2-di(ethoxycarbonyl)ethyl *OO*-dimethyl phosphorothiolothionate; malaonon, *S*-1:2-di(ethoxycarbonyl)ethyl *OO*-dimethyl phosphorothiolate; parathion, diethyl *p*-nitrophenyl phosphorothionate; methyl parathion, dimethyl *p*-nitrophenyl phosphorothionate; paraoxon, diethyl *p*-nitrophenyl phosphate; tetraethyl pyrophosphate (TEPP); dimethyl 2:2-dichlorovinyl phosphate (DDVP); dimethoate (American Cyanamid Co.), *OO*-dimethyl *S*-(*N*-methyl carbamoyl-

methyl) phosphorodithioate; diisopropyl phosphorofluoridate (DFP).

Studies in vivo

Flies were treated on the ventral tip of the abdomen with the predetermined 1280 min. LD₅₀ of the organophosphate compound in acetone. These were as follows (μg./g.): DFP, 6.0; TEPP, 5.0; paraoxon, 0.75; malathion, 15; parathion, 1.0; dimethoate, 0.45; DDVP, 1.5; methyl parathion, 1.0. At various periods up to 1280 min. after treatment the surviving flies were decapitated and the cholinesterase activity was determined by a standard manometric technique (Mengle & Casida, 1958) and by the following protective technique: fly heads were homogenized at 0° at a concentration of 1.5 heads/ml. in 0.01M-acetylcholine bromide in buffer (0.025M-NaHCO₃, 0.15M-NaCl, 0.04M-MgCl₂). A portion (2 ml.) of this preparation was added at zero time to Warburg flasks, which were then transferred to the bath at 38°, gassed with CO₂+N₂ (5:95), equilibrated and readings were begun 24 min. from zero time. In a few cases 4 heads/ml. were used, and it was then necessary to tip in an additional amount of acetylcholine (1.6 mg. in 0.4 ml. in the side arm) just before the zero readings.

Studies in vitro

Solutions of organophosphates in dilute (1% or less) ethanol were added to homogenates of fly heads in buffer (as described above, without acetylcholine) at concentrations predetermined to inhibit 95% or more of the cholinesterase activity after incubation for 1 hr. at 38°. The excess of inhibitor in the homogenate was then removed by adding a preparation of rabbit-plasma A-esterase (Aldridge, 1953) and incubating additionally for 1 hr. at 38°. (This A-esterase preparation was made by adding 1 ml. of 0.1 mM-DFP to 10 ml. of heparinized rabbit plasma, incubating for 1 hr. at 38° and storing at 5° until used; it contained no cholinesterase activity even after 24 hr. storage or after addition of oximes.) Experiments showed that this treatment totally eliminated excess of inhibitor.

At 1, 4, 12 and 24 hr. after the end of the inhibition period groups of samples were removed: one pair of samples was assayed for cholinesterase to determine spontaneous recovery. Each sample was assayed in triplicate. To other pairs were added various oximes, and cholinesterase was determined 1 hr. later; this gave the oxime-reactivable cholinesterase. In these experiments magnesium was omitted from the buffer since it inhibits A-esterase (Aldridge, 1953). Also investigated was the recovery *in vitro*, both spontaneous and oxime-induced, of fly-head cholinesterase inhibited *in vivo*. In this study flies were treated as described above, but at the predetermined time of maximum cholinesterase inhibition they were decapitated, the heads were homogenized and the resulting brei was studied for spontaneous and oxime-induced recovery of enzyme activity.

RESULTS AND DISCUSSION

Cholinesterase inhibition and recovery in vivo

The possibility that the previously reported inhibitions *in vivo* (Mengle & Casida, 1958) were high owing to artifacts was examined by comparing the protective with the standard procedure

at the time of maximum cholinesterase inhibition. As Table 1 shows, the protective technique did not give higher estimates of the cholinesterase for the three phosphorothionates studied.

Another approach was used: the possibility of an excess of inhibitor in the heads from treated flies was examined by homogenizing these heads with those from untreated flies. Preliminary experiments showed a linear relationship between enzyme concentration and activity under these conditions. As Table 2 shows, excess of inhibitor was found only at $10 \times LD_{50}$ of DDVP and parathion. At the LD_{50} for DDVP and TEPP and at $10 \times LD_{50}$ for TEPP the activities were higher than expected, suggesting that the heads of the untreated flies contained some form of reactivating factor. A similar conclusion may be drawn from the data of Rasmuson & Holmstedt (1958) for parathion and tabun (*O*-ethyl *NN*-dimethylphosphorocyanamidate) with the housefly.

With this technique, excess of inhibitor was shown by Van Asperen (1957) in houseflies with DDVP: the concentration of inhibitor fell to one-half after 22 hr. Colhoun (1959) found excess of

TEPP for 7 hr. after treatment of American cockroaches. Rasmuson & Holmstedt (1958) found excess of paraoxon in houseflies at 'knock down'.

We conclude that at the LD_{50} phosphorothionates have not shown the homogenization artifact. Phosphates may show artifacts when used at high concentrations. Probably our technique of treatment on the tip of the abdomen and assay of cholinesterase only in the head minimizes the artifact: Van Asperen (1957) found that the head contained far less inhibitor than the body after vapour treatment by DDVP.

The studies on the time course of inhibition *in vivo* (Mengle & Casida, 1958) were extended to three more compounds: DFP, TEPP and paraoxon at the LD_{50} (Fig. 1). All showed similar effects. It was particularly surprising that the cholinesterase of DFP-treated flies recovered so readily. A check with the protective method showed that the severe inhibition and recovery was genuine. When DFP at the LD_{75} was used, recovery in the survivors to only 15–20% of normal was noted in 1280 min. Recovery could have been more extensive later on.

Table 1. *Cholinesterase activity in fly heads at various times after treatment in vivo with malathion, parathion or dimethoate*

Each figure is the mean of triplicate Warburg assays. The LD_{50} dose was given to each fly. Cholinesterase was determined as described in the Materials and Methods section.

Insecticide	Time after treatment (min.)	Heads/ml.	Percentage of pretreatment cholinesterase activity	
			Protective method	Standard method
Malathion	40	1.5	23, 25	26, 32
	40	4.0	16, 21	27, 28
Parathion	160	1.5	8, 15	11, 17
	160	4.0	25, 27	23, 29
Dimethoate	160	4.0	21, 29	22, 24

Table 2. *Examination for excess of inhibitor in heads of treated flies*

Flies were decapitated 1 hr. after treatment with the topical LD_{50} dose or 10 times the LD_{50} dose. Equal numbers of treated and untreated heads were mixed, homogenized by the standard (non-protective) technique at 1.5 heads/ml., and the homogenate was kept for 1 hr. at 38°. Cholinesterase activity was assayed as described in the Materials and Methods section.

Compound	Percentage of normal cholinesterase activity					
	At LD_{50}			At $10 \times LD_{50}$		
	Treated	Treated + untreated (observed)	Treated + untreated (predicted)*	Treated	Treated + untreated (observed)	Treated + untreated (predicted)*
DDVP	6	63	53	5	7	53
TEPP	5	91	53	0	65	50
Parathion	5	55	53	6	33	53
Malathion	26	61	63	15	59	58

* Prediction based on the assumption of no excess of inhibitor in heads of treated flies. If prediction exceeds observation, excess of inhibitor is present. If observation exceeds prediction, reactivation is suggested.

*Cholinesterase recovery in vitro after
inhibition in vitro*

The observation by Van Asperen (1958*b*) that housefly cholinesterase did not recover spontaneously *in vitro* after treatment with DDVP was confirmed and extended to four other inhibitors. Human-plasma cholinesterase was shown to reverse readily, as is well known (Table 3). Our data on plasma show the spontaneous recovery to depend on the alkoxy substituent in the descending order ethyl, methyl, *isopropyl*. Aldridge (1953) showed that the order was methyl, ethyl for rabbit whole-blood cholinesterase.

Fig. 1. Comparison of time mortality and symptomatology curves with fly-brain cholinesterase activity of surviving flies, with three organophosphates. Symptoms observed at predetermined times after treatment were: n, normal; h, hyperactivity; p, paralysis. ●, Percentage of pretreatment brain-cholinesterase activity; ▲, percentage of survivors.

The phenomenon of 'ageing' has been found with both mammalian cholinesterases: true and pseudo-phosphorylated cholinesterase slowly changes into a form which reactivates neither spontaneously nor under the influence of oximes (Hobbiger, 1956; Davies & Green, 1956). To study the possibility that rapid ageing accounted for the failure of spontaneous recovery in fly cholinesterase, the effect of oximes was studied with fly-head cholinesterase inhibited *in vitro* by excess of organophosphate (0.1 mM) subsequently removed by A-esterase.

Fig. 2. Recovery *in vitro* of fly-brain cholinesterase inhibited *in vitro*. The various organophosphate inhibitors were added at 0.1 mM and excess was later removed with an A-esterase preparation. ▲, After 1 hr. with 2 mM-pyridine 2-aldoxime methiodide; ●, no oxime added.

Table 3. *Spontaneous recovery of cholinesterase in vitro in 24 hr.*

Values expressed are percentages of normal (uninhibited) cholinesterase activity observed after 1 hr. incubation with 0.1 mM inhibitor at 38° with subsequent removal of excess of inhibitor and holding at 38° for 24 hr. Excess of inhibitor was removed by adding the A-esterase preparation at the rate of 1 ml. of A-esterase to 9 ml. of the inhibited tissue homogenate. After the holding period cholinesterase activity was determined by the standard method. These values were compared with the cholinesterase activity of uninhibited homogenates treated only with the A-esterase preparation and held for 24 hr. before assay. Before the holding period the cholinesterase activity of the inhibited homogenate was in all cases less than 5% of that found in the uninhibited homogenate.

Origin of enzyme	Inhibitor				
	DFP	TEPP	Paraoxon	Malaoxon	DDVP
	Percentage of uninhibited cholinesterase activity				
Human plasma	2-7	41-46	54-58	19-21	40-46
Fly head	0	1	0	0	0

Fig. 2 shows results with pyridine 2-aldoxime methiodide: similar but far less effective results were found with pyridine 2-aldoxime dodecaiodide. For instance, a portion of one sample of cholinesterase which had been freshly inhibited by DFP could be restored to 94 % of normal by pyridine 2-aldoxime methiodide, but another portion of this sample could be restored only to 6 % of normal by pyridine 2-aldoxime dodecaiodide. With diisotritroacetone monodibutylamine salt, no recovery was obtained. The behaviour with pyridine 2-aldoxime methiodide was as with the mammalian enzyme: the enzyme could be largely reactivated at first, but slowly became less capable of reactivation on standing. 'Ageing' with respect to oximes therefore occurs. The 'ageing' rate depends on alkoxy substituents, being in the descending order methyl, ethyl, isopropyl. The corresponding order in erythrocyte cholinesterase is methyl (= isopropyl), ethyl (Davies & Green, 1956). 'Ageing' of housefly cholinesterase *in vitro* is not an extremely rapid process, and cannot account for the failure of the inhibited enzyme to recover spontaneously.

Comparison of recovery *in vivo* and *in vitro*

We have an interesting paradox: after organophosphate poisoning, cholinesterase *in vivo* recovers more rapidly than does that of the mammal (Davison, 1955). Yet after inhibition *in vitro* housefly cholinesterase shows no spontaneous recovery, in contrast with the mammal.

Two hypotheses are possible: (a) The nature of the inhibited cholinesterase *in vivo* may be different from that inhibited *in vitro*, being more reversible. This was tested by homogenizing treated flies at the time of maximum cholinesterase depression.

Fig. 3. Recovery *in vitro* of fly-brain cholinesterase inhibited *in vivo*. The organophosphates were applied at the LD₅₀ dose and the flies were decapitated at the pre-determined time of maximum cholinesterase inhibition. Time from treatment to decapitation for parathion was 160 min.; for malathion the time was 40 min. ▲, After 1 hr. with 2 mm-pyridine 2-aldoxime methiodide; ●, no oxime added.

This inhibited cholinesterase behaved precisely as did enzyme inhibited *in vitro*: it did not recover at all spontaneously; it was readily reactivated by oximes at first; and it 'aged' to a less reactivatable form (Fig. 3). This hypothesis is therefore not tenable.

(b) There is in intact flies a reactivating factor, which is partially destroyed by homogenization. Such a factor has been shown in mammalian sera by Neubert, Schaefer & Kewitz (1958). There are two pieces of evidence for the occurrence of this factor in flies. First, as shown in Table 2, homogenates of mixed heads from treated and untreated flies may give cholinesterase activity in excess of that predicted on the assumption that the inhibited cholinesterase is not affected by the process. Secondly, fly-head cholinesterase was treated *in vitro* with various inhibitors to obtain total inhibition. Then after various times fresh preparations of untreated fly (whole insects or heads only) were added. As Table 4 shows, these preparations were quite effective in reactivating the inhibited cholinesterase. As with oximes, the capacity of the inhibited enzyme to be reactivated decreased with time; by 12 hr. little reactivation was possible. The reactivating capacity of the untreated fly preparations was rapidly lost on standing; after 30 min. in the refrigerator it was completely lost.

Experiments showed that the cholinesterase of flies killed by organophosphate poisoning was almost totally inhibited, and did not recover upon standing at room temperature, e.g. with the LD₅₀ of parathion it was 6 % of normal, and 15 hr. later was 2 %; with malathion the figures were 5 and 0 %, with TEPP 4 and 0 %. In earlier work (Mengle & Casida, 1958) with Dipterex (dimethyl 2:2:2-trichloro-1-hydroxyethylphosphonate) and Thimet (OO-diethyl S-ethylthiomethyl phosphorodithioate) cholinesterase in dead flies was not so substantially inhibited at sampling and showed a little spontaneous reactivation, e.g. from 15 to 27 % in 15 hr. with Thimet. For malathion O'Brien (1956) found that 24 hr. after the LD₅₀ of malathion the total population (dead and alive) had 50 % of normal cholinesterase; taken with Mengle & Casida's observation that under similar conditions the live flies had 100 % of their normal cholinesterase, this suggests that at 24 hr. the cholinesterase of the dead flies was totally inhibited; since inhibition was maximal at 20 min., the cholinesterase of the dead flies cannot have recovered at all.

We can conclude that there exists in living flies a factor which can reactivate inhibited cholinesterase. This factor is very labile after homogenization and is presumably of reduced effectiveness in flies killed by organophosphates, since their cholinesterase fails to recover.

Table 4. *Determination of reactivation of inhibited fly-brain cholinesterase by homogenates of untreated heads and whole flies*

Data are expressed as $\mu\text{l. of CO}_2$ evolved/20 min. in excess of predicted value, which is the mean of that for inhibited homogenate and that for untreated homogenate. The predicted value for each concentration of fly homogenate is shown in the bottom line. Treated homogenate contained 1.5 fly heads/ml. of solution. Inhibition was with 0.1 mM inhibitor for 1 hr. at 38°. After inhibition the excess of inhibitor was removed by adding A-esterase preparation and incubating at 38°. At periods of 1, 12 and 24 hr. after adding A-esterase the treated homogenates were added at the rate of 1 ml. to 1 ml. of homogenate of untreated fly heads or whole flies, the concentration of which is given in the Table. The inhibited and fresh tissue homogenates were mixed at 38° for 1 hr., after which cholinesterase determinations were made upon 1.8 ml. portions of the mixture.

Treatment <i>in vitro</i> (hr. after inhibition)	Untreated homogenate			
	1.5 heads/ml.	1.5 flies/ml.	0.15 head/ml.	0.15 fly/ml.
DFP				
1	46	51	31	27
12	5	23	11	10
24	0	5	0	0
TEPP				
1	34	26	24	28
12	0	5	4	7
24	0	1	0	1
Paroxon				
1	25	17	16	12
12	1	5	5	8
24	0	4	0	0
Malaoxon				
1	46	54	11	9
12	13	8	5	0
24	0	1	0	0
Predicted values ...	72	81	11	13

If endogenous reactivating factors assist in the reversal of cholinesterase inhibition *in vivo*, administered oximes might possibly hasten the process and thus reduce the toxicity of organophosphates. Parathion, methyl parathion, paroxon, malathion, DFP and TEPP were applied to the abdomen at the LD_{50} , $2 \times \text{LD}_{50}$ and $8 \times \text{LD}_{50}$ doses. The oximes injected simultaneously were pyridine 2-aldoxime methiodide, pyridine 2-aldoxime dodecaiodide or diisonitrosoacetone at 50 $\mu\text{g./g.}$ and 1000 $\mu\text{g./g.}$ No effect on the mortality at 24 hr. was found except for DFP, whose LD_{50} was increased 1.5-fold by both levels of pyridine 2-aldoxime methiodide. Symptoms were delayed for about 1 hr. with parathion, malathion and DFP at both concentrations of pyridine 2-aldoxime methiodide. No substantial changes were induced by pyridine 2-aldoxime methiodide (500 $\mu\text{g./g.}$) in the pattern of cholinesterase inhibition *in vivo* after poisoning with malathion and parathion. This was equally true if the oxime was injected 2 hr. before the organophosphate, simultaneously with it, or near the pre-established period of maximal inhibition.

Winteringham (1957) and Winteringham, Harrison, McKay and Weatherley (1957), using DFP and pyridine 2-aldoxime methiodide (PAM), have reported alleviation of the symptoms of paralysis

and respiratory stimulation, but no effect on LD_{50} . Our different finding for LD_{50} is not seriously in conflict with theirs, since a 1.5-fold increase in LD_{50} is of little importance.

Winteringham (1957) concluded: 'It seems, therefore, that PAM successfully reversed the anti-cholinesterase action in the insect as indicated by its recovery from paralysis but failed to protect the insect from the slower lethal effect.' He therefore assumes that paralysis is due to cholinesterase inhibition, but that death is not. Our data suggest the contrary: since the oximes have no marked effect *in vivo* either upon cholinesterase or mortality, cholinesterase inhibition must still be considered a reasonable cause of death. Pyridine 2-aldoxime alleviates paralysis, which may therefore be caused by inhibition of some system other than cholinesterase. At 'knock down' Van Asperen (1957) showed that aliesterase was severely inhibited; D. Stegwee (personal communication) finds that aliesterase is located principally outside the nerve cord of the housefly and would therefore be accessible to ionized compounds such as pyridine 2-aldoxime. It is therefore possible that paralysis and respiratory stimulation are caused by inhibition of aliesterase (or related enzymes) but that this inhibition is not important in causing death.

The above-described effects of oximes *in vivo* are smaller than those found for mammals (Kewitz, Wilson & Nachmansohn, 1956; Hobbiger, 1957). The discrepancy is probably attributable to ionization in pyridine 2-aldoxime methiodide and diisonitrosoacetone; ionized compounds fail to penetrate to the nervous system of insects (Twarog & Roeder, 1957; Winton, Metcalf & Fukuto, 1958; O'Brien & Fisher, 1958). Diisonitrosoacetone was found to have pK_a 10.5, and therefore is 99.9% ionized at pH 7.0. Although pyridine 2-aldoxime dodecaiodide is much more soluble in lipids than is the methiodide (Wilson, 1958), this property did not facilitate its action in the fly, in fact the use of dodecaiodide did not show the small alleviation found with the methiodide.

SUMMARY

1. With the six organophosphates tested, the brain cholinesterase of poisoned houseflies is rapidly inhibited. In the survivors, this cholinesterase recovers almost to normal within 1 day; in dead flies, it remains almost fully inhibited. These results are not due to homogenization artifacts.

2. The cholinesterase of the housefly differs from both true and pseudo-cholinesterase of mammals in that spontaneous recovery does not occur after inhibition by organophosphates *in vitro*. It can be reactivated by oximes. Its capacity to be reactivated slowly disappears at a rate depending upon the alkoxy substituents of the organophosphates: methyl > ethyl > isopropyl. This order differs from that found with mammalian cholinesterase.

3. Housefly-brain cholinesterase inhibited *in vivo* behaves *in vitro* precisely as that inhibited *in vitro*.

4. The discrepancy between ready recovery of cholinesterase *in vivo* and failure to recover *in vitro* is attributed to a labile reactivating factor present in living flies and fresh homogenates of untreated flies.

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