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DDT:
EFFECTS ON SOME MEMBRANE-RELATED FUNCTIONS

by

DAVID ALEXANDER JACKSON, B.Sc.

A thesis submitted to the Faculty of
Graduate Studies in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Carleton University

Ottawa, Ontario

April, 1976

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ABSTRACT

The adenosine triphosphate hydrolase (ATPase) activity of membrane fractions isolated from brain tissue was investigated as a possible site of toxic action of DDT in salmonid fish. When added to the in vitro assay medium, DDT and several other organochlorines inhibited both the $(\text{Na}^+ + \text{K}^+)$ - and Mg^{2+} -activated ATPases, but their in vitro effects did not correspond well with their generally observed in vivo toxicities.

Kinetic studies of the Mg^{2+} -ATPase indicated that a single step, of activation energy 15.4 kcal/mole, was rate-limiting from 5 to 40°C. The basis of organochlorine inhibition appeared to be a dose-dependent reduction in the ability of the Mg^{2+} -ATPase to lower the activation energy of ATP hydrolysis. This effect, combined with an apparent increase in the frequency factor of the reaction, gave rise to biphasic dose-response curves, with low doses of organochlorine stimulating Mg^{2+} -ATPase activity and larger doses inhibiting it. The inhibition caused by a constant dose of organochlorine was an inverse function of temperature.

Substrate competition studies showed that DDT was an allosteric inhibitor of the Mg^{2+} -ATPase. However, it is suggested that this inhibition is unlikely to give rise to the observed symptoms of acute in vivo DDT intoxication.

Also investigated was the ability of DDT to antagonize the valinomycin-induced increase in K^+ -conductance of artificial lecithin-decane bilayer membranes. Although DDT did antagonize valinomycin when both compounds were added to the aqueous phase adjacent to the membrane, no such antagonism was observed when they were added directly to the membrane-forming lipid solution, nor did DDT cause any significant change in membrane specific capacitance. It is concluded that valinomycin is inappropriate as a probe of possible DDT-induced changes in membrane structure and ionic conductance.

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ABBREVIATIONS AND DEFINITIONS

DDT, DDD etc.: p,p'-isomers, unless otherwise specified
(see Table 1, p. 34)

OC: organochlorine

LD₅₀, ED₅₀, I₅₀: lethal dose 50, effective dose 50, and
inhibitory dose (concentration) 50:
dose required for 50% response

CNS: central nervous system

ϵ_{Na} , ϵ_K , ϵ_m : sodium ion, potassium ion and membrane
specific conductances (conductance G per
unit area of membrane A_m)

BLM: bilayer lipid membrane

Mg^{2+} -ATPase: ouabain-insensitive ATPase requiring Mg^{2+}
or Mn^{2+} for activity

$(Na^+ + K^+)$ -ATPase: ouabain-sensitive, requiring Na^+ and
 K^+ as well as Mg^{2+} for activity

PMR: proton magnetic resonance

E_{340}^{mM} : millimolar extinction coefficient at 340 nm

A_{340} : absorption (optical density) at 340 nm

P_i : inorganic phosphate (orthophosphate)

%I: percent inhibition

TBM: trout brain mitochondria(1)

OM: oligomycin

VM: valinomycin

$[E]_0$: total enzyme concentration, here defined as TBM
protein content (mg) of ATPase assay medium

v: velocity of reaction ($\mu\text{mole} \cdot \text{min}^{-1}$); subscripts i and
o denote presence and absence, respectively, of inhib-
itor; $v/[E]_0$ thus has units of specific activity,
 $\mu\text{mole} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$

E_a : activation energy

(log) A: "frequency factor" of Arrhenius relationship

kcal: kilocalories

C_m : specific capacitance (capacitance per unit area
membrane)

μF , nF: 10^{-6} , 10^{-9} Farad

[X]: the concentration of X

INTRODUCTION

DDT - a contraction of its earlier chemical designation, dichlorodiphenyltrichloroethane - was a spectacularly successful insecticide when its use first became widespread in the mid-1940's. Its attractiveness was due to the combination of several desirable properties in a single molecule:

- (a) it was lethal in very low doses to most insects
- (b) its low volatility, high chemical stability and virtual insolubility in water made it extremely persistent
- (c) it was relatively innocuous to man and domesticated animals

and (d) it was inexpensive and easily manufactured on a large scale.

The world-wide use of DDT for the control of disease vectors and agricultural pests has undoubtedly saved millions of human lives and improved the existence of millions more, but its miraculous nature was short-lived. The first sign of trouble was the appearance of resistant strains of insects one or two years after exposure to the pesticide. Doses were increased, but this acted as a selective force in the evolution of yet more resistant strains, and the increased doses began to have obvious side-effects. An early example of such side-effects in Canada was the decimation of the Atlantic salmon in New Brunswick rivers following forest spraying for spruce budworm in the 1950's (Kerswill and Elson, 1955). These effects continue today: the eggs of birds

nesting around the Great Lakes (especially Lake Ontario) are among the most contaminated with DDT and other organochlorines of those found anywhere in the world (Gilbertson, 1975). Yet its manufacture and use continued to increase. It has been estimated that in the three decades following 1944 over 2.8 million metric tons of DDT have been produced and distributed as an intentional environmental contaminant, and that about 5.4 million kilograms were probably in the world biota in the late 1960's. Even with a drastic decrease in its use in the 1970's, the extraordinary persistence of the compound ensures that the levels of DDT in the atmosphere and mixed layers of the oceans will continue to be high - greater than one part in 10^{12} - until the year 2000 (Woodwell et al., 1971).

This one-part-per-trillion concentration may seem insignificant, but its effects are magnified by bioaccumulation. Trout, for instance, can directly accumulate a whole-body concentration of DDT more than 8500-fold higher than its concentration in the surrounding water, although the food chain appears to be the major source of contamination (Macek and Korn, 1970); and Metcalf (1972) has measured a bioaccumulation factor for DDT and metabolites in mosquitofish* which was an order of magnitude larger still.

If DDT and other organochlorine (OC) insecticides were toxic only to the target insect, such bioaccumulation

*Gambusia affinis, a small freshwater fish of the topminnow family.

would be of little consequence. Unhappily, such is not the case: insecticidal OCs are extremely toxic to fish, the most susceptible of the vertebrate classes (O'Brien, 1967, p.302) - and to other lower vertebrates; they are also toxic to most non-target insects, many of which are directly or indirectly beneficial to man through pollination of flowering plants, predation and parasitism of other insects, and contributions to general ecosystem diversity and stability. Finally, even though much less acutely toxic to birds and mammals, OCs may induce in these vertebrates insidious chronic changes in physiology, behaviour and reproduction, as is evidenced by the striking decline in predatory bird populations due to nonviable eggs. Consequently an environmental dose which is sublethal for an individual may eventually be fatal for the population.

The recent evidence that DDT and other OCs accumulate in, and affect the photosynthetic processes of, marine phytoplankton (Wurster, 1968; Menzel et al., 1970; Bowes & Gee, 1971; Cox, 1972) is even more alarming, since these organisms form the base of the marine food web and produce most of the world's oxygen.

It is therefore disconcerting to note the continued large scale use of DDT and other highly residual insecticides in the developing nations, where economic expediency precludes environmental considerations. Even more disturbing is the fact that such an enormous quantity of DDT could have been released into the environment with

little or no knowledge of its long-term effects or of its mechanism of acute toxicity. In spite of the large quantity and variety of DDT-related data which has been published in the last three decades, there is still no consensus for a unifying hypothesis of toxic action. Largely responsible for this lack of consensus are the inconsistencies and contradictions in the DDT literature. One recurrent approach to gaining some information about the site of action of DDT has been to modify the DDT molecule and observe the effects on toxicity. Such structure-activity correlations are useful both for characterizing the DDT receptor and for the design of new insecticides, but these correlations may be very difficult to interpret depending on the complexity of the system studied. For clarity of discussion, these studies may be considered to fall into four broad categories, in order of decreasing complexity: whole-organism responses, neurophysiological effects, biochemical lesions, and organochlorine chemistry.

1. Whole-organism responses.

In investigations on whole organisms, the effectiveness of a toxicant may be statistically quantified in two basic types of study - lethal and sublethal. A commonly used measure of acute lethality is the LD_{50} (lethal dose, 50), the dose of toxicant required to kill 50% of a treated population within a specified time period. In sublethal studies, a similar statistic - the ED_{50} (effective dose, 50) - is used to express the efficacy of a toxicant in eliciting some non-lethal change in 50% of a treated population.

In general, any alterations in the structure of the DDT molecule tend to reduce acute in vivo toxicity. Examples of such alterations include

1. elimination of ethane chlorines, e.g. DDE (XX)*, DDMU (XXV), and DDNU (XXVI).
2. reductive dechlorination of the ethane moiety, e.g. DDD (VII), DDMS (XVII) and DDNS (XVIII).
3. oxidation to more hydrophilic compounds, e.g. dicofol (XIV), DDOH (XIX), DDA (IX) and DBP (XXIII).

All of the above-mentioned derivatives have been detected as transformation products of DDT metabolism in various microorganisms, plants and animals (Brooks, 1974b, p. 72). In vertebrates, liver microsomal enzymes are largely responsible for these conversions, the trend in the biodegradative pathways being successive substitution or elimination of chlorines, and oxidation to more polar compounds which may then be excreted.

It is important, however, to note that any correlation of toxicity with such structural changes requires extremely well-controlled test conditions since there are so many possible barriers and diversions which could affect the movement of toxicant toward its site of action in the organism, and which are unrelated to the toxicant's intrinsic toxicity (defined in Fig. 1). The result may be anomalous structure-activity correlations, such as the finding that o,p'-DDT (X), a "non-toxic" isomer generally

*See Table 1 (p.34) for structural formulae of DDT analogues.

Fig. 1 Pharmacodynamics of a toxicant. The vertical arrow represents movement of a toxic compound toward its site of action within an animal. Horizontal bars indicate possible barriers or rate-limiting steps in this movement, while horizontal arrows denote possible losses of toxicant en route. The barriers and diversions need not all be significant, nor need they all occur in the order shown. Modified from Brooks (1974b, p. 64).

comprising about 20% of technical grade DDT*, can be quite toxic to certain DDT-resistant strains of insects - apparently because it was less susceptible to enzymic inactivation by "DDT-ase" (Kimura et al., 1965).

Although the whole-organism symptoms which result from an acute lethal dose of DDT - hyperexcitability, tremors, and convulsions eventually leading to paralysis and death - suggest destabilization of nerves, the immediate cause of death is often obscure. In mammals, it is known that ventricular fibrillation and consequent brain anoxia, due to interrupted blood flow can be the immediate cause of death, but death can also be the end result of gradual depression and coma following a series of convulsions (Brooks, 1974b, p. 131). However, brain anoxia due to interrupted blood flow is obviously not the cause of death in insects which do not rely on blood flow for oxygen distribution.

The large increase in respiration and depletion of energy stores which accompany the hyperexcitability symptoms in insects prompted an early hypothesis that DDT had an uncoupler-like action, but it was later shown that these changes in respiration and energy consumption could be eliminated by preventing hyperactivity with general anaesthesia (Metcalf, 1955).

*Another minor (4%) component of technical grade DDT is p,p' - DDD, which is insecticidal. The remaining 5% consists of a variety of chlorinated and sulphonated benzene derivatives (Brooks, 1974a, p. 55) of unknown toxicity.

2. Neurophysiological symptoms

In organisms possessing complex nervous systems, death following the administration of a lethal dose of some toxicant is usually due either directly or indirectly to effects on the nervous system. This simply reflects this system's intolerance of even transitory changes in its integrity. Thus pesticides which have been shown to interfere with nervous function via some biochemical reaction with a particular nerve component (target) need not be specific for the nervous system: the latter may simply be the most sensitive of several tissues which are so attacked.

The neurotoxic symptoms of acute DDT poisoning are coincident with the whole-organism symptoms, and are indicative of nerve destabilization. DDT-treated nerves, whether isolated or in situ, tend to become hypersensitive to external stimuli, and show a "multiplication effect" whereby single impulses arriving at a DDT-poisoned region give rise to multiple high-frequency discharges (volleys). This axonal - as opposed to synaptic - impairment of nerve function is characteristic of very few neurotropic drugs, the best known of which is veratrine (O'Brien, 1967, p.112; Brooks, 1974b, p.131).

When isolated tissues are treated with DDT, and one excludes those effects which are given equally by DDT and nontoxic analogues, only nervous tissue is sensitive to very low doses (O'Brien, 1967, p.111). Yet even isolated nerves can present barriers to movement of DDT:

the isolated cockroach central nervous system (CNS), for example, is relatively insensitive to DDT perfusion unless it is first desheathed (Smyth, 1960). The intact preparation is, however, a good deal less complex than the whole organism, and several of the initial barriers and diversions outlined in Fig. 1 can obviously be eliminated.

As a result of residual barriers such as a protective sheath, nerves show a range of sensitivities to DDT. Among the peripheral nerves of the cockroach, motor nerves are much less sensitive than sensory; and among sensory nerves, the cercal nerve is less sensitive than the crural (Roeder and Weiant, 1948; Eaton and Sternburg, 1967).

However, repetitive discharges from sensory nerves alone do not account for whole-organism toxic symptoms, since the former can occur in the absence of the latter; CNS high-frequency discharges, conversely, do seem to coincide with the gross toxic symptoms (Narahashi, 1971). One particularly striking way of demonstrating the dependence of gross toxic symptoms on CNS events is to make use of the unusual negative temperature coefficient of DDT toxicity: at appropriate doses of DDT, insects can appear quite normal at 35°C, but be thrown into violent symptoms of DDT poisoning at 15°C. A return to higher temperatures alleviates the symptoms, and the cycle can be repeated many times. The fact that repetitive firing in the insect CNS coincides with the appearance of these

toxic symptoms at low temperature (and their disappearance at high temperature) was shown by Eaton and Sternburg (1964). They found that the sensory crural nerve, in contrast, continued to fire repetitively at both temperatures, and in fact showed an apparent positive temperature coefficient, but Narahashi (1971) has suggested that the latter finding was a result of the DDT dose being well above sensory-nerve threshold at both high and low temperatures.

This negative temperature coefficient has also been observed in fish. Cope (1965) reported that DDT was more toxic to rainbow trout and bluegill sunfish at 13°C than at 18.5 and 23°C, and similar results were later obtained for methoxychlor (Macek et al., 1969). Bridges et al. (1963) found that the inverse temperature effects levelled off below 7°C and above 29.5°C. And Anderson (1968) noted an inverse correlation between temperature and electrical activity of the lateral line (peripheral sensory) nerve of DDT-poisoned brook trout.

The basis for the negative temperature coefficient is not obvious, since temperature changes can have such diverse effects. For instance, a rise in temperature may increase the rates of OC absorption, diffusion and transport, which would tend to accelerate intoxication; but concomitant increases in the rates of volatilization, desorption, inactivation and excretion may keep pace with, or even surpass, the intoxication processes.

Sophisticated electrophysiological and pharma-

cological techniques have been used to study the effects of DDT on the sodium and potassium conductances g_{Na} and g_K associated with action potentials in isolated axons. DDT has been found to slow the kinetics of the on-process of g_{Na} , the off-process of g_{Na} ("sodium inactivation"), and the on-process of g_K . By far the most drastic change is in sodium inactivation: its slowing, combined with the lesser slowing of g_K increase, causes a prolongation of the falling phase of the action potential - a prolonged "negative after-potential" - during which time the nerve is hyperexcitable and subject to repetitive firing (Narahashi, 1971).

However, a prolonged negative after-potential cannot be the sole cause of hyperexcitability, since a sustained depolarizing pulse applied to an isolated axon does not produce the prolonged repetitive firing which is characteristic of DDT (Yamasaki and Narahashi, 1959). Also required is a reduction in the sodium inactivation (i.e. an increase in the proportion of sodium channels which are free from inactivation at the steady-state potential), and/or an increase in the time constant of sodium inactivation: either or both of these changes would delay accommodation to a prolonged depolarizing stimulus and would allow repetitive firing.

Investigations into the molecular mechanisms underlying DDT neurotoxicity are currently limited by lack of knowledge of the ion gating mechanisms themselves. Attempts have therefore been made to study interactions of OCs with ion conductance mechanisms in a simpler,

better characterized membrane system - the artificial bilayer (or "black") lipid membrane (BLM).

Hilton and O'Brien (1971) found that the K^+ conductance induced by valinomycin, a K^+ -selective ionophorous antibiotic, in a lecithin-decane BLM was qualitatively antagonized by DDT, but not by two cyclo-diene insecticides. In a subsequent study, however, Hilton et al. (1973) found poor correlation between antagonism of valinomycin action and the in vivo neurotoxicities of DDT and thirteen toxic and nontoxic analogues. A further study on the effects of DDT and twelve other OCs on the physical properties of a phospholipid monolayer (Hilton and O'Brien, 1973) failed to explain the action of these compounds on either nerves or BLMs.

3. Biochemical lesions

Several attempts have also been made to isolate a specific enzyme, enzyme system, or other subcellular component which is sensitive to DDT both in vivo and in vitro - analogous to the in vitro acetylcholinesterase assay which is used for testing organophosphate and carbamate insecticides. This would not only eliminate most of the variables shown in Fig. 1, thus allowing one to study intrinsic toxicity and molecular mechanisms, but it would also provide a simple bioassay system for testing the toxicity of new OC pesticides.

Johnston (1951) found DDT and several analogues to have little effect on succinic dehydrogenase of rat tissue, and although most of the OCs inhibited

succinoxidase activity, they did so only at very high concentrations (10-100mM). Similarly, Sacklin et al. (1955) found that cytochrome oxidase activity of housefly mitochondria was inhibited only at very high doses of DDT. Acetylcholinesterase also seems to have been abandoned as a possible site of action of DDT (Colhoun, 1959). Ela et al. (1970) showed that DDT administered in vivo did not inhibit any of the enzymes of glycolysis, pentose cycle or Krebs cycle of the American cockroach, but had an uncoupler-like action causing reduced ATP and increased orthophosphate (P_i) concentrations. Working with isolated mouse liver mitochondria, Settlemire et al. (1974) reached a similar conclusion for heptachlor, and mentioned (without data) similar results obtained with DDT, dieldrin and aldrin; but their "uncoupling" was unusual in that respiration was stimulated by OC only in the presence of ADP.

Apparently contradictory results were reported by Waddill and Keeley (1971), who could detect no changes in respiratory rate or ADP ratio of mitochondria isolated from DDT-poisoned roaches, whereas both parameters were decreased by in vitro DDT treatment of mitochondria isolated from untreated roaches. Unaccountably, their radiolabelling (^{14}C -DDT) data seems to indicate that both in vivo- and in vitro-treated mitochondria contained similar quantities of DDT. Finally, neither inhibition of respiratory functions nor the level of DDT achieved in the in vivo-treated mitochondria correlated with appearance

of toxic symptoms.

In vitro inhibition of respiration and/or oxidative phosphorylation has also been reported by Sacklin et al. (1955), Gonda et al. (1959) and Gregg et al. (1964) for insect mitochondria, and by Parker (1960) for rat liver mitochondria. Particularly interesting were the results of Gonda et al. dealing with the effects of DDT and nine analogues on the in vitro ATP- $^{32}\text{P}_i$ exchange reaction catalyzed by mosquito mitochondria; they found that all of the OCs tested (whether toxic or nontoxic in vivo) inhibited the reaction when their concentration in the mitochondria reached the same level (their LC_{50} values, i.e. their concentrations in the aqueous medium at 50% inhibition, were - as expected - markedly different). The authors concluded that either the effect of DDT on phosphorylation has no physiological significance, or that unknown factors selectively impede access of the nontoxic analogues to the in situ mitochondria.

The effects of DDT on oxidative phosphorylation have been further investigated, indirectly, by the Cutkomp-Koch group (Koch, 1969/70, 1969; Koch et al., 1971; Cutkomp et al., 1971; Desai et al., 1974), who have reported in vitro sensitivity of the mitochondrial Mg^{2+} -activated adenosine 5'-triphosphate hydrolase (Mg^{2+} -ATPase) activity to DDT and toxic analogues.

Mg^{2+} -ATPase is bound to the inner mitochondrial membrane, requires Mg^{2+} or Mn^{2+} for activity, and is thought to catalyze the terminal transphosphorylation

reaction in oxidative phosphorylation:

The ATPase reaction ($X = H_2O$, and $X \sim P = P_i$) is normally latent in intact mitochondria and is considered to be an artifact arising from mitochondrial damage during isolation (Penefsky, 1974). Inhibition of reaction (1) in vivo and consequent reduction in oxidative phosphorylation has been proposed by Cutkomp et al. (1971a, b; 1972) and Koch et al. (1971) as the basis of DDT neurotoxicity.

Inhibition of the plasma membrane ($Na^+ + K^+$)-ATPase, generally accepted as the Na^+/K^+ pump, has also been proposed as the site of DDT toxicity (Janicki and Kinter, 1971a, b; Davis and Wedemeyer, 1971; Campbell et al., 1974; Leadem et al., 1974). Its inhibition in osmoregulatory tissue could cause severe electrolyte imbalance, while in nerve tissue it could interfere with maintenance of the ionic gradients which are the basis of nerve excitability.

Yet another ATPase, neurostenin (reviewed by Berl et al., 1973), has been proposed as a possible site of action of DDT (Doherty and Matsumura, 1975). This enzyme is bound to nerve cell membranes, is activated by Ca^{2+} and Mg^{2+} , and demonstrates properties which are characteristic of contractile proteins such as actomyosin. It is hypothesized to be involved in exocytosis, which in nerves takes the form of neurosecretion and transmitter release at nerve endings; it has also been implicated in the membrane conductance changes associated with nerve excitation (Bowler and Duncan, 1966).

Finally, the discovery that the blood of DDT-poisoned cockroaches contains a toxin (not DDT) that causes paralysis and death when injected into untreated insects (Sternburg and Kearns, 1952), and the later discoveries of a variety of stressful treatments which induce production of this and other toxins (O'Brien, 1967, p. 116), have been used as evidence that DDT causes death indirectly, through autointoxication. However, these toxins have been poorly characterized, and are in any case secondary to the actual DDT lesion - presumably axolemmal destabilization.

4. Organochlorine chemistry

Some of the physical properties of DDT have already been mentioned - low volatility, virtual insolubility in water and high chemical stability. As might be expected, the chemical stability of DDT is dependent upon environmental factors, ranging from an indefinite shelf life to a half-life of a few hours in reducing environments such as sewer sludge (Zoro et al., 1974). In other environments, DDT has a half-life between these two extremes e.g. a few years in soil (Menzie, 1972) and a few weeks in birds (Brooks, 1974a, p. 74).

Although DDT must obviously enter into chemical reactions, there is no evidence that such reactions are involved in the poisoning process; the consensus seems to be that DDT and related OCs exert their toxic effect via relatively weak, non-covalent interactions. Evidence for

the formation of a weak complex between DDT and components of cockroach nerve was presented by O'Brien and Matsumura (1964; 1966a, b) who monitored changes in UV absorption and fluorescence spectra and binding of ^{14}C -DDT to components of cockroach nerve cord. They hypothesized that DDT and some unspecified DDT receptor formed a charge-transfer complex.

The significance of these results has, however, been placed in doubt by subsequent studies. Hatanaka et al. (1967) failed to find evidence for the formation of specific complexes in homogenates of nerve and other tissues from rat and cockroach. Also Wilson et al. (1971) observed spectral shifts of aqueous DDT suspensions - in the absence of biological material - which were strikingly similar to those cited by O'Brien and Matsumura as evidence of complex formation: Wilson et al. concluded that the shifts were due to self-association of DDT. Using proton magnetic resonance (PMR) spectra, Wilson (1972) determined that the trichloromethyl group of DDT caused increased polarity of the C-H bond of the benzylic proton, and considerably decreased the electron density of the aromatic rings. She detected two corresponding types of complexes, one involving the benzhydryl trichloromethyl grouping of DDT with polar complexing agents, and the other interaction of the DDT aromatic π - electron system with π electrons in complexing agents. In another PMR study, Wilson and Wilson (1972) showed that DDT could complex with a variety of agents, and concluded

that the phosphate and ester groups of phospholipids and aromatic substituents of proteins were the most likely important binding sites of DDT in biological tissue. Direct interaction of a phospholipid (lecithin) with DDT and several toxic and nontoxic compounds has been reported in other PMR studies (Tinsley et al., 1971; Haque et al., 1974), Haque et al. finding a correlation between insecticidal activity and the presence of an acidic proton in the region of the ethane moiety of DDT analogues. The applicability of these PMR studies to in vivo mechanisms is not clear, however, since complex formation was only observed in solutions of a low molecular weight organic solvent (e.g. carbon tetrachloride); it is not known if these interactions also occur in biological tissue.

Several attempts have been made to characterize the DDT receptor by extrapolating back from the structure of insecticidal analogues. Structure-activity studies carried out before 1950 were reviewed by Brown (1951), who concluded that "any attempt to obtain any complete quantitative correlation with any single property ends in failure", and that symmetry was the only feature common to all active DDT analogues. More recent studies have shown that symmetry is also not a prerequisite of toxicity (Metcalf et al., 1972).

Mullins (1955) suggested that the DDT receptor was a lattice of cylindrical lipoprotein molecules between which pass the ions responsible for action potentials. He

hypothesized that analogues of appropriate size and shape interfere with ion passage by plugging or distorting the membrane pores.

DDT toxicity has also been related to the ability of the DDT phenyl rings to assume a coplanar configuration (Rogers et al., 1953), and to rotate freely (Riemschneider, 1958), which may be a prerequisite for attaining coplanarity. The significance of this observation is not known, although it may be speculated that if an acidic benzylic hydrogen on the DDT molecule (Haque et al., 1974) is involved in formation of the hypothetical DDT-receptor charge-transfer complex, the complex would be stabilized by delocalization of benzylic carbon charge in the ring π -orbital system: such delocalization requires coplanarity of separate orbitals. Furthermore, if very polar intermediates were involved in the formation of the complex, the ability of the DDT rings and ethane chlorines to delocalize benzylic carbon charge might also lower the free energy of activation, thus increasing the rate of formation of the complex. However, this mechanism cannot explain the toxicity of halogen-free DDT analogues such as dianisyl neopentane (Rogers et al., 1953) or the completely methylated DDT isostere 3,3-bis(p-methylphenyl)-2,2-dimethylpropane (Metcalf et al., 1972), which lack acidic benzylic carbons and are much less polar than DDT. Nor can it explain the synergized toxicity of α -fluoro-DDT and other α -fluoro analogues (Fahmy et al., 1973) which have no

benzylic hydrogen: one would not expect these compounds, having an α -group so different from hydrogen in size and electronegativity, to interact with the same receptor as DDT.

In another structure-activity study, Holan (1969) tested the insecticidal activity of a series of cyclopropane derivatives of DDT. In a modification of the Mullins (1955) hypothesis, Holan proposed that DDT-type molecules distribute themselves at the protein-lipid interface of nerve membranes, the DDT base (phenyl rings) forming a complex with overlying protein and the apex (trichloromethyl group) fitting into a channel or pore: DDT would thus act as a "molecular wedge". A later report by Holan and Spurling (1974) indicated that there is a good correlation between apex charge density and insecticidal activity for DDT analogues of similar shape and size, but data was available for only five analogues.

Fahmy et al. (1973) used multiple regression analysis and a series of empirical substituent constants to examine the relationship between the structure of a series of DDT analogues and their insecticidal activity. They concluded that the steric substituent constant was the single most important parameter, and visualized the DDT receptor as a flexible cavity which could accommodate DDT analogues of varying dimensions (even asymmetrical ones) as long as the overall volume of the molecule did not deviate substantially from that of DDT. Metcalf and

Fukuto (1968) had reached a similar conclusion after testing the insecticidal activity of over one hundred DDT analogues.

This thesis report attempts to determine a biochemical-biophysical basis for the intrinsic toxicity (Fig. 1) of DDT and related compounds.

A preliminary series of experiments (total ATPases) was undertaken using (a) an ATPase system isolated from a tissue (brain) and species (trout and salmon) which are known to be very sensitive to OCs, to confirm some recently-published data on the in vitro inhibition of ATPases by OCs (b) several DDT analogues, to attempt to compare their in vitro effects with their generally observed in vivo toxicities and (c) a surface-active agent in the ATPase assay medium to prevent OC precipitation and make the in vitro dose-response data more quantitative.

A subsequent series of ATPase experiments

- (a) focussed on the mitochondrial Mg^{2+} -activated ATPase, since this had been shown by Koch et al. (1971) and Cutkomp et al. (1971a, b; 1972) to be the most sensitive of the ATPases to in vitro inhibition by OCs

and (b) extended the initial studies to include effects of OCs on enzyme kinetics, an area not yet investigated by other laboratories.

Another possible site of action of DDT - nerve membrane ionic conductance - was investigated indirectly by using the artificial lipid bilayer membrane (BLM) system reported by Hilton et al. (1971, 1973). Although they found that DDT antagonized the valinomycin (VM)-induced rise in BLM conductance g_m , their results remain equivocal as they added both VM and DDT to the aqueous phase; under these conditions diffusion of VM into the membrane should have been the rate-limiting step in g_m (Hladky, 1973), and Hilton et al. may have detected interaction of the two lipophilic compounds in the aqueous phase rather than a DDT-membrane interaction. The final section of this thesis therefore reports the effect of DDT on the VM-mediated g_m of membranes formed from lipid solution already containing VM and/or DDT. This modification was expected not only to eliminate interaction of drugs outside the membrane, but also to improve reproducibility and possibly to allow extension of the study into an assay for membrane structural transitions (Stark et al., 1972) which may be involved in DDT toxicity.

II. MATERIALS AND METHODS

1. ATPases, preliminary experiments: total ($\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$)-ATPase*.

- (i) Preparation of ATPase.

Atlantic salmon (Salmo salar) parr and brook trout (Salvelinus fontinalis) were obtained from the Fisheries Research Board Biological Station, St. Andrews, New Brunswick. The average weights of the parr and trout were 25 and 190 grams respectively; both species were maintained in running fresh water at $12 \pm 3^\circ\text{C}$, and fed commercial fish pellets.

Fish were stunned with a sharp blow to the supraoccipital region, then decapitated and the whole brain excised. The brains of several parr or trout were pooled and a crude mitochondrial/nerve ending (membrane) fraction was isolated according to Koch (1969); (see Fig. 2). Following osmotic shock and resedimentation at 20,000 g x 10 min., the loosely pelleted membrane material was rehomogenized, frozen and stored in liquid nitrogen in droplet form.

- (ii) Assay of ATPase.

Just prior to assay, aliquots of mitochondrial preparation were thawed and dispersed in an ATPase incubation medium (100 mM NaCl, 20 mM KCl, 6mM MgCl_2 and 30 mM Tris-HCl, pH 7.6) to give a 100 μg protein/ml stock suspension. Protein content was determined using the method of Bailey (1962), with bovine serum albumin

*See list of abbreviations and definitions, p.xi.

Fig. 2 Preparation of a crude mitochondrial fraction from fish brain.

fraction V as standard.

All reactions were carried out in 10-ml disposable glass culture tubes. To each tube was added 0.2 ml (i.e. 20 μ g membrane protein) of the enzyme stock suspension, followed by 1.6 ml ATPase incubation medium which, where applicable, contained the surfactant Corexit 7664. The ATPase reaction was started by adding 0.2 ml 5mM Na_2ATP , and terminated at various times thereafter by addition of 3 ml ice-cold 4% trichloroacetic acid (TCA). The reaction product (orthophosphate) was then determined by the colourimetric method of Lowry and Lopez (1946); standards contained various concentrations of orthophosphate in place of ATP. Also run through each experiment were various controls, including ATP and/or enzyme-free media, and reaction media stopped at zero time.

(iii) Treatment of ATPase with OC

After (or immediately prior to) dilution of the mitochondrial suspension with ATPase assay medium, a 25 μ l aliquot of OC in 95% ethanol was added; specific aqueous concentrations were achieved by using appropriate concentrations of OC in ethanol. Since considerable precipitation of OC occurred at higher concentrations, the reaction medium was clarified for the colourimetric orthophosphate assay by the addition of an appropriate quantity of Corexit 7664 with the reaction-terminating trichloroacetic acid.

II.2. Mg^{2+} -ATPase*

Only brook trout (1-2 years old) were used in this series. Fish were obtained from the provincial fish hatchery at Petawawa, Ontario and were maintained in running dechlorinated tapwater ($9 \pm 4^{\circ}C$) and fed commercial trout pellets.

(i) Preparation of ATPase

Whole brains were excised over ice and homogenized in a Ten-Broeck ground glass homogenizer (ten strokes) at $0-1^{\circ}C$. The homogenizing medium was 320 mM sucrose* buffered with 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-NaOH, pH 7.4 at $25^{\circ}C$; resuspensions were also made with this medium. Double glass-distilled water was used for this and all other aqueous solutions.

The supernatant remaining after centrifuging at 900 g for 10 minutes was spun at 13,000 g for 20 minutes. The resulting pellet was resuspended by expelling through a 26-gauge hypodermic needle, tip pressed tightly to the wall of the centrifuge tube, spun once more at 900 g for 10 minutes, and the supernatant again pelleted at 13,000 g for 20 minutes. The final mitochondrial pellet was resuspended through the hypodermic needle, then frozen and stored in liquid nitrogen in droplet form.

(ii) Assay of ATPase activity

Reactions were carried out in standard optical glass spectrophotometer cells, maintained at constant

*See list of abbreviations and definitions, p. xi.

temperature in a Lauda K-2/RD constant temperature ($\pm 0.01 - 0.02^{\circ}\text{C}$) circulator; during spectrophotometer readings, cell temperature was maintained by circulating water from the K-2/RD through a water-jacketed cell holder in the spectrophotometer.

Each cell contained a miniature Teflon-coated magnetic stirbar for mixing during addition of reagents. Before each experiment, the cells were rinsed sequentially with water, hot ethanol, hot concentrated nitric acid, hot saturated tribasic sodium phosphate, and several changes of hot distilled water.

The enzymic hydrolysis of ATP was coupled to an ATP-regenerating system, phosphoenol pyruvate (PEP) plus pyruvate kinase (PK); this was in turn coupled to a spectrophotometric indicator system, β -NADH plus lactic dehydrogenase (LDH). The sequence is shown in Figure 3 (Pullman et al., 1960).

Oxidation of NADH, at steady-state stoichiometrically coupled to ATP hydrolysis, was monitored at 340 nm in a Beckman DU-2 spectrophotometer, with attached Coleman Hitachi Perkin-Elmer model 165 chart recorder for a continuous permanent record of absorbance changes.

The 3 ml Mg^{2+} -ATPase assay medium contained the following components (some variable, depending on the particular experiment), in 50 mM HEPES buffer (Sigma), pH 7.5:

NaCl, 25-100 mM (Fisher)

KCl, 5-20 mM (Fisher), $[\text{Na}^+]/[\text{K}^+] = 5$

Fig. 3 Coupled reaction sequence in the enzymic ATPase assay. The ATPase is coupled to an ATP-regenerating system, phosphoenol pyruvate (PEP) plus pyruvic kinase (PK), which is in turn coupled to a spectrophotometric indicator system, NADH plus lactic dehydrogenase (LDH). The concentration of PK, LDH, PEP and NADH were maintained at levels which caused ATPase to be rate-limiting.

PEP, 1-2 mM (Sigma, potassium salt)
PK, 20-25 units, and LDH, 24 units (Sigma, lyophilized salt-free powder, from rabbit muscle)
ouabain, 0.5 mM (Sigma, octahydrate)
 MgSO_4 , equimolar with Na_2ATP (Fisher)
 Na_2ATP or MgATP (Sigma, from equine muscle)
BSA, 0.02% (Sigma, bovine serum albumin fraction V)
 β -NADH, up to 0.5 mM (Sigma grade III)
trout brain mitochondria (TBM), up to 10 μg protein

Organic reagents and ancillary enzymes were stored at -20°C over P_2O_5 in air tight containers.

A stock solution of each reagent was made in HEPES buffer and stored up to 3 days at $0-1^\circ\text{C}$. The PK, LDH and ATP stock solutions were filtered (0.22 micron Millipore) before use to remove particulate matter.

Albumin was included as an ATPase-stabilizing agent: it absorbs inhibitory fatty acids (Nicholls et al., 1974; Sharp et al., 1974), and has some surface-active properties which aid the dispersal of lipophilic material in aqueous media.

The concentrations of PK, LDH, PEP and NADH were set empirically such that release of ADP was the rate-limiting step in the coupled reaction sequence (Fig. 3). Initial NADH concentration was normally set at 0.5 mM, and NADH was replenished when required during the reaction by addition of solid reagent. The signal-noise ratio of the recorded ΔT trace set the upper limit of NADH

concentration at about 0.5 mM, which gave less than 0.1 % transmittance at 340 nm.

Protein content of the mitochondrial suspension was determined colourimetrically according to Bailey (1962), using BSA fraction V as the standard, and correcting for the presence of HEPES. Lipophilic light-scattering material was extracted with chloroform just before reading absorbance.

Prior to addition of TBM, ATP added to the otherwise complete assay medium gave rise to a NADH oxidation transient, as the minor (1-2%) ADP content of the substrate was phosphorylated. The size and time course of this transient was a convenient indicator of the integrity of the ancillary (PK + LDH) reactions.

The ATPase reaction was then started by adding TBM suspension. Only after the 340 nm absorbance (A_{340}) had reached a constant rate of change (i.e. the coupled reactions had reached steady-state) were inhibitors added or reaction conditions otherwise changed; da_{340}/dt was converted to rate of NADH oxidation (and hence rate of ATP hydrolysis) using the NADH extinction coefficient $E_{340}^{mM} = 6.1$.

Rates of ATP hydrolysis were corrected for the background da_{340}/dt , arising from changes in absorbance not associated with TBM-catalysed ATP hydrolysis (e.g. spontaneous PEP hydrolysis, NAD reduction by TBM respiratory enzymes), and from changes in the scattering coefficient (e.g. TBM accretion, organochlorine

precipitation). Total background was calculated by algebraic summation (n equations in n unknowns) of the rates of a series of reaction media lacking one or more components, each component being assumed to contribute to the total background independently of the others (i.e. assuming no synergism).

For kinetic studies, reaction (i.e. water bath and spectrophotometer cell jacket) temperature was varied from the initial 25°C , the NADH oxidation rate recorded after the new steady-state had been reached, and the temperature then returned to 25°C to check for irreversible changes in rate which might have occurred during the temperature displacement.

(iii) Inhibitors

I	NaN_3 :	sodium azide
II	oligomycin:	15% oligomycin A, 85% oligomycin B
III	DCCD:	N,N'-dicyclohexylcarbodiimide
IV	atractyloside:	K^+ salt
V	dicumarol:	bis-hydroxycoumarin
VI	ouabain:	strophanthidin G octahydrate
VII	DDT:	1,1,1,-trichloro-2,2-bis(p-chlorophenyl) ethane
VIII	DDD:	1,1-dichloro-2,2-bis(p-chlorophenyl) ethane
IX	DDA:	bis (p-chlorophenyl) acetic acid
X	o,p'-DDT:	1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl) ethane
XI	o,p'-DDD:	1,1-dichloro-2(o-chlorophenyl)-2-(p-chlorophenyl) ethane

XII	Cl-DDT:	1,1,1,2-tetrachloro-2,2-bis(p-chlorophenyl) ethane
XIII	F ₃ -DDT:	1,1,1-trifluoro-2,2-bis(p-chlorophenyl) ethane
XIV	dicofol	1,1,1-trichloro-2-hydroxy-2,2-bis(p-chlorophenyl) ethane
XV	Perthane:	1,1-dichloro-2,2-bis(p-ethylphenyl) ethane
XVI	methoxychlor:	1,1,1-trichloro-2,2-bis(p-methoxyphenyl) ethane
XVII	DDE:	1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene
XVIII	o,p'-DDE:	1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl) ethylene
XIX	DBP:	4,4'-dichlorobenzophenone
XX	methoxychlor-DDE	1,1-dichloro-2,2-bis(p-methoxyphenyl) ethylene
XXI	dieldrin (HEOD):	1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo,exo-5,8-dimethanonaphthalene

Compound I was a purified Fisher reagent; II-VI were obtained from Sigma; VII, VIII and XVII were from Aldrich (99⁺%); XXI was donated by Dr. J.M. Anderson, Fisheries Research Board Biological Station, St. Andrews, New Brunswick; the remaining DDT derivatives were chromatographically pure samples, generously provided by Dr. H.V. Morley, Department of Agricultural Central Experimental Farm, Ottawa, Ontario.

Structural formulae of DDT analogues are shown in Table 1.

Table 1. DDT derivatives.
Compounds XVII-XIX, XXV and XXVI were not
used, but are discussed in the text.

DDT DERIVATIVES

(a) Saturated benzylic carbon:

		U	V	W	X	Y	Z
VII	DDT	Cl	H	H	CCl ₃	H	Cl
VIII	DDD	Cl	H	H	CHCl ₂	H	Cl
IX	DDA	Cl	H	H	COOH	H	Cl
X	o,p'-DDT	H	Cl	H	CCl ₃	H	Cl
XI	o,p'-DDD	H	Cl	H	CHCl ₂	H	Cl
XII	Cl-DDT	Cl	H	Cl	CCl ₃	H	Cl
XIII	F ₃ -DDT	Cl	H	H	CF ₃	H	Cl
XIV	dicofol (Kelthane)	Cl	H	OH	CCl ₃	H	Cl
XV	Perthane	CH ₂ CH ₃	H	H	CHCl ₂	H	CH ₂ CH ₃
XVI	methoxychlor	OCH ₃	H	H	CCl ₃	H	OCH ₃
XVII	DDMS	Cl	H	H	CH ₂ Cl	H	Cl
XVIII	DDNS	Cl	H	H	CH ₃	H	Cl
XIX	DDOH	Cl	H	H	CH ₂ OH	H	Cl

(b) Unsaturated benzylic carbon:

		U	V	X	Y	Z
XX	DDE	Cl	H	CCl ₂	H	Cl
XXII	o,p'-DDE	H	Cl	CCl ₂	H	Cl
XXIII	DBP	Cl	H	O	H	Cl
XXIV	methoxychlor-DDE	OCH ₃	H	CCl ₂	H	OCH ₃
XXV	DDMU	Cl	H	CHCl	H	Cl
XXVI	DDNU	Cl	H	CH ₂	H	Cl

Organochlorines, DCCD and oligomycin were dissolved in absolute ethanol and stored in the dark in glass vials with Teflon-lined caps. Up to 3 μ l of these stock solutions was added per 3-ml reaction medium, via polyethylene tubing (Clay-Adams Intramedic PE 10) affixed to the needle of a Hamilton 5 or 10 μ l syringe. A fresh length of tubing was used for each injection, and was filled such that (i) there was no air in either the tubing or syringe (to ensure precise delivery), and (ii) inhibitor solution did not enter the syringe (to avoid contamination from previous injections). Inhibitors were injected as quickly as possible under the surface and into the vortex of the rapidly stirred reaction medium; controls received the same volume of absolute ethanol.

(iv) Other ATPase reaction conditions

To ensure that TBM-catalyzed hydrolysis of ATP was the rate-limiting step in the coupled sequence of Fig. 3 the concentration of TBM protein in the reaction mixture was varied above and below the usual 2 μ g/ml, and the resulting rates of NADH oxidation plotted as a function of protein concentration.

The integrity of the ancillary reactions in the presence of the various inhibitors was monitored by pulsing with ADP substrate. The initial rates of change of the resulting NADH oxidation transients (neglecting the brief acceleration phase during mixing) were measured up to four hours after addition of inhibitor and compared with inhibitor-free controls.

Reactions were also carried out at several substrate (MgATP) concentrations in the presence and absence of inhibitors to determine the type of inhibition - competitive, noncompetitive or un(anti)competitive - as indicated by Lineweaver-Burk plots. ATP concentration was determined spectrophotometrically, using the 259 nm millimolar extinction coefficient, $E_{259}^{\text{mM}} = 15.4$ (Merck).

To determine the pH spectrum of ATPase activity, pH was varied during the reaction by adding aliquots of HCl or NH_4OH , with controls receiving similar aliquots of neutral NH_4Cl . Reaction replicates were tested at various times during the reaction, using a Corning combination electrode No. 476115.

II.3. Artificial membrane

(i) Membrane apparatus (Fig. 4A)

The membrane apparatus consisted of two open-top vessels, one of Teflon (4 ml capacity) inside one of glass (40 ml capacity). When both vessels were filled with electrolyte solution, the only contact between inner and outer solutions was via a small circular aperture (usually $1-2 \text{ mm}^2$) punched in a Teflon disc. The disc was mounted on a threaded insert in the wall of the inner vessel: this design permitted interchanging of discs with apertures of various diameters. For long-term experiments, two discs were mounted in tandem; this allowed the maintenance of a reservoir of bulk lipid solution between the discs which acted as a mechanical buffer and greatly increased BLM stability (Fig. 4B).

Fig. 4. Artificial membrane apparatus

A. Thin section through assembled apparatus

1. electrolyte reservoir and electrode housing
2. threaded plunger
3. electrode (calomel or Ag/AgCl)
4. electrolyte
5. bridge
6. outer chamber (glass)
7. inner chamber (Teflon)
8. stirbar
9. sidearm aperture assembly

B. Sidearm assembly, cross section

(i) Exploded view

1. threaded hole in wall of inner chamber
2. sidearm
3. aperture discs (in tandem)
4. end cap

(ii) Details of tandem apertures

5. bulk lipid solution
6. Plateau-Gibbs border
7. bilayer membrane

C. Electronics schematic

1. square pulse generator
2. series calibration resistance, switch-selectable in decade steps to 10^{10} ohms
3. equivalent circuit of artificial membrane
4. electrometer amplifier
5. trace-storing oscilloscope

Both glass and Teflon chambers were cleaned by soaking in concentrated KOH, followed by alternate washes in ethanol and distilled water. Teflon components were also washed and stored in n-heptane.

The electrolyte solutions on either side of the membrane were not in direct contact with the recording calomel electrodes: the latter were inserted into separate reservoirs of electrolyte which were in turn in electrical contact, via salt bridges, with the two electrolyte solutions. Threaded plungers mounted on the reservoirs allowed limited transfer of reservoir electrolyte to the corresponding membrane chamber; thus any transmembrane hydrostatic pressure difference (indicated by membrane bowing) could be neutralized.

A d.c. motor-driven magnetic stirrer was used to drive a small Teflon-coated stirbar inside the inner chamber.

(ii) Electrical apparatus (Fig. 4C)

A Medistor microvolt calibrator (Medistor model Cl-A, Medistor Instrument Co., Seattle, Wash.) was used as an electrical square-pulse source. In series with this source of potential was a resistance unit consisting of high-megohm Victoreen resistors (up to 10^{10} ohm, Victoreen Instrument Co., Cleveland, Ohio), mounted in electrical isolation from one another on ceramic rotary switches, all enclosed in a fully shielded, dustproof chassis. Two sets of switch-selectable resistances were arranged in series with the membrane. A square-wave calibration pulse applied across this circuit then

gave rise to a series of voltage drops which were recorded with a high-impedance (greater than 10^{14} ohms, shunted by 10 pf) electrometer amplifier (PAR 134, Princeton Applied Research Corp., Princeton, N.J.).

Capacitance-modified square-pulse transients were displayed on a trace-storing oscilloscope (Tektronix 564B, Tektronix Inc., Portland, Oregon).

The calomel electrodes were occasionally replaced with chlorided silver wire; pairs were matched for minimum potential difference (less than 1mV).

(iii) Formation of membranes

Phospholipids (vegetable lecithins, Eastman) or pure phosphatidyl choline (dipalmitoyl, Fluka) were "dissolved" in a straight-chain alkane (usually n-decane, Eastman). In most experiments an antioxidant, BHT (butylated hydroxytoluene: 2,6-ditert-butyl-p-cresol, Sigma), was included to retard phospholipid peroxidation (Van Zutphen and Cornwell, 1973).

Immediately prior to immersion in this electrolyte solution (90 mM KCl buffered to pH 7 with 10 mM K_2HPO_4 - KH_2PO_4), membrane apertures were preconditioned with a drop of the membrane-forming lipid solution. Another drop of the latter was added to the immersed aperture to form a thick film of lipid solution; alternatively, an air bubble was moved slowly across the preconditioned aperture, giving rise to a thin, coloured lamella.

A collimated light beam, the membrane and a

stereoscopic microscope were arranged so that light was reflected from the membrane into one of the microscope eye pieces; the latter was equipped with a reticle for measuring membrane diameter.

(iv) Electrical measurements

Membrane conductance was calculated from the steady-state proportion of a calibrated step pulse (applied across the membrane and a series calibration resistance) which dropped across the membrane resistance. Where significant, the electrode/bridge resistance was also included in the calculations; the output resistance of the microvolt calibrator was insignificant.

Unless otherwise indicated, the transmembrane potential difference was kept below 30 mV so that the membrane current-voltage relationship would remain linear (Andreoli et al., 1967).

Membrane capacitance was calculated from the time constant* of the membrane potential transient which followed step pulse onset or offset. True membrane capacitance was derived from the time constant ($R \times C$) either by plotting C as a function of BLM area, or by subtracting the stray (system) capacitance from C ; stray capacitance was measured with the membrane aperture plugged with silicone grease. The R term in the time constant was the parallel combination of membrane and calibration resistances.

*The system was regarded as a parallel-plate capacitor in which the electrolyte solutions on either side of the membrane are the conducting plates, and the membrane is dielectric (Läuger et al., 1967)

(v) Membrane modifiers

For some experiments, valinomycin (A grade, Calbiochem) and p,p'-DDT (99⁺ % Aldrich) were dissolved to appropriate concentrations in absolute ethanol. Microliter volumes of these solutions were injected into the stirred inner chamber electrolyte.

Alternatively, DDT and/or valinomycin were dissolved in the membrane-forming lipid solution. Various valinomycin concentrations were obtained by serial dilution of a 10^{-3} molal stock solution; the molality of the stock solution was based on a valinomycin molecular weight of 1111, assuming 100% purity.

III RESULTS

1. ATPases, preliminary experiments

The results of these preliminary experiments, dealing with effects of OCs on the total ATPase activity of the mitochondrial/nerve-ending fraction of fish brain, are shown in Figs. 5 (salmon) and 6 (trout).

Since inhibitions occurred over such a wide range of OC concentrations, a logarithmic scale is used for the abscissa. The ordinate is expressed as % of control activity since neither ethanol nor 0.2% Corexit caused any detectable change in trout brain ATPase activity. The higher Corexit concentration (5% v/v) used in the assay of the salmon brain ATPase did however, result in a 10-15% reduction in activity: controls for the combined (OC + Corexit) treatments were therefore of lower activity than the controls for OC treatment alone.

Figures 5 and 6 (crosses) show that the OCs had qualitatively similar effects on the two enzyme preparations, many giving sigmoid dose response curves. The only striking difference between salmon and trout preparations was that dieldrin partially inhibited the former (Fig. 5F) but had no detectable effect on the latter (Fig. 6F).

In the absence of surfactant, most of the OCs caused marked decreases in the ATPase activity, but the OCs differed from one another in the concentration ranges which produced inhibition. The OC concentration causing 50% inhibition (I_{50}), for instance, was about 0.6 mM for

Fig. 5 Inhibition of the total ($\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$)-ATPase activity of a salmon brain mitochondrial fraction by organochlorines in the presence (circles) or absence (crosses) of the nonionic surfactant Corexit 7664. ATPase activity is expressed as percent of organochlorine-free control activity. Points represent the average of 5 to 10 experimental values; standard deviations were 1-4%. Since a precipitate became evident at about 30 μM for most of the organochlorines, the term "Molar" is not strictly applicable as a measure of organochlorine dose.

Fig. 6 Inhibition of the total ($\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$)-ATPase activity of a trout brain mitochondrial fraction by organochlorines in the presence (circles) or absence (crosses) of Corexit. Points are averaged from at least 6 trials; standard deviations were under 5 %.

DDA (Fig. 6G), but was only 10 μM for DDT (Fig. 6A).

Attempts at obtaining linear dose-response curves by the use of probit transformation were not particularly successful (Figs. 7 and 8), although there is some improvement in linearity at low OC concentrations. At higher OC doses there appears to be a saturation effect which was more abrupt for the salmon enzyme (Fig. 7). Note the steep slope of the curves for dicofol and DDA, and the position of the methoxychlor curves.

With the exception of the less hydrophobic compounds (DDA and dicofol), immediate precipitation of OC followed injection of the ethanolic OC solution, the light-scattering precipitate being visually and/or spectrophotometrically evident at OC concentrations above 30 μM . The presence of the nonionic surfactant Corexit 7664 in the ATPase assay medium reduced or eliminated not only the precipitation of OCs but also their inhibitory action (Figs. 5 and 6, circles).

III.2 Mg^{2+} -ATPase

(i) Ancillary reactions

Using the millimolar extinction coefficients ($E_{340}^{\text{mM}} = 6.1$ for NADH, $E_{259}^{\text{mM}} = 15.4$ for ADP), and the anhydrous molecular weight of pyruvate (desiccated over P_2O_5), at least 98% stoichiometry was calculated for the NADH-oxidation following addition of ADP or pyruvate to the ATPase assay medium.

Fig. 9 (circles) shows the fraction of the NADH (and hence ADP pulse) still to be oxidized, plotted as a

Fig. 7 Percent inhibition (%I) of salmon brain ATPases, no Corexit. Symbols represent DDT (x), DDE (□), DDD (▲), Cl-DDT (○), DDA (●), methoxychlor (●), dicofol (+), and dieldrin (▼). Percent inhibition values obtained from Fig. 5 have been plotted on a probit scale. Note the atypical slopes of the dose-response curves for dicofol and DDA.

Fig. 8. Percent inhibition (%I) of trout brain ATPases (probit transformation of the data in Fig. 6). Symbols are as defined in Fig. 7. Data for dieldrin and DDA are not included since their %I values fall outside the range of the probit function.

Fig. 9 Time course of the ADP-pulse-induced NADH oxidation transient catalyzed by the ancillary enzymes PK and LDH shown in Fig. 3. The 3-ml assay medium contained 25 units PK, 24 units LDH, 30 mM Na^+ , 6 mM K^+ , 0.5 mM ouabain, 0.02 % w/v bovine serum albumen, and (initially) 2mM PEP and 0.5 mM NADH. The reaction temperature was 25°C.

At zero time, 0.3 μmole ADP was added, and the ensuing NADH oxidation transient was plotted as the apparent fraction f (circles) of the substrate ADP remaining, versus time. The value of f was calculated using equation 1, and was assumed to be synonymous with the change in NADH content and A_{340} . Also plotted is the negative logarithm of f (crosses); the best-fit curve to these points gives an estimate of the first-order rate constant $k = 0.013/\text{sec}$.

function of time after addition of ADP. The concentration of ADP at a particular time was assumed to be a linear inverse function of A_{340} at that time. The fraction f of the ADP pulse remaining at time t is given by

$$f = \frac{[\text{ADP}]_t}{[\text{ADP}]_i} = \frac{A_t - A_f}{A_i - A_f} \quad (2)$$

where $[\text{ADP}]_i$ and A_i are the initial values of ADP concentration and optical absorbance (A_{340}) at time zero, $[\text{ADP}]_t$ and A_t are the corresponding values at time t , and A_f is the final value of A_{340} . The value of $[\text{ADP}]_f$ is effectively zero (Appendix 1).

Also plotted in Fig. 9 (crosses) is negative log f versus time. The slope of the linear portion is $-k_{\text{anc}}/2.303$, where k_{anc} is the apparently first-order rate constant of the ancillary reactions considered as a unit.

The lack of effect of various ATPase inhibitors on this gross rate constant k_{anc} of the ancillary reactions is shown in Table 2. Values of k_{anc} were determined from the slope of plots similar to Fig. 9, where the inhibitor-treated ancillary system was pulsed with ADP at various times after addition of inhibitor.

That the ancillary reactions were never rate-limiting during ATPase assay is illustrated in Fig. 10: rate of NADH oxidation was a direct linear function of trout brain mitochondrial (TBM) protein concentration even up to 5 μg TBM protein per ml (over twice the usual concentration).

Table 2 Lack of inhibition of ancillary reactions by ATPase inhibitors, as indicated by the values of the apparent first-order rate constant k_{app} (sec.⁻¹).

Values of k_{app} were determined from the linear portion of semilog plots similar to Fig. 9. At zero time, just before addition of inhibitors, reaction media were pulsed with 0.3 mole ADP to ascertain initial uniformity of activity. A similar ADP pulse was given at three hours.

Pyruvate (rather than ADP) pulses also gave rise to apparently first-order NADH oxidation transients whose k values (0.022-0.026) were similarly unaffected by these inhibitors.

Other reaction conditions were as described in Fig. 9.

k_{anc} (sec⁻¹)

time (min.)	inhibitor				
	none	3 μ l ethanol	1 mM NaN ₃	1.5 μ g OM	10 μ M DCCD 100 μ M DDT
0	0.013	0.014	0.013	0.014	0.013
180	0.009	0.010	0.013	0.014	0.011

Fig. 10 Rate-limiting step in the ATPase assay sequence (Fig. 3), shown by plotting the steady-state, background-corrected rate of change of absorbance at 340 nm as a function of TBM protein content of the assay medium. Reaction conditions were as described in Fig. 9 except for the variable concentration of TBM preparation and the presence of 2.5 mM MgATP in place of ADP.

The slope of the curve, $5.8 \times 10^{-3} \text{ min}^{-1} \cdot \text{g}^{-1} \cdot \text{ml}$, corresponds to 0.95 μmole NADH oxidized per minute per mg TBM protein (= the specific activity of the MgATPase at 25°C and 2.5 mM MgATP.)

(ii) Background changes in absorbance

Algebraic summation of the rates of change of absorbance dA_{340}/dt , estimated from the slope of tangents to the A_{340} -time curve) of a series of incomplete reaction media, yielded a composite background rate (Fig. 11, open circles) which was an inverse function of time. The single incomplete medium which most closely approximated this calculated background rate was one lacking PEP. (open triangles). Fig. 11 also shows the dependence of this PEP-free control rate on the concentration of TBM protein: the open squares indicate the rate of a PEP-free control which contained twice as much TBM protein as the other two. However, when inverse rate values were plotted after normalization with respect to TBM content, both PEP-free controls (closed triangles and squares) closely approximated the calculated background (closed circles).

Thus from each experimental reading of dA_{340}/dt taken during an ATPase assay, a simultaneous reading taken from a PEP-free control was subtracted from the experimental to give the rate due to Mg^{2+} -ATPase alone. Controls received the same treatment (inhibitors, temperature, etc.) as experimentals.

(iii) The ATPase reaction

Fig. 12 shows the gross rate of NADH oxidation as a function of time after initiation of the ATPase reaction, that is after addition of TBM preparation.

Fig. 11 Background absorbance changes, expressed as (open symbols) apparent rate of NADH oxidation, following addition of TBM to the otherwise complete ATPase assay medium.

Open circles show the time course of a composite background, calculated by algebraic summation of the rates of individual reactions lacking one or more key components (PEP, NADH etc.). Open squares and triangles show the background changes for assay media lacking only PEP, and the effect of doubling the TBM content from the normal 7 μg (open circles and triangles) to 14 μg (open squares).

Closed symbols show the inverse of these three background rates when the latter are normalized with respect to TBM content.

Fig. 12 Apparent rate of NADH oxidation (i.e. ATP hydrolysis), as a function of time after start of the ATPase reaction by addition of TBM.

Circles show the rate in the presence of 0.5 mM ouabain, which inhibits the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ but not the $\text{Mg}^{2+}\text{-ATPase}$. Squares show the ouabain-free rate. Open symbols indicate the presence of a low concentration of monovalent salts (30 mM Na^+ , 6 mM K^+) in the assay medium, while closed symbols indicate high salt concentration (100 mM Na^+ , 20 mM K^+). Other reaction conditions were as described in Fig. 10, with 10.2 μg TBM protein per 3 ml assay medium.

The total ($\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$) ATPase activity (squares) was initially quite high, but dropped non-linearly with time: the decline was quicker in total ATPase assay medium (100 mM NaCl, 20 mM KCl: closed squares) than in Mg^{2+} -ATPase assay medium (20 mM NaCl, 6 mM KCl: open squares).

Addition of ouabain to the total ATPase assay medium eliminated both the ($\text{Na}^+ + \text{K}^+$) - activated portion and also the abrupt decline in activity (closed circles), whereas in low-salt medium, the Mg^{2+} -ATPase activity actually increased during the first three hours (open circles). All subsequent assays of Mg^{2+} -ATPase were done in this low-salt medium in the presence of 0.5 mM ouabain.

Since Mg^{2+} -ATPase activity was unaffected by changes in pH between 7.1 and 7.8, no attempt was made to compensate for the temperature regression, $-0.014/^{\circ}\text{C}$ (Good et al., 1966), of the HEPES buffer.

(iv) Standard inhibitors

Figs. 13, 15 show dose-response curves for inhibition of the TBM enzyme by three inhibitors of mitochondrial ATPase: maximal inhibition was about 90% for all three. Fig. 16 shows the time course of these inhibitions: when present in great excess, the three inhibitors caused very similar rates of decline in ATPase activity.

Neither the uncoupler dicoumarol nor the energy-transfer inhibitor atractyloside had any detectable effect on the Mg^{2+} -ATPase activity.

Fig. 13 Percent inhibition (%I) of Mg^{2+} -ATPase as a function of oligomycin (OM) dose. Reaction conditions were as described in Fig. 9, with 2.5 mM MgATP replacing ADP, and 2.3 μ g TBM protein per ml medium.

The molar concentration of OM was based on a weighted average of the molecular weights of oligomycins A and B, 425 and 397 respectively (Lardy et al., 1965).

Fig. 14 Percent inhibition of Mg^{2+} -ATPase as a function of DCCD dose. Reaction conditions were as described in Fig. 13.

Fig. 15 Percent inhibition of Mg^{2+} -ATPase as a function of sodium azide concentration. Reaction conditions were as described in Fig. 13.

Fig. 16. Time course of inhibition of Mg^{2+} -ATPase by 1 mM azide, 1.25 μ M oligomycin (OM), and 10 μ M dicyclohexylcarbodiimide (DCCD). Inhibitors were added to the steady-state ATPase reaction at zero time. Plotted against time after addition of inhibitor is $\log f_s$, the logarithm of the fraction of inhibitor-sensitive activity remaining. Reaction conditions were as described in Fig. 13.

(v) Sensitivity of Mg^{2+} -ATPase to organochlorines

Table 3 shows the effects of various DDT analogs on the activity of one of the earlier TBM preparations, assayed at high salt concentration. This preparation was more sensitive to OCs and less sensitive to oligomycin, DCCD and azide than were later preparations.

The OCs in Table 3 are arranged (from left to right) in approximate order of decreasing effectiveness as inhibitors of the enzyme. Values for inhibition at $10^{\circ}C$ are expressed as percent inhibition of the $10^{\circ}C$ control activity, while those for 25° are compared to the 25° control activity. The figures in parentheses indicate the percent inhibition which would be expected if both members of a pair of inhibitors were acting independently on the same enzyme (Woolfolk and Stadtman, 1964); for instance, if DDT and azide inhibit independently, one would expect azide to inhibit 80% of the DDT-insensitive activity, to give a combined inhibition of $71.5 + 0.8 \cdot (100 - 71.5) = 94.3\%$, a figure very close to the observed 92%.

Table 4 shows similar data for a later TBM preparation, assayed in low-salt medium, which was less sensitive to OCs and more sensitive to oligomycin, DCCD and azide than was the earlier preparation reported in Table 3.

With OC concentration held constant, the degree of inhibition of Mg^{2+} -ATPase was found to vary inversely with

Table 3 Sensitivity of Mg^{2+} -ATPase to inhibitors, preliminary data.

These data were obtained from one of the earlier TBM preparations assayed in the presence of 100 mM NaCl and 20 mM KCl. Numbers are % inhibition, obtained by comparing the activity of inhibited reactions with the spontaneously decreasing activity of inhibitor-free controls (Fig. 12, closed circles). Because the inhibition was superimposed on this continuous spontaneous decrease in activity, these figures should be considered qualitative.

Inhibitor concentrations in the reaction medium were 10 μ M for the OCs and DCCD, 1 mM for azide and 0.5 μ g/ml for oligomycin (OM).

Numbers in parentheses indicate the cumulative inhibition one would expect of two inhibitors which act simultaneously on an enzyme at two distinct non-interacting sites. Thus if oligomycin and azide were independent inhibitors, their combined inhibition should be $80 + 80(100-80) = 96\%$ (Woolfolk and Stadtman, 1964).

secondary treatment			
25°C	10°C	azide, 25°C	
80.5	80.5	93 (96)	dicofol
80	88.5	87 (87.5)	o,p'-DDT
71.5	79	92 (94.5)	DDT
68.5	82	90 (93.5)	Cl-DDT
66	76	87 (93)	o,p'-DDD
64	77.5	89.5 (93)	DDE
63.5	74	89.5 (92.5)	DDD
61	74.5	91 (92)	Perthane
53	71.5	96.5 (90.5)	F ₃ -DDT
52	69.5	91.5 (90.5)	o,p'-DDE
14.5	48.5	85 (83)	methoxychlor-DDE
-20	49	84 (80)	methoxychlor
-1.5	-1.5	81 (80)	DBP
-1.5	-	82 (80)	DDA
80	80	79.5 (96)	oligomycin
80	80	-	azide
80.5	79	89 (96)	DCCD

Table 4 Sensitivity of Mg^{2+} -ATPase to inhibitors. These data were obtained from a later TBM preparation assayed in the presence of 30 mM Na^+ and 6 mM K^+ . Numbers are percent inhibition caused by addition of inhibitor after steady-state activity (Fig. 12, open circles) had been attained. Other conditions and the significance of the numbers in parentheses are as described in Table 3.

primary inhibitor

75 μ M DDT 75 μ M DDE 100 μ M Cl-DDT 1 mM NaN_3 1.5 μ g OM⁺ 1 μ M DCCD

none

secondary
inhibitor

1 mM
 NaN_3

46.7 43.8 43.7 94 92 90

97.7 97.9 98.1 - 94 95
(96.8) (96.6) (96.6) (99.5) (99.4)

2 3

OF/DE

the TBM protein concentration* $[E]_0$ (Fig. 17); OC dose is therefore expressed in the following data as micromole OC per milligram TBM protein, rather than OC molarity.

Dose-response curves for four representative DDT analogues are depicted in figures 18 to 21. These curves resemble those obtained for the total ATPase, except:

- (a) The Mg^{2+} -ATPase curves (apart from methoxychlor, Fig. 21, which once again was found to be ineffective) drop more abruptly in the inhibitory range of OC concentrations, resembling the earlier curve for dicofol (Fig. 6H). For this reason linear rather than log scale was used for the abscissae of Mg^{2+} -ATPase dose-response plots.
- (b) Inhibition of the Mg^{2+} -ATPase generally occurred at lower OC concentrations. The OC-induced stimulation of Mg^{2+} -ATPase activity which occurred at low OC doses and/or high temperatures was not detected in the preliminary total ATPase experiments.

The time course of inhibition by DDE (no suitable data was available for DDT) is shown in Fig. 22.

(vi) Substrate concentration

The dependence of Mg^{2+} -ATPase activity on the concentration of substrate ($MgATP$) is illustrated in a Lineweaver-Burk plot (Fig. 23, circles). The y-intercept

*Note that the units of $[E]_0$ are mg TBM protein per ml, rather than the more conventional molecular (e.g. molar) units.

Fig. 17. Effect of varying the TBM content $[E]_0$ of the ATPase assay medium on the inhibition of Mg^{2+} -ATPase caused by a constant ($3 \mu M$) concentration of DDT. The symbols v_i and v_o denote Mg^{2+} -ATPase activity in the presence and absence, respectively, of the organochlorine. The other reaction conditions were as described in Fig. 10.

Fig. 18 Effect of DDT on Mg^{2+} -ATPase at 25°C (circles) and 15°C (crosses). The ATPase activity v_i remaining at either 25°C or 15°C in the presence of the organochlorine is expressed as a fraction of the control (organochlorine-free) activity v_o at that temperature. Other reaction conditions were as described in Fig. 13. Since the TBM protein content of the assay medium was 2.3 $\mu g/ml$, 1 $\mu M/mg$ corresponds to 2.3 μM DDT. Points show the relative rates of individual reaction media; for a given set of reaction conditions (monovalent salt concentration, particular enzyme preparation, etc.) rates were reproducible to within 2% of control activity (usually 0.5-1.5%).

OC dose, $\mu\text{m}/\text{mg prot.}$

p,p'-DDT

Fig. 19 Effect of DDE on Mg^{2+} -ATPase. Units, symbols and reaction conditions are described in Fig. 18.

Fig. 20 Effect of o,p'-DDE on Mg^{2+} -ATPase. Units, symbols and reaction conditions are described in Fig. 18.

OC dose $\mu\text{m/mg prot.}$

Fig. 21 Effect of methoxychlor on Mg^{2+} -ATPase.
Units, symbols and reaction conditions are
described in Fig. 18.

OC dose, $\mu\text{m}/\text{mg prot.}$

Fig. 22 Time course of inhibition of Mg^{2+} -ATPase by DDE. Plotted against time after addition of DDE ($31.6 \mu M$) to the steady-state ATPase reaction is the logarithm of f_s , the fraction of the DDE-sensitive activity remaining; only 59% of the Mg^{2+} -ATPase activity was inhibited by saturating concentrations of DDE. The background changes in absorbance included a large scattering transient which was subtracted from the apparent ATPase rate. Other reaction conditions were as described in Fig. 13.

Fig. 23 Double-reciprocal (Lineweaver-Burk) plot of Mg^{2+} -ATPase activity as a function of substrate (MgATP) concentration c , in the presence (crosses) or absence (circles) of DDT (10 μmole per mg TBM protein). Horizontal and vertical lines through the points indicate the precision of the measurement. The y-intercept indicates a maximum reaction rate $V = 0.97 \mu\text{m min}^{-1}\text{mg}^{-1}$ protein at 25°C . The x-intercept indicates a steady-state rate constant $K_A = 0.08 \pm 0.003 \text{ mM}$. $[E]$ units are mg TBM protein, so that $v/[E]$ is specific activity rather than molecular activity. Reaction conditions were as described in Fig. 9, with variable concentrations of MgATP in place of ADP, and $2 \mu\text{g}$ TBM protein per ml.

is 1.03, corresponding to a maximum reaction rate of 0.97 micromole ATP hydrolyzed per minute per milligram of TBM protein. The x-intercept corresponds to an enzyme-substrate dissociation constant $K_A = 0.08 \pm .003$ mM (Appendix 2).

The effect of DDT on enzyme-substrate interactions is also shown in Fig. 23 (crosses): increasing concentrations of DDT caused increases in the slope of the double-reciprocal plot, but no change in the x-intercept, that is, the percent inhibition at a given dose of DDT was independent of MgATP concentration.

(vii) Temperature

Table 3 contains inhibition data for the Mg^{2+} -ATPase at 10°C as well as 25°C which shows that the OCs were more effective at lower temperature. This is also illustrated in figures 18-21 for 15°C (crosses).

To more fully investigate this negative temperature correlation of ATPase inhibition, the temperature dependence of the inhibitor-free enzyme activity was first studied. Fig. 24 (circles) shows that this dependence followed the Arrhenius relationship, $\log Mg^{2+}$ -ATPase activity being a linear function of inverse absolute temperature from 5 to 40°C. The slope of the function is $-E_a/4.57$, where E_a is the activation energy of the ATPase reaction; the y-intercept ($1/T = 0$) is $\log A$, where A is a constant virtually independent of temperature (Appendix 3).

DDT had two effects on the ATPase kinetics (Fig. 24, crosses). One was an increase in the slope,

Fig. 24 Arrhenius plot of Mg^{2+} -ATPase specific activity as a function of reaction temperature, in the presence (crosses) or absence (circles) of DDT. Step changes in reaction temperature were made above and below $25^{\circ}C$; periodic returns to $25^{\circ}C$ served as a check for irreversible changes in rate. The DDT dose was $14 \mu\text{mole/mg TBM protein}$ ($32 \mu\text{M}$).

indicating an apparent increase in E_a ; the other was an upward shift of the y-intercept, indicating an apparent increase in A. When values of E_a and $\log A$, determined from plots similar to Fig. 24, are plotted against OC dose, both kinetic parameters are seen to increase to plateau values as OC doses increase; although the effects of DDT, DDE, o,p'-DDE and methoxychlor are qualitatively similar, the OCs differ in the doses required for saturation (plateau) and also in the final plateau values attained (Figs 25 and 26).

Oligomycin, DCCD and azide caused no significant changes in the slope of the Arrhenius plot: the y-intercept simply shifted downward, proportional to the degree of inhibition. Some enzyme preparations showed apparent changes in degree of inhibition at a given dose of one of these three inhibitors following a change in reaction temperature. The proportion of control activity inhibited by azide or DCCD often increased at higher temperatures, but such changes, unlike those for OCs, were found to be irreversible. For this reason, treatment with azide and DCCD was carried out at higher temperatures - at least 25° - to ensure complete inhibition.

Oligomycin exhibited some anomalous behaviour at higher temperatures. Following a temperature step increase from 25 to 35°C, for instance, the oligomycin-insensitive activity showed a continuous long-term increase (7-8% of control activity per hour); a return to 25°C reversed the process. These changes were quite distinct from the

Fig. 25

Secondary plots of activation energy E_a of the Mg^{2+} -ATPase reaction, as a function of organochlorine dose. E_a values were derived from Arrhenius plots similar to Fig. 24 for TBM treated with various doses of DDT ($\bullet$), DDE ($\blacktriangle$), o,p'-DDE ($\times$), or methoxychlor ($\circ$).

Fig. 26

Secondary plots of $\log A$ of the ATPase reaction as a function of organochlorine dose. Log A values were calculated from Arrhenius plots similar to Fig. 24. Symbols are defined in Fig. 25.

behaviour of ATPase in the presence of OCs, for which temperature-related changes in percent inhibition seemed to be instantaneous functions of temperature.

III.3. Artificial membrane

(i) Physical appearance

Following the application of a drop of lipid solution to the membrane aperture (about $5\ \mu\text{l}$ lipid solution for a $2\ \text{mm}^2$ aperture with reservoir groove), the resulting thick film normally underwent a series of spontaneous changes.

(a) The film first thinned to a partly-structured "multilayer" state, characterized by reflectance of intense interference colours when illuminated with white light. These colours progressed with time through the series yellow-blue-gold-silver, indicating progressive thinning of the lipid lamella; gold-silver portions typically developed first at the lower edge of the lamella, gradually displacing other colour upwards.

(b) One or more "black" (nonreflective) areas (first appearing in the silver-grey regions in the lower portion of the lamella) then displaced coloured areas upwards and outwards into the bulk phase annulus. The membrane was most fragile at this stage.

(c) Following displacement of all the multilayer areas, the bilayer lipid membrane (BLM) then normally underwent additional expansion at the expense of the bulk phase annulus. This process was greatly accelerated if no reservoir groove were present around the aperture, in

which case the BLM normally ruptured shortly after all of the annulus had become displaced. Slow expansion of the BLM in a grooved aperture could be accelerated or reversed by appropriate horizontal (i.e. normal to the plane of the membrane) repositioning of the BLM with hydrostatic pressure, achieved by introducing electrolyte into (or withdrawing it from) one of the electrolyte chambers; change in BLM area was facilitated when the apertures of the two tandem aperture discs were of different sizes, and several cycles of BLM expansion and shrinkage could be studied.

(d) Occasionally an island of bulk lipid solution became trapped in the black area during thinning, in which case electrical measurements were postponed until the island had drifted to and fused with the annulus.

(e) When stationary, the BLM was virtually invisible, but with careful positioning of light, membrane, and microscope a faint silver-grey shimmer could be seen as the membrane responded to environmental vibrations. The BLM became much more visible during stirring of the electrolyte, which induced peripheral portions of the BLM to revolve about the centre. These eddy currents in the plane of the membrane were obvious because the black lamella was not homogeneous: invariably present were minute (5-10 micron) lenses which reflected silver-grey light as they revolved about the centre of the membrane. These microlenses, accounting for up to 2% of the total BLM area, were present throughout most experiments; only

in the most prolonged experiments were their numbers observed to diminish, disappearing completely between 3 and 4 hours after initial BLM formation.

(ii) Electrical properties

(a) Capacitance

The electrical capacitance of mature (greater than 1/2 hour old) lecithin-decane membranes was a direct linear function of BLM area (Fig. 27). The straight lines through the points of this and subsequent figures are best-fit (least squares) regressions of the form

$$y = a + bx \quad (3)$$

The slope, b , of Fig. 27 indicates an average specific capacitance ($C_m = C/A_m$) of $0.39 \mu\text{F}/\text{cm}^2$, while the y -intercept, a , gives the stray capacitance - about 130 pF. BLMS formed from DDT-doped lipid solution (equimolar DDT-lecithin) yielded a similar capacitance - area relationship (Fig. 28), with a slightly lower average C_m , $0.37 \mu\text{F}/\text{cm}^2$. The difference between these two values of C_m is not statistically significant (Appendix 4,A)

Also shown in Figs. 27 and 28 (open symbols) are values for coloured lamellae, the regression coefficients being 0.07 and $0.05 \mu\text{F}/\text{cm}^2$ respectively. The point scatter is obviously much larger than for BLMS.

Limited observations made on membranes formed from other lipid solutions indicated that specific capacitance was affected not only by the type of phospholipid used but also by the type of solvent. For instance membranes formed with 2% dipalmitoyl phosphatidyl choline (+ 0.1%

Fig. 27 Total electrical capacitance plotted against membrane area. Capacitance was calculated from the time constants of the exponential charge/discharge transients resulting from application of step pulses across the membrane and a calibrated seriesresistance. Membranes were formed from the standard lipid solution (2% vegetable lecithin + 0.5% BHT in n-decane).

Closed circles indicate readings taken from bilayer (black)membranes; open circles indicate those taken from multilayers (coloured). The best-fit line gives an average specific bilayer capacitance of $0.39 \mu\text{F cm}^2$, and a multilayer capacitance of $0.07 \mu\text{F cm}^2$.

Fig. 28 Total electrical capacitance plotted against membrane area. Conditions were as described in Fig. 27 except the lipid solution also contained 2% DDT. Open triangles indicate capacitance of coloured lamellae.

α -tocopherol + trace of methanol to effect solubilization in n-decane; formed at 45°C) had a somewhat higher specific capacitance, 0.44 $\mu\text{F}/\text{cm}^2$. And a vegetable lecithin - butylated hydroxytoluene (2% - 0.5% w/w, respectively) membrane solution in which the alkane solvent was n-hexadecane rather than n-decane, gave rise to unstable BLMs of very high capacitance - about 0.8 $\mu\text{F}/\text{cm}^2$.

Specific capacitance also appeared to increase with BLM age: one large (6.6 mm^2) BLM, for instance, achieved 0.45, 0.50 and 0.55 $\mu\text{F}/\text{cm}^2$ at 3, 6 and 7 hours respectively after initial black. For most membranes, electrical measurements were completed before such long-term changes became evident.

(b) Conductance

The d.c. conductance (G) of "unmodified" (ionophore-free) lipid films showed no consistent correlation with developmental stage (thick film, coloured lamella, BLM), nor did it vary consistently with BLM area. The conductance of the whole membrane was typically about 10^{-10} mho (range, 2 - 30 $\times 10^{-11}$ mho).

Addition of the K^+ - ionophore valinomycin (VM) to the aqueous phase on one side of an unmodified BLM caused the expected increase in G. An example of this phenomenon is shown in Fig. 29(A), where the addition of 8×10^{-12} M valinomycin is seen to have increased the specific conductance (conductance per unit area membrane, $g_m = G/A_m$) from 2.5×10^{-8} mho/ cm^2 to 6.2×10^{-7} mho/ cm^2 . Although such changes were not reproducible from one membrane to another,

Fig. 29 Effect of valinomycin (VM) and DDT on BLM specific conductance. Using a 5% w/w solution of vegetable lecithin in n-decane, a membrane was formed across a 1.6 mm^2 aperture separating two identical phases of electrolyte ($0.09 \text{ M KCl} + 0.01 \text{ N K}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$, pH 7).

VM in ethanol was injected (A) into the stirred inner chamber electrolyte to give a concentration of $0.8 \times 10^{-11} \text{ M}$. DDT in ethanol was later injected (B) to give a concentration of $2 \text{ }\mu\text{M}$. Ethanol alone had no effect.

a statistical approach (fitting of least-squares curves) showed that g_m was a direct function of VM concentration (Fig. 30): the linear regression coefficient is 1.03, and is not significantly different from unity. (Appendix 4,B).

In addition to the poor reproducibility of g_m of individual membranes, another problem was encountered. Fig. 31 (open circles) shows that at higher VM concentrations (in this case, 0.1 μ M), g_m increased rapidly to a maximum, then slowly declined, failing to reach a plateau value during the time course of most experiments: it is this peak g_m that was plotted as a function of VM concentration in Fig. 30. The rate of decline of g_m was also unpredictable from one membrane to another, making it difficult to analyse the effects of subsequent treatment of the membrane with DDT.

Following the rise to peak g_m induced by addition of VM to the electrolyte, addition of DDT to the same electrolyte caused a decline in g_m which was once again not quantitative in either rate or magnitude. For those membranes whose g_m reached a plateau after VM addition, DDT caused an abrupt decline (Fig. 29, B); otherwise DDT caused a less distinct acceleration of the normal decline in conductance. DDT added to the aqueous phase prior to VM appeared to have a buffering effect on VM action: the abrupt rise in g_m to a transient peak was transformed into a gradual rise to a plateau value (Fig. 31, squares). Membranes formed with a lipid solution already containing DDT (equimolar with respect to lecithin) gave a transient peak conductance which was indistinguishable from DDT-free

Fig. 30 Dependence of BLM peak specific conductance g_m on the molal concentration of VM in the inner chamber electrolyte.

Fig. 31 Effect of VM and DDT on BLM specific conductance. Membranes were formed as described in Fig. 29.

(i) Open circles: VM was injected (A) into the inner chamber electrolyte to give a concentration of $0.1 \mu\text{M}$.

(ii) Closed circles: same as open circles except membrane was formed from a lipid solution containing DDT (equimolar with lecithin); VM was again added at A.

(iii) Squares: DDT was first injected (A) into the stirred inner chamber electrolyte to give a concentration of $10 \mu\text{M}$; VM was later added (B) to give $0.1 \mu\text{M}$.

membranes, but the subsequent decline in g_m was slower (Fig. 31, closed circles).

Another series of experiments was done using valinomycin-doped lipid solution to form the membranes. This method allowed a more precise correlation of g_m with valinomycin concentration than had been possible when the ionophore was added to the aqueous phase. Specific conductance g_m of these VM-doped membranes was determined from the slope of $G:A_m$ curves (such as that shown in Fig. 32) wherever a sufficiently wide range of readings could be taken from a single membrane; otherwise, g_m was calculated by simply dividing each G by the membrane area at that time.

Figs. 33 and 34 show that g_m was a direct linear function of VM concentration in both DDT-free and DDT-doped BLMS. The slopes b of Figs 33 and 34 are 1.083 and 1.049, neither being significantly different from unity (Appendix 4, C and D) nor are the two slopes or two y-intercepts (a_1 and a_2) significantly different from each other (Appendix 4, E and F). The ionophore also increased the conductance of thin coloured lamellae (Fig. 33, triangles), but only to values about one order of magnitude below those of BLMS. When DDT was added to the aqueous phase adjacent to a VM-doped BLM, there were no detectable changes in g_m , even when needle-like inclusions (presumably precipitated DDT) were visible in the plane of the membrane.

Specific conductance was particularly sensitive to

Fig. 32 Dependence of VM-doped membrane conductance G on BLM area. Points represent readings taken from a single membrane whose area was varied by horizontal repositioning of the membrane. The slope of the curve gives $g_m = G/A_m$, the specific conductance of the bilayer, while the y-intercept gives the thick-film conductance.

Fig. 33 Dependence of specific conductance g_m on concentration of VM in the membrane-forming solution. Circles represent readings taken from bilayers, while triangles show multilayer readings. Electrolyte temperature was $25 \pm 1.5^\circ\text{C}$.

Fig. 34 Dependence of g_m on concentration of VM in the membrane-forming solution. Conditions were as described in Fig. 33, except that the membrane-forming solution contained 2% DDT in addition to the usual components.

two variables - stirring and temperature. Fig. 35 shows the effect of stirring the inner chamber electrolyte; g_m of the VM-doped membrane fell continuously but non-linearly during stirring, and rose again when stirring was stopped. An opposite effect (not shown) was noted when VM had been added to the inner chamber electrolyte rather than to the lipid solution: in this case, onset of stirring caused an increase in g_m which was reversed upon cessation of stirring. Unless otherwise noted, all conductance readings were taken in the absence of stirring.

Conductance was also temperature-dependent. Fig. 36 shows an Arrhenius plot of g_m , over the temperature range 22-45°C, for a single VM-doped BLM. The slope of the best-fit (least-squares) line is -3.26, indicating an apparent energy of activation of 14.9 kcal. mole⁻¹. Over a lower temperature range (13-33°C), another VM-doped BLM gave a biphasic curve (Fig. 37), the slopes indicating an activation energy of 15.5 above 22°C and 26.8 at lower temperatures.

Fig. 35 Effect of stirring on BLM conductance.
The magnetic stirrer in the inner chamber electrolyte was activated at 45 minutes and deactivated at 62 minutes.

Fig. 36 Arrhenius plot of variation of BLM specific conductance over the temperature range 22-45°C. BLM area was 3.45 mm² or less throughout the experiment. The membrane-forming solution contained 1 millimolar VM in addition to the usual components.

Fig. 37

Temperature dependence of BLM specific conductance, 13-33°C. The membrane was formed over a 6.15 mm² aperture, otherwise conditions were as described in Fig. 36.

IV. DISCUSSION

1. ATPases, preliminary experiments:

total ($\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$)-ATPase

To summarize the results, the OCs tested (excepting DDA and dicofol) caused inhibition of ATPase activity which (a) was incomplete, even at 1 mM OC; (b) was reduced or abolished by addition of Corexit; and (c) only occurred at concentrations of OC which exceeded the limits of OC solubility in water.

Although in the absence of Corexit a precipitate was not visible at inhibitory DDT concentrations below about 30 μM , it is inferred that one was present, since its limit of solubility in water is only about 1.2 ppb or 3.4 nM (Bowman et al., 1960); inhibition of ATPase, by comparison, required well over 100 nM DDT.

It therefore seemed possible that OC precipitation was a prerequisite of ATPase inhibition, which would make this inhibition an artifact of the in vitro system (precipitation being unlikely in vivo). The much higher concentrations of DDA required to cause inhibition might therefore reflect this compound's higher aqueous solubility, precipitation occurring only at very high concentrations. Similarly Corexit might raise the concentrations of OC required to cause inhibition by increasing aqueous OC solubility, i.e. by raising the concentration at which OC precipitation begins.

A similar precipitation-artifact theory has been advanced by Pocker *et al.*, (1971) to explain the *in vitro* inhibition of carbonic anhydrase by DDT. Sharp *et al.*, (1974) have also reported an inactivation of $(\text{Na}^+ + \text{K}^+)$ -ATPase which was the result of interaction of the enzyme with oil-in-water dispersions of fatty acids or OCs: clear aqueous phases obtained by centrifugation of oleate, PCBs or DDT suspensions were not inhibitory. Sharp *et al.* further reported that the inactivation by DDT, deoxycholate and a PCB was reversed by 0.2% BSA (a 10-fold higher concentration than was used in the present study), presumably due to the latter's surface-active properties.

The precipitation-artifact theory fails however to explain the lack of effect of methoxychlor (Figs 5E and 6E) and dieldrin (Fig. 6F) which appeared to precipitate as strongly as DDT. Cutkomp *et al.*, (1971) similarly found methoxychlor and several epoxide cyclodienes to be poor inhibitors of ATPases from fish tissues. However, if inhibition were a precipitation artifact, one would not *a priori* expect similarities between these *in vitro* effects and the *in vivo* toxicities of the analogues; such dissimilarities are evident, where

(a) DDE and Cl-DDT are generally nontoxic *in vivo* (Metcalf, 1955, 1968), yet were effective ATPase inhibitors. (Figs 5 and 6, B and C).

(b) Methoxychlor and dieldrin are about as toxic as DDT to fish (Muirhead-Thompson, 1971, p.55; Gardner, 1973), yet were ineffective inhibitors of ATPase (Figs 5E, 6E, 6F).

Cutkomp et al., (1971a) reported a similar lack of correlation for inhibition of the brain Mg^{2+} -ATPase of a freshwater sunfish by a variety of OCs. Whereas neurotoxic methoxychlor was found to be an ineffective ATPase inhibitor, "nontoxic" o,p'-DDT was as effective an ATPase inhibitor as (and DDE only slightly less effective than) DDT. Cutkomp et al. also tested several cyclodienes, finding that the nonepoxy analogues such as aldrin were effective inhibitors of the Mg^{2+} -ATPase, while the epoxy analogues such as dieldrin were not. They were at a loss to explain this, since both types are toxic to fish. Indeed, the epoxy analogues are more toxic than the non-epoxy, in that they act more quickly (Brooks 1974b, p. 119).

However, these discrepancies need not be significant. For instance, DDE and Cl-DDT may never reach the site of action in vivo due to some of the barriers and diversions outlined in Fig. 1. And no single factor need be responsible for in vivo resistance to these analogues: as Brooks (1974b, p. 67) points out, a combination of modest resistance factors may give virtual immunity.

The lack of in vitro effectiveness of methoxychlor and dieldrin is less easily explained. Dieldrin, belonging to a completely different family of OCs, may owe its toxicity to a different mechanism (even though the symptoms of intoxication closely resemble those of DDT), but there is no a priori reason to assume that this is true for the DDT analogue, methoxychlor. The substitution of

the p-chloro groups of DDT with methoxy groups is, chemically speaking, a conservative change: both groups have electromeric and inductive effects on the ring system, and are of similar size. Furthermore, there is no evidence for the direct involvement of the para groups in any chemical reactions in the intoxication process. Methoxychlor is admittedly more easily metabolized (Brooks, 1974b, p.79), but no biodegradation was detectable (by gas chromatography) in the present reaction medium.

The significance of the effects of DDA and dicofol is not clear. The very high concentration of DDA required to cause half-inhibition ($I_{50} = 0.57$ mM, Fig. 6G) is not inconsistent with its innocuous in vivo nature (Metcalf, 1955). As for dicofol, there seems to be no data available on its toxicity to fish, and its toxicity to other organisms is too diverse to make any generalizations (it is noninsecticidal, but is a potent acaricide: Metcalf, 1955; Brooks, 1974a, p.50).

As an aid to comparison of dose-response curves, several papers (Matsumura and Patil, 1969; Cutkomp et al., 1971b and 1972; Desai et al., 1974) have included probit analysis which can convert certain semilog dose-response functions to linear relationships. The probit is no more than a convenient mathematical device for solving otherwise intractable problems (Finney, 1964): by expanding the %I scale at either extreme, it tends to straighten sigmoid curves, allowing more precise interpolations and extrapolations from best-fit curves.

Apart from dicofol and DDA, the shape of the curves obtained for total ATPase (Figs 7 and 8) - reasonably linear at low OC doses and tending to level off at high doses - suggests (a) there were at least two classes of ATPase, OC-sensitive and OC-insensitive; and (b) the OC-sensitive portion may have had a normally-distributed range of sensitivities to OC. The atypically abrupt dose-probit response curves of dicofol and DDA (Figs 7 and 8) and the fact that only these two OCs were capable of inhibiting close to 100% of the ATPase suggest that these compounds bring about inhibition by a mechanism different from that of the other OCs.

The fact that only total ATPase activity was assayed in these preliminary experiments makes their significance even less clear, since several papers from Cutkomp's laboratory have reported a selective inhibition of the oligomycin-sensitive mitochondrial Mg^{2+} -ATPase by DDT and related compounds (Koch et al., 1971; Cutkomp et al., 1971; Desaiah et al., 1974). I_{50} values for this component were reported to be at least an order of magnitude lower than those derived from Figs 5 and 6, and inhibition was reported to occur in the absence of OC precipitation (Desaiah et al., 1974). This selectivity however appears not to be a universal effect of DDT: Matsumura and Narahashi (1971), for instance, found the $(Na^+ + K^+)$ -ATPase of lobster nerve to be twice as sensitive to DDT and DDE as was the Mg^{2+} -ATPase.

If the oligomycin-sensitive component had comprised

only a small portion of the total ATPases, its inhibition at low OC concentrations might have gone unnoticed, being masked by the continued activity of the less-sensitive components. The experiments discussed next were designed to investigate this possibility, and to extend the studies into areas such as reaction kinetics which had not yet been explored by other laboratories.

IV.2. Mg^{2+} -ATPase

(i) Preparation

The specific activity of the TBM- Mg^{2+} ATPase, $0.95 \mu\text{m min}^{-1}\text{mg}^{-1}$ protein (Fig. 10), is quite low compared to the activity of the highly purified soluble mitochondrial ATPase: the " F_1 coupling factor" isolated by Horstman and Racker (1970), for instance had a specific activity 70 times higher than this. However, continued use of the minimally-purified membranous TBM preparation was deemed preferable because:

1. OCs are not known to affect any soluble enzymes.
2. The purified soluble Mg^{2+} -ATPase is cold-labile, dissociating into subunits and losing enzymic activity at $0-10^\circ\text{C}$, whereas the membrane-bound enzyme is stable and amenable to kinetic studies at low temperatures.
3. Associated with the membrane-bound enzyme are various proteins and other components through which control may be exerted on the ATPase proper. One polypeptide is an intrinsic inhibitor of the enzyme, and its presence in the TBM preparation

contributed to the latter's low activity. More importantly, other proteins are required to confer sensitivity to the specific ATPase inhibitors oligomycin, rutamycin, aurovertin and DCCD: the soluble F_1 ATPase is insensitive or only slightly sensitive to these compounds.

(References for the Mg^{2+} -ATPase properties above may be found in the excellent review by Penefsky, 1974).

(ii) Properties

Isolated by preparatory techniques similar to those used in the preliminary experiments, but omitting the osmotic shock step (since the assay medium was hypotonic anyway), ATPase preparations were consistently found to have a ouabain-insensitive component (Mg^{2+} -ATPase) of about 75% (Fig. 12). This is much higher than has been reported (19-47%) for the fish brain preparations of Cutkomp and associates (Koch, 1969/70; Koch *et al.*, 1971; Cutkomp *et al.*, 1971a), and may be indicative of lower contamination by plasma membrane fragments, the major source of the ouabain-sensitive component.

Although not required for Mg^{2+} -ATPase activity, Na^+ and K^+ were initially included in order to compare results with the Cutkomp-Koch group who use 100 mM Na^+ and 20 mM K^+ in their assays. After later finding that the Mg^{2+} -ATPase was more active at lower salt concentrations (Fig. 12), Na^+ and K^+ concentrations were reduced for the Mg^{2+} -ATPase assay to 30 and 6 mM respectively. They were not totally eliminated from the assay medium partly for

convenience ($\text{Na}^+ + \text{K}^+$ reagent salts were used) and partly because both ions are required in other reactions - Na^+ and K^+ for irreversible inhibition by ouabain (Aker and Brody, 1970), and K^+ as an activator of pyruvate kinase.

The significance of the initial slow increase in activity (to a plateau value) of Mg^{2+} -ATPase in low-salt medium (Fig. 12, open circles) is not apparent; it may be an example of the "opening phenomenon" (Green and Baum, 1970, p.62) characteristic of mitochondrial activities in vitro.

(iii) Standard Inhibitors

Ouabain (strophanthin G) is a well-known cardiac glycoside that specifically inhibits the cell membrane Na^+/K^+ pump, i.e. the $(\text{Na}^+ + \text{K}^+)$ -activated ATPase. It has no known effect on mitochondrial functions.

Both oligomycin and DCCD are potent inhibitors of the mitochondrial ATPase and also of ADP-stimulated respiration. They also block ATP-driven partial reactions in submitochondrial particles (reduction of NAD^+ by succinate; NAD^+ -NADP transhydrogenase), and stimulate the ATP-driven reduction of NAD^+ by succinate in "EDTA particles" (Robertson et al., 1968). They are both effective at extremely low concentrations - of the same order of magnitude as the concentration of electron transport components, about 1 micromole per gram membrane protein.

As was mentioned earlier when discussing the rationale for use of a minimally-purified ATPase, neither of these inhibitors is effective on the soluble Mg^{2+} -ATPase, although DCCD can inhibit the soluble F_1 form very slowly,

and only at high concentrations. Both inhibitors require the presence of intermediary membrane components in order to inhibit the ATPase: the oligomycin sensitivity-conferring protein (OSCP) has been isolated and purified, and tentatively identified with a morphological subunit of the mitochondrial membrane (Kagawa and Racker, 1966a). DCCD requires, in addition to the OSCP, two other membrane components in order to bind to F_1 (Penefsky, 1974).

Oligomycin and DCCD are not entirely equivalent, however. Oligomycin is thought to prevent the energized intermediate from reacting with water (spontaneous hydrolysis) or with ADP (phosphorylation): it does not prevent either the generation or dissipation of the intermediate in other reactions (Racker and Horstman, 1972). DCCD, on the other hand, is thought to prevent energization of the intermediate; binding somewhere between the respiratory chain and the ATPase complex, it is an inhibitor of energy transduction (Azzi *et al.*, 1974). DCCD and oligomycin further differ in that the former binds covalently, while the latter binds noncovalently (Azzi *et al.*, 1974); oligomycin is probably somewhat less specific than DCCD, since the former can inhibit the $(Na^+ + K^+)$ -ATPase in addition to the Mg^{2+} -ATPase of fish brain (Koch *et al.*, 1971).

The data in Tables 3 and 4 support these differences: OM and azide appear to inhibit a portion of the Mg^{2+} -ATPase slightly different from that inhibited by DCCD. The 80-90% proportion inhibited by OM was, incidently,

much larger than the 40-49% reported by Cutkomp et al. (1971b, 1972). The significance of the OM-insensitive component is not known: it is probably the result of preparation heterogeneity, such as contamination with nuclear membranes which also contain a Mg^{2+} -ATPase (Zbarsky et al., 1969), but may also reflect lack of mitochondrial membrane integrity.

Azide is commonly used as an inhibitor of mitochondrial respiration, and is thought to be more or less specific for electron transport complex IV which contains cytochrome a and a_3 plus associated proteins (Green and Baum, 1970, p. 103). Although its interaction with cytochrome oxidase is well documented (Wever et al., 1973), its precise mode of toxic action is not definitely established.

Two facts suggest that azide may owe at least part of its toxicity to inhibition of oxidative phosphorylation: (a) it preferentially blocks the state 3 respiration of mitochondria, that is, it inhibits the stimulation of respiration normally induced by addition of ADP to state 4 (resting) mitochondria (Wilson and Chance, 1967), and (b) it inhibits some Mg^{2+} -ATPases (Harold, 1972).

The fact that OM and azide caused a combined inhibition indistinguishable from (Table 3) or very similar to (Table 4), their individual inhibitions suggests that these compounds inhibit a common fraction of the Mg^{2+} -ATPase, for which DCCD is seen to be only partly specific.

The I_{50} values interpolated from the dose-response

curves for oligomycin, DCCD and azide (Figs 13-15) are 0.15 nmole/mg TBM protein, 17 nmole/mg TBM protein, and 13 μ M respectively. The value for azide is quite close to 60 μ M, the I_{50} found by Wilson and Chance (1967) for inhibition of state 3 mitochondrial respiration. Similarly the I_{50} for OM (Fig. 12) is very close to 0.5 nmole/mg protein, the approximate theoretical dose required for stoichiometric inhibition of mitochondrial ATPase (Robertson et al., 1968; Azzi et al., 1974). The I_{50} for DCCD (Fig. 14) is much larger than this.

The unusually high dose of DCCD required may reflect its instability at higher temperatures. DCCD and other carbodiimides are normally incubated with mitochondrial preparations for 18 to 48 hours at 0°C, but for the TBM preparation this was not convenient due to the spontaneous loss of activity (50% in 18 hours at 0°C) that occurred during refrigeration. To avoid this spontaneous loss of activity, DCCD inhibition was carried out at 25°C.

The inhibition kinetics of oligomycin, DCCD and azide were investigated by adding saturating quantities of these inhibitors: if the interaction of ATPase and inhibitor is regarded as a bimolecular reaction, the presence of one reactant - the inhibitor - in great excess should make the reaction rate a simple function of ATPase concentration.

In the presence of high concentrations of inhibitor the rate of loss of inhibitor-sensitive ATPase activity

was found to be first order with respect to time, following a function of the form:

$$-\frac{dc}{dt} = kc \quad (4)$$

$$\text{i.e. } -\ln c = kt + \text{a constant} \quad (5)$$

where c - the concentration of inhibitor-sensitive ATPase remaining active (not yet inhibited) - is replaced with v_i , the specific activity of inhibitor-sensitive enzyme (because the enzyme concentration is not known).

Fig. 16 shows plots of $\log v_i$ versus time; from equation (5) one can see that the apparent first order rate constants can be derived from the slopes ($= k/2.303$).

Atractyloside is a plant glycoside which inhibits the mitochondrial membrane-bound transphosphorylase that transfers phosphate from intramitochondrial ATP to extramitochondrial ADP (Green and Baum, 1970, p.102). Its lack of effect on the TBM-ATPase activity indicates that transphorylation was not rate-limiting, that is, that ATP had free access to the ATPase. This is not unexpected, since the mitochondrial membrane integrity must have been disrupted by preparative techniques, assay medium hypotonicity and the presence of surface-active serum albumin. Atractyloside does not affect disrupted mitochondria (Bruni *et al.*, 1962).

Similarly, the lack of effect of the uncoupler dicumarol also indicates TBM membrane disruption: as previously mentioned, low ATPase activity is used as a criterion of intact mitochondria, and the lack of effect of uncoupler is interpreted as meaning the preparation

is already completely uncoupled.

(iv) Organochlorines

As in the preliminary experiments, a poor relationship was obtained between the in vivo toxicities of DDT analogues and their in vitro effectiveness as Mg^{2+} -ATPase inhibitors.

(a) Analogues which have (generally) a low in vivo toxicity such as o,p'-DDT, o,p'-DDE, F_3 -DDT and Cl-DDT (Metcalf et al., 1968, 1972) were able to inhibit the Mg^{2+} -ATPase in vitro.

(b) Methoxychlor, which has high in vivo toxicity, actually stimulated Mg^{2+} -ATPase activity at 25°C, although some inhibition occurred at 10°C (Table 3).

As was mentioned in the discussion of the preliminary experiments, discrepancy (a) may be explained as the result of one or more of the barriers and diversions outlined in Fig. 1 preventing these OCs from reaching the mitochondria, whereas discrepancy (b) is inexplicable if Mg^{2+} -ATPase is the target.*

In comparison with the preliminary experiments on total ATPase, the Mg^{2+} -ATPase gave dose-response curves which dropped more abruptly, that is, over a narrower range of OC concentrations (Figs 18-21). This may have been due to a more specific inhibition - the response of the total ATPase having been an average value for fractions

*unless methoxychlor must first be activated (Fig. 1) to become toxic in vivo, but there is no evidence for such a mechanism.

of different sensitivities - and/or to the provisions made in the Mg^{2+} -ATPase assay for equilibrium conditions to be reached.

The discovery that OC effects were a function of quantity of OC per unit enzyme, rather than of OC aqueous concentration, is not surprising, considering the large partition coefficient of these compounds (Appendix 5). If the preliminary data is thus normalized with respect to TBM protein concentration, the OC doses causing inhibition of total ATPase are found to be comparable with those required to inhibit the Mg^{2+} -ATPase, with one notable exception: Mg^{2+} -ATPase was much less sensitive to DDE than was the total ATPase. Figures 6B and 19 are not directly comparable due to the different units used for OC dose and also to the difference in maximum inhibition. However, the dose of DDE required to cause half-maximal inhibition is found to be more than an order of magnitude higher for the Mg^{2+} -ATPase.

Conversions of Figs 18-21 to probit-response form were not included since they would be even less useful than Figs 7 and 8:

(a) In order to use %I/probit transformation tables, the limits of %I should be 0 and 100; but since none of the OCs used in the Mg^{2+} -ATPase assay even approached 100% inhibition, the %I values must first be converted to % of maximum inhibition (i.e. % of the OC-sensitive portion, $\%I_{OC-S}$). Unfortunately, plots of probit $\%I_{OC-S}$ versus log OC concentration are of little practical value,

since the OC-S portion varies so widely.

(b) The stimulation of Mg^{2+} -ATPase activity that occurs at low OC concentrations and/or high temperatures is incompatible with probit analysis.

One obvious conclusion to be drawn from the dose-response data is that OCs cannot have a strong specific affinity for the ATPase: whereas the dose of oligomycin required to inhibit 50% of the OM-sensitive fraction was only 0.15 nmole/mg TBM protein (Fig. 13), the corresponding value for DDT (at 25°C) was 1.6 μ mole/mg or a factor of 10^4 larger than for OM. Assuming this dose of DDT to be quantitatively taken up by the TBM membrane phase, it is equivalent to 57% of the protein content of the membranes, at only half-maximal inhibition.

Similarly massive doses of DDT were required in a quite different system, chloroplast: Bowes and Gee (1971) found that close to a 1:1 molar ratio of DDT to chlorophyll a was required to bring about a 50% reduction in photosynthetic electron transport in the isolated chloroplasts of two marine algae. When such drastic changes occur in the lipid environment of membrane-associated functions, it seems unnecessary to hypothesize specific interactions between DDT and nonlipid membrane components.

From the present dose-response data there is little that can be concluded about the significance of the OC-insensitive portion of the ATPase activity, which varied greatly depending on the OC (compare dicofol and methoxychlor in Figs 5 and 6 and Table 3) and on the enzyme

preparation (compare Tables 3 and 4). For instance, if one assumes a 1:1 interaction between DDT and enzyme to form a complex DDT·E, a maximum inhibition of 50% could mean that:

- (a) only 50% of E is DDT-sensitive, and DDT·E is completely inactive.
- or (b) 100% of E is DDT-sensitive, and DDT·E is 50% less active than E
- or (c) a situation intermediate between (a) and (b) exists.

But there is no way to differentiate among these possibilities from Figs 18-21 since all three would give the same dose-response curve.

Were it not for the low-dose/high temperature stimulation of Mg^{2+} -ATPase by OCs (Figs 18-21), these sigmoid curves might be amenable to analysis using Hill plots:

$$\frac{Y}{100} = \frac{K_D [DDT]^n}{1 + K_D [DDT]^n} \quad (6)$$

where Y is the % saturation of the ATPase complex with DDT. If a plot of $\log \frac{Y}{100-Y}$ versus $\log$ DDT concentration were linear, the slope would be n - the empirical reaction order (not necessarily an integer) - and the y-intercept $\log K_D$ would give the dissociation constant. Unfortunately, apart from the indirect indication given by ATPase activity, there is no accurate method of measuring Y, and one would expect such a parameter to be especially difficult to measure in a heterogeneous membrane system.

The system heterogeneity is represented schematically in Fig. 38, where the assay medium and TBM preparation form a two-phase system. At high OC doses, precipitated OC forms a third phase, easily detectable by spectrophotometric scattering. Even a solution of only 1.7 ppb* DDT in water was found by Biggar et al. (1966) to develop, upon standing for 200 hours, an upper phase containing 3.4 ppb DDT. This self-association is illustrated as the first equilibrium constant P_1 in Fig. 38.

The partition coefficients P_1 and P_2 in Fig. 38 may be instrumental in determining the efficacy of OCs as inhibitors of membrane-associated functions (Appendix 5), but since no data on partition coefficients is available for biological systems, P values can be only roughly established from scattered solubility data. For instance, the microparticulate (saturated) concentrations of o,p'-DDT and p,p'-DDE in water are 3.4 and 4.8 fold, respectively, higher than that of DDT (Biggar et al., 1966). The aqueous solubility of methoxychlor is comparatively large, about 0.1 ppm (Brooks 1974a, p.57), or 80 times that of DDT; this is consistent with the bioaccumulation factor of methoxychlor being only 1/67 that of DDT (Brooks 1974b, p.77). If the lipid solubilities of these analogues are not similarly increased, they will have smaller P values than DDT, and higher aqueous concentrations will therefore be required in order to

*parts per billion (10^9).

Fig. 38 Multiple-phase system in the ATPase assay medium. A dynamic equilibrium forms after addition of excess hydrophobic organochlorine (OC) to an aqueous phase containing dispersed membrane material. P_1 and P_2 are partition coefficients (Appendix 5). K_D is the enzyme-OC dissociation constant, and $OC \cdot E$ is the organochlorine-enzyme complex which gives rise to enzyme inhibition. Direct interaction between precipitate phase and membrane phase (adsorption, not shown) is also possible.

reach inhibitory concentrations in the membranes.

However, there are some complications which make interpretation of these figures difficult.

1. These OC solubilities in water may differ greatly from their solubilities in an aqueous biophase which contains many solutes, often surface-active.
2. The temperature-dependence of OC solubilities may differ. The solubility of DDT in water appears to be a linear function of temperature, increasing less than 1 ppb per $^{\circ}\text{C}$ (Biggar *et al.*, 1966); methoxychlor solubility however appears to be a geometric function of temperature, doubling every 10°C (Richardson and Miller, 1960). Thus if methoxychlor's aqueous solubility is 80 times that of DDT at 25°C , it should be over 200-fold more soluble at 45°C ; this is consistent with the fact that methoxychlor only causes inhibition of the ATPase at low temperatures (Fig. 21, crosses; Table 3), and is selectively toxic to poikilotherms as opposed to homeotherms.
3. DDT analogues may be more lipid-soluble than DDT: DDE, for instance, is twice as soluble as DDT in olive oil (Metcalf, cited in Kenaga, 1972). The differences in P values per se may therefore not be able to account for the differences in toxicities.

It should also be noted that in contrast to the results reported by Cutkomp and colleagues (Koch *et al.*, 1971; Cutkomp *et al.*, 1971; Desai *et al.*, 1974), there appeared to be no specificity of inhibition by DDT for either the azide (i.e. OM, since they inhibit the same fraction*)-sensitive or -insensitive components of the TBM Mg^{2+} -ATPase (Tables 3 and 4). This independence of inhibition suggests that the OCs attacked a target distinctly different from that attacked by the three standard inhibitors. This is further supported by the difference in inhibition kinetics, DDE inhibition being about 50% slower (Fig. 22) than that caused by oligomycin, DCCD or azide (Fig. 16).

The only OC that showed any specificity was dicofol, which in combination with either DCCD or oligomycin caused the same inhibition as any one of these three compounds alone. This was unusual, because azide inhibited the dicofol-sensitive and -insensitive fractions to about the same extent (Table 3). Dicofol was also distinctive in being the most effective (at low doses) of all the OCs tested (Figs 5H and 6H; Table 3), and in being the only OC whose degree of inhibition was independent of temperature.

(v) Substrate concentration

The Lineweaver-Burk plot (Fig. 23) conforms to the steady-state rate equation

*For co-inhibition studies, azide was used in preference to oligomycin in order to avoid the possibility of direct lipophilic interactions with OCs in the aqueous phase.

$$v_f = \frac{V_A [A]}{K_A + [A]} \quad (\text{A4, Appendix 2})$$

or, inverted to linear form for plotting,

$$\frac{1}{v_f} = \frac{K_A}{V_A} \frac{1}{[A]} + \frac{1}{V_A} \quad (7)$$

where v_f is the forward velocity of the ATPase reaction, V_A is the maximum forward velocity, $[A]$ is the MgATP concentration and K_A the Briggs-Haldane constant (Appendix 2). Since K_A and V_A are constants (for a given set of reaction conditions), equation (7) is seen to be linear, with y-intercept $(V_A)^{-1}$ and x-intercept $-(K_A)^{-1}$.

From Fig. 23, the y-intercept is 1.03, indicating a maximum rate (under these conditions) of 0.97 micromole ATP hydrolyzed per minute per milligram mitochondrial protein; the x-intercept, 12.5 ± 0.5 , gives $K_A = 0.08 \pm .003$ mM.

For most experiments, substrate concentration was 2.5 mM, which gave a reaction rate of 0.95, i.e. 98% maximum. Setting $[A] \gg K_A$ allows equation (A4) to simplify to

$$v_f \approx V_A = k[E]_0 \quad (\text{A5, Appendix 2})$$

that is, at high substrate concentrations, reaction rate should be a linear function of enzyme concentration $[E]_0$. This was found to be true for the Mg^{2+} -ATPase (Fig. 10).

Fig. 23 also illustrates the response of DDT-treated Mg^{2+} -ATPase to variations in substrate concentration.

DDT inhibition was independent of substrate concentration, causing changes in the slope but not the x-intercept of the Lineweaver-Burk plot; this is a clear indication that DDT effects are noncompetitive and allosteric.

(vi) Temperature

The enhanced inhibition of Mg^{2+} -ATPase by OCs at low temperatures ($10^{\circ}C$, Table 3; $15^{\circ}C$, Figs 18-21 crosses) is reminiscent of the negative temperature coefficient of DDT toxicity in vivo, and suggests that this may be a general feature of OC action.

As was outlined in the introduction, (Section I.2), the basis of the in vivo negative temperature correlation is not clear since so many variables are involved in in vivo toxicity (Fig. 1), each variable having its own dependence on temperature. In these ATPase studies, however, most of the variables have been eliminated. For instance, since ATPase rates were measured at steady-state, diffusion and transport variables are no longer significant. And although volatilization and chemical inactivation are still possible in a cell-free system, gas chromatographic analysis of one of the ATPase assay media (methoxychlor*) revealed no change in OC content during the reaction.

Another variable present in the in vitro system that may have a negative dependence on temperature is the partition of OC among the phases outlined in Fig. 38. If, for instance, the partition coefficient

*methoxychlor stability was of particular interest because of its lack of effectiveness as an ATPase inhibitor.

$$P_2 \quad (= \frac{[OC \cdot E]}{[OC] \cdot [E]} \quad \text{at equilibrium})$$

were inversely dependent on temperature, and $OC \cdot E$ were less active than free enzyme E , then measured enzyme activity would have a negative temperature coefficient. Unfortunately, data on the temperature dependence of partition coefficients is unavailable, and must be calculated where possible from fragments of solubility data scattered throughout the literature.

The solubility of OCs in hydrocarbon solvents, while not a simple function of temperature, does invariably increase as solvent temperature rises. Gunther (1945) plotted the temperature dependence of DDT solubility in several low-molecular weight organic solvents, obtaining fairly linear regressions (at least in the range 10-40°C) which varied from 0.075 weight per cent/°C for 95% ethanol, up to 0.75 weight per cent/°C for acetone. Using these two values as limits (since similar data is not available for large, complex solvents like phospholipids), a 10°C rise in temperature should increase DDT lipid solubility by 0.75-7.5 g/l; since DDT solubility in most aliphatic hydrocarbons is about 11% w/v (Frear, 1955) at 27-30°C, the solubility of DDT at 37-40°C should rise to 12-18% w/v (lower and upper limits respectively).

These rough estimates give rise to DDT lipid-water partition coefficients of 4.0×10^5 at 27-30°C, and somewhere between 3.2×10^5 and 4.7×10^5 at 37-40°C. The difference cannot be considered significant: a

definite conclusion will require more precise OC solubility data, especially for solubility in phospholipids.

At the molecular rather than macroscopic phase level, the theory advanced by Matsumura and O'Brien (1964) might also explain the negative correlation with temperature. If DDT toxicity requires formation of a charge-transfer complex,

one would expect $\text{DDT}_n \cdot \text{E}$ to be heat-labile: increasing thermal agitation should favour dissociation, i.e. an increase in the dissociation constant:

$$K_D = \frac{[\text{DDT}_n \cdot \text{E}]}{[\text{DDT}]^n \cdot [\text{E}]} \quad (9)$$

In plots of enzyme activity (a direct function of $[\text{E}]$) versus log DDT concentration, an increase in K_D should be qualitatively evident as a more abrupt drop in the log dose-response curve at lower temperatures. But no such change is evident for DDT, DDE and o,p'-DDE (Figs. 18-21, crosses).

Of course, the DDT-enzyme interaction need not be as simple as indicated in equation (8). There may, for instance, be co-operative effects among several DDT molecules influencing a common enzyme molecule: the binding of one DDT molecule may enhance or diminish the enzyme's affinity for more DDT or other ligands (ligands other than substrate: DDT inhibition is non-competitive, allosteric). The molecular activity of the ATPase may thus

vary nonlinearly as the order n of equation (8) increases, analogous to the control of activity in regulatory enzymes (Monod, 1966); the sigmoidicity of dose-activity plots for these enzymes is thought to be due to conformational changes. Slater (1974) reported such co-operativity for the binding of aurovertin to the ATPase of rat liver mitochondria, further noting that the properties of the in situ ATPase changed in response to coupled electron transport; the importance of the membrane environment in the allosteric control of ATPase activity is obvious.

Despite the possible complications mentioned above, and despite the fact that the rate constant k (equation A6, Appendix 2) of the ATPase reaction is a composite of several individual reaction constants (each of which may vary independently with temperature), the dependence of Mg^{2+} -ATPase specific activity on temperature appeared to be quite straightforward. In either the presence or absence of inhibitors, Arrhenius plots of specific activity over the temperature range 5-40°C were linear, indicating that a single reaction step was rate-limiting under all conditions (Appendix 3).

Fig. 24 shows the kinetics of untreated (circles) and DDT-treated (crosses) Mg^{2+} -ATPase. Except for DDA, DBP and dicofol, all of the DDT analogues tested caused similar changes in the Arrhenius plot: the increases in the absolute value of the slope and upward shifts in the y-intercept indicate that both activation energy E_a

and the frequency factor A are increased by these OCs (Appendix 3). Note in Fig. 24 the differential inhibition, which varies from about 69% at 15°C to no inhibition at all at 40°C; at lower concentrations of DDT, the two curves intersect at lower temperatures, corresponding to stimulation of activity at temperatures above the intersection point, and inhibition at temperatures below it.

Secondary plots of E_a and $\log A$ were subsequently found to be continuous functions of OC dose, both parameters increasing to plateau values at saturating doses of OC (Figs. 25 and 26). It is important to note, however, that the Arrhenius plots are empirical, in that the ordinate scale is not in units of $\log k$ (equations A5 and A6, Appendix 2) but rather $\log$ specific activity. What is tacitly assumed in the secondary plots (Figs. 25 and 26) is that $[E]_0$, the total concentration of active Mg^{2+} -ATPase (equation A5), is constant. Unfortunately, there was no obvious means of verifying this last assumption. The most accurate determination of ATPase concentration would involve isolation of a homogeneous, pure enzyme, but as was discussed earlier (section IV.2.i), the properties of such a preparation differ dramatically from those of the membrane-bound complex.

A method of estimating $[E]_0$ that requires no prior modification of the enzyme is titration with a site-specific irreversible inhibitor such as DCCD, but even this allows the determination of only relative values of $[E]_0$ due to the lack of knowledge of

- (a) the relationship between binding sites of different inhibitors: does DDT-inhibited enzyme still bind DCCD?
- (b) the activity of individual ATPase molecules: is inhibition by OCs and DCCD all-or-none; do all ATPase molecules have the same activity to begin with?
- (c) the specificity of DCCD binding: does latent ATPase (that portion inhibited by the intrinsic inhibitory polypeptide) also bind DCCD?

The problem with using DCCD and oligomycin for the determination of ATPase concentration is that they are allosteric inhibitors, whereas what is actually required is an isosteric irreversibly-binding substrate analogue. The latter, binding only to the active site of the ATPase, would allow the selective titration of only those ATPase molecules which are accessible to ATP. But ATP binding is not synonymous with hydrolytic activity: titration of an allosterically-inhibited enzyme with an active-site ligand may indicate no change in the concentration of available active sites, even though the concentration of active enzyme molecules has decreased (or the activity of each enzyme molecule has decreased).

To conclude this section on kinetics, the significance of the changes in E_a and $\log A$ will be discussed. Although E_a and $\log A$ appear to be continuous functions of OC concentration, and the Arrhenius plots contained no discontinuities, there is at present insufficient evidence

to state unequivocally that DDT and analogues act by raising the activation energy and frequency factor of the Mg^{2+} -ATPase reaction. If, for instance, one of the equilibria of Fig. 38 were a continuous inverse function of temperature, the OC-inhibited enzyme might appear to have an increased activation energy. One equilibrium not illustrated in Fig. 38 is adsorption (direct interaction between OC precipitate and membrane) which is known to be favoured by low temperatures (Metcalf, 1955): the hypothesis of coprecipitation of enzyme with high doses of OC is therefore compatible with the kinetic behaviour of this in vitro system.

Since data on ATPase activation energy E_a appear to be lacking for fish, some comparisons will be made with data from mammalian systems. Kimelberg and Papahadjopoulos (1974), while studying the effects of phospholipid acylchain fluidity, phase transitions and cholesterol on the $(Na^+ + K^+)$ -ATPase of rabbit kidney medulla, also assayed Mg^{2+} -ATPase. In agreement with the present results for TBM Mg^{2+} -ATPase, they found no discontinuities in the Arrhenius plot (5-50°C). More significantly, Kimelberg and Papahadjopoulos found that E_a was very sensitive to the presence and composition of associated lipids, varying from 4.0 kcal/mole for delipidized enzyme + dioleoyl phosphatidyl glycerol, to 16.0 kcal/mole for delipidized enzyme + dipalmitoyl phosphatidyl glycerol. Their data, although limited, suggested that E_a was raised by increasing chain length and degree of saturation of the fatty

acid chains. Long, saturated fatty acid chains favour the formation of stable bilayers rather than hydrophobic interdigitation with proteins (Lenaz et al., 1971).

Interaction of membrane-bound ($\text{Na}^+ + \text{K}^+$)-ATPase with lipophilic compounds was also reported by Sharp et al. (1974), who concluded that acidic phospholipids were necessary for stabilization of the enzyme, and that OCs (DDT and PCBs) and anionic amphiphiles interfered with this stabilization process.

The next section of the discussion deals with the results of the artificial membrane experiments, which were undertaken to investigate this DDT-membrane interaction in greater detail.

IV. 3. Artificial membrane

(i) Appearance

The development and physical characteristics of the lecithin-decane membranes were in general agreement with published observations. Visible heterogeneity in the "black" membrane, though not routinely mentioned in BLM studies, has been reported several times. Henn and Thompson (1968) studied transverse sections of fixed BLMs, noting the presence of pockets of non-staining material enclosed within the two dark-staining bands. They also noted that bilayer regions varied considerably in thickness, and suggested that their BLMs had an appreciable core of neutral hydrocarbon (about 10 molecules of n-decane for each phospholipid molecule).

Pagano et al. (1972) developed an ingenious method

of sampling the black region of unfixed BLMs formed from radioactively labelled components, and reached similar conclusions. They noted the inherent irreproducibility of BLM structure and composition, individual BLMs containing quite different proportions of hydrocarbon solvent. They concluded that even when lenses of bulk lipid were not visible on the bilayer area, the latter must have contained microscopic lenses of solvent, so that the membranes were not truly bimolecular in their transverse dimension. Even when the hydrocarbon was hexadecane - the alkane solvent which gives highest BLM capacitance and allows closest approximation of BLM to the bilayer state (Fettiplace et al., 1971) - the solvent was determined by Pagano et al. to comprise about one half the membrane structure.

In addition to these structural and radiometric observations, evidence for the presence of considerable hydrocarbon solvent in BLMs is also provided by the latter's electrical characteristics. Membrane thickness can be estimated from the relationship

$$d = \frac{A\epsilon}{C} \quad (10)$$

where d is the bilayer thickness in meters, A is the bilayer area in meters², C is the capacitance in farads, and ϵ is the absolute dielectric constant of the bilayer in coulombs² . newton⁻¹ . meter⁻² (the product of ϵ_r , the relative dielectric constant, and ϵ_0 , the permittivity of free space),

(ii) Electrical properties

(a) Capacitance

Whereas the capacitance of natural (cellular) membranes is typically about $1 \mu\text{F}/\text{cm}^2$, that of most artificial bilayer membranes is less than $0.4 \mu\text{F}/\text{cm}^2$. BLMs are therefore inferred to have a much thicker dielectric (hydrocarbon core) than do natural membranes. On the basis of capacitance measurements, Fettiplace et al. (1971) and Stark et al. (1972) found that BLM thickness did not depend strongly on the fatty acid chain length of the lipid, and concluded that the hydrocarbon solvent comprises a significant portion of the bilayer. Fettiplace et al. also found that BLM thickness was determined in part by the chain length of the solvent.

Using more refined measuring techniques which allowed very precise ($\pm 0.3\%$) calculation of C_m , White and Thompson (1973) found that C_m was not reproducible for BLMs formed from the same lipid solution, and concluded that variability in the amount and distribution of solvent trapped in the BLM was the source of this irreproducibility.

The regression constants (slopes) of the capacitance-area curves obtained for lecithin-decane (Fig. 27) and DDT-lecithin-decane (Fig. 28) BLMs are 3.9 and $3.7 \text{ nF}/\text{mm}^2$, i.e. 0.39 and $0.37 \mu\text{F}/\text{cm}^2$ respectively. These values are very similar to those obtained by Hanai et al. (1964) for egg lecithin-decane membranes, which ranged from 0.373 to $0.405 \mu\text{F}/\text{cm}^2$, depending on the

electrolyte used. Using equation (10), and assuming $\epsilon_r = 2.05$ (Hanai et al., 1964), the specific capacitances 0.39 and 0.37 $\mu\text{F}/\text{cm}^2$ gave rise to estimated BLM core thicknesses of 46.5 and 49 Å respectively. By way of comparison, fully extended 18-carbon fatty acid (e.g. stearic) chains arranged tail-to-tail in a bilayer might be expected to give a BLM core thickness of about 40 Å.

Due to point scatter, the differences in slope of the regressions in Figs 27 and 28 cannot be considered significant (Appendix 4,A). BLM structural variability probably accounts for much of the point scatter (White and Thompson, 1973), other contributing factors being membrane asymmetry, difficulty of determining BLM margins, and imprecision of time constant readings. Variation of C_m with applied voltage may be considered a minor source of error since applied voltage was kept below 30 mV, which according to White and Thompson (1973) should cause an increase in C_m of only 1.3%.

During long-term experiments, the tendency for visible microlenses to disappear from the BLM and the tendency for BLM capacitance to increase might have been related, since removal of microlenses should give a smaller average BLM thickness and hence larger capacitance (equation 10). There were, however, insufficient observations for a proper correlation.

(b) Conductance

The inherently irreproducible nature of specific conductance ($g_m = G/A_m$) of unmodified membranes has been

well documented, conductance apparently being a function of structural imperfections (dust, water droplets) in the membrane, border leakage, or other unknown factors.

Addition to the aqueous phase of a lipophilic ion carrier such as valinomycin causes an increase in G (Figs. 29-31). The slow decline in g_m following a peak conductance (Fig. 31, circles) was explained by Hladky (1973) as being the result of a continuous diffusion of VM into the BLM and its accumulation in the bulk lipid annulus. This would gradually lower the aqueous concentration of VM, thereby reducing g_m which is a linear function of aqueous VM concentration (Fig. 30). Hladky estimated that the distribution of VM between electrolyte and lipid might take over 15 hours to reach equilibrium, so that the concentration of VM in the BLM would at best only approximate a steady-state.

At sufficiently high carrier concentrations, the irreproducible background conductance becomes insignificant, and G becomes a linear function of carrier concentration (Fig. 30) and of membrane area (Fig. 32). Part of the point scatter in Fig. 30 may be attributed to the presence of microscopic lipid droplets dispersed in the aqueous phase as a result of membrane rupture (typically, formation of a stable BLM required more than one attempt); these droplets would compete with the BLM for aqueous VM, thus reducing g_m (Pagano *et al.*, 1972).

The point scatter for g_m plotted as a function of concentration of VM in the lipid (Figs. 33 and 34), while

somewhat less pronounced than that of Fig. 30, is still appreciable, so that the difference between their slopes and unity is not significant. The regressions of g_m on VM concentration are quite similar to those reported by Stark and Benz (1971), who interpreted the linearity of the regression as evidence that K^+ transport is mediated by single VM molecules, with no apparent interaction among the latter.

As had been reported by Hilton and O'Brien (1970), addition of valinomycin to the aqueous phase adjacent to a lecithin-decane BLM caused poorly-reproducible increases in g_m , and subsequent addition of DDT to the same phase caused variable decreases in g_m . However the absence of a similar effect

- (a) when DDT was added to the aqueous phase adjacent to a VM-doped membrane, i.e. a membrane formed from lipid solution containing VM
- (b) when VM was added to the aqueous phase adjacent to a DDT-doped membrane (Fig. 31, closed circles) and
- (c) when membranes were formed from a lipid solution containing both VM and DDT (compare Figs. 33 and 34)

suggests that the DDT-VM interaction must have been extramembranal.

One possibility is that when both VM and DDT are added to the same aqueous phase, the membrane must compete with precipitated DDT for adsorption of VM (antagonism of

VM conductance requires the addition of quantities of DDT far in excess of the latter's limit of aqueous solubility). This might explain the lack of correlation between VM antagonism and in vivo toxicity of the DDT analogues tested by Hilton et al. (1973) were it not for the exception of o,p'-DDD (XI) which had no effect on g_m , and DDMU (XXI), which increased g_m . All of the other hydrophobic analogues decreased g_m , whereas analogues containing hydrophilic groups (and therefore, presumably, being less prone to precipitation in the aqueous phase) increased g_m .

In the same paper, Hilton et al. reported finding no evidence of complex formation between VM and DDT. However, since they tested for this in organic solvents but not in aqueous media, the results do not exclude the possibility of a hydrophobic DDT-VM interaction in the aqueous phase.

The effects on g_m of stirring the aqueous phase (Fig. 35) have been reported by Hladky (1973), who attributed the g_m increase during stirring (when VM had been added to the aqueous phase) to thinning of an aqueous unstirred layer adjacent to the membrane. Hladky hypothesized that diffusion of carrier through this unstirred layer into the BLM determined the concentration of VM within the membrane and hence determined g_m . It is not clear whether this unstirred layer is synonymous with the thin (4 micron) water layers adjacent to BLMs which show anomalous low-frequency dielectric dispersion (Coster and Simons, 1970).

Above 22°C the dependence of VM-mediated BLM

conductance on temperature gave an apparently linear Arrhenius plot (Fig. 36). Its slope may be interpreted as evidence for a single rate-limiting step in K^+ conductance in this temperature range, with an activation energy of about 15 kcal/mole. Johnson and Bangham (1969) calculated a similar value (15.2 ± 0.1) for ΔH^* for the K^+ permeability of liposomes over the temperature range 21 - 60°C. But this similarity may be fortuitous, considering the differences in lipid content: whereas the BLMs contained perhaps 10 times more decane than lecithin (Henn and Thompson, 1968), the liposomes contained only phosphatidic acid and phosphatidylcholine (4:96). This is further supported by the data of Stark et al. (1972), who observed the temperature dependence of VM-mediated conductance in BLMs formed from decane solutions of several pure lecithins, and found a different E_a value for each lecithin.

The discontinuity in the low-temperature Arrhenius plot (Fig. 37) marks the temperature (20 - 21°C) at which the activation energy increases to about 27 kcal/mole. Similar increases in E_a at low temperatures were noted by Stark et al. (1972) for three different lecithins, each having its own discontinuity (transition) temperature. Stark et al. suggested that these discontinuities were the result of structural transitions in the BLM, perhaps indicative of a phase separation between the phospholipid and solvent. They further showed that the breaks reflected a property of the BLM itself (rather than a

peculiarity of the VM system brought about by a combination of temperature-dependent rate constants), since carriers other than VM gave the same transition temperature despite changes in the E_a values.

In contrast to Hladkey's (1973) conclusion that the rate-limiting step in VM-mediated K^+ conductance was diffusion of VM across an aqueous unstirred layer adjacent to the membrane, Stark et al. (1972) concluded that the predominant rate constant in the observed temperature dependence was k_R , the recombination (association) constant for the reaction

where a and m denote aqueous and membrane phases, respectively and k_D is the dissociation constant. At constant salt concentration, g_m will be a direct function of the concentration of $VM \cdot K^+_{(m)}$, which will in turn depend on the concentration of $VM_{(m)}$. Upon application of a potential field across the membrane, $VM \cdot K^+_{(m)}$ would migrate away from the more positive interface and be unavailable for dissociation at that interface, g_m then becoming a function of $k_R \cdot [VM_{(m)}]$.

However, while k_R may be rate-limiting for BLMS formed from VM-doped lipid, it seems more likely that diffusion of VM into the BLM is rate-limiting when VM is added to the aqueous phase.

The lack of effect of DDT on VM-mediated K^+ conductance when both drugs are included in the membrane-

forming solution argues against the formation of an inactive DDT-VM complex in the membrane, and against any alteration of membrane structure which would give rise to changes in k_R . It is therefore concluded that the antagonism of VM activity by DDT was probably an artifact resulting from addition of both drugs to the aqueous phase where their interaction reduced the rate of diffusion of carrier into the membrane; this in turn reduced the VM concentration within the BLM, thus reducing g_m .

The applicability of the limited antagonism of VM action by DDT to the latter's neurotoxic action is made even more tenuous by the fact that K^+ conductance during nerve activity exhibits "channel" or "pore" kinetics, whereas VM acts as a monomolecular shuttle (Pressman, 1969).

Although valinomycin thus appears to be of limited use as a probe of DDT-induced changes in artificial membranes, other analytical methods such as differential scanning calorimetry, X-ray diffraction, ESR and fluorescent probe relaxation kinetics, and the use of solvent-free bilayer systems such as liposomes and double, monolayer BLMs (Montal and Mueller, 1972), may yet elucidate the mechanism of DDT-membrane interaction.

IV. 4. General conclusions

To enable one to state unequivocally whether or not mitochondrial Mg^{2+} -ATPase is the site of action of DDT in either acute or chronic intoxication, two vital questions must first be answered:

1. Do toxic OCs accumulate in the mitochondria in situ in the same massive concentrations which are required for in vitro inhibition of the ATPase? There is some evidence that they do not. Waddill and Keeley (1971) injected cockroaches with lethal doses of ring-labelled ^{14}C -DDT, then isolated the mitochondria from two tissues, fat body and coxal muscle. They found that DDT did reach the mitochondria within 1 hour, but the mitochondria did not have a high affinity for DDT; in fact, by 24 hours the mitochondrial DDT concentration had fallen to half the value it had been one hour after injection. More significantly, at no time during the sampling period (up to 48 hours after injection, at which time the insects were moribund and showing typical DDT tremors) did mitochondria isolated from either of the tissues show any changes in states 3 or 4 respiration, ADP:O ratio or respiratory control. In contrast, mitochondria treated with DDT in vitro showed reduced respiratory control and inhibited state 3 respiration. Waddill and Keeley concluded that, although DDT has the

potential to affect mitochondrial functions, it does not affect mitochondria during the poisoning process in vivo.

2. If inhibition of Mg^{2+} -ATPase does occur in vivo, is it deleterious? One would intuitively expect the opposite to be true, since very low ATPase activity - no higher than 0.7 nanomole ATP hydrolyzed per milligram mitochondrial protein per hour - is considered a criterion of mitochondrial integrity (Wainio, 1970, p.58).

One would expect the inhibition to have deleterious effects only if it also applied to the reverse reaction, oxidative phosphorylation, but this need not be the case. Aurovertin, for instance, is an extremely potent inhibitor of oxidative phosphorylation, yet is a poor ATPase inhibitor (Robertson et al., 1968); and the intrinsic inhibitory polypeptide of mitochondrial ATPase preparations actually stimulates phosphorylation in F_1 -deficient submitochondrial particles (Penefsky, 1974).

The hypothesis of coprecipitation of enzyme with OC seems inadequate to explain the in vitro inhibition of ATPases, partly because of the lack of effect of methoxychlor and dieldrin which do precipitate, and partly because the enzyme is already in particulate form. It seems more likely that precipitation of OC is an incidental consequence of the high concentration of membrane particles in the assay medium: in order to saturate a large lipid phase, large quantities of OC must be added to the aqueous phase (Appendix 5).

A hypothesis of DDT action that is particularly appealing because of its general applicability is that DDT may bind to membrane phospholipids in a way that makes them less fluid; decreased fluidity would in turn affect the activity of transport macromolecules which require a fluid environment within the membrane (McConnell, 1970). For instance, the activity of plasma membrane ($\text{Na}^+ + \text{K}^+$)-ATPase is known to be a direct function of acyl chain fluidity, with discontinuities in the Arrhenius plots of enzyme activity occurring at the endothermic transition temperatures (Grisham and Barnett, 1973; Kimelberg and Papahadjopoulos, 1974). Such modifications might prove to be the bases not only for the variety of effects that DDT can elicit in membrane-associated functions, but also for some of the contradictory data in the literature. Like cholesterol, which can fluidize the rigid structure of membranes composed of saturated lipids but has the opposite effect on the liquid-crystalline structure of unsaturated membranes (March and Smith, 1973), DDT may exert a variety of effects on a particular membrane function due to natural variability in the lipid components. Incidentally, these changes might also be expected to alter the longitudinal viscosity of membranes, thus perhaps affecting such functions as electron transport: cytochrome c and ubiquinone are thought to be mobile electron carriers which move along "molecular tracks" in the plane of the inner mitochondrial membrane (Green and Baum, 1970, p.69). Furthermore, in order to alter perpendicular and lateral transport, DDT need not affect the entire membrane: it

may act locally, for instance in the fluid areas of a fluid-ordered mosaic (Barnett et al., 1974) structure thus having selective effects.

It had been hoped that artificial lipid bilayer membranes would provide a very simple model system in which to study DDT-membrane interaction, and that valinomycin could be used as a probe of membrane structural changes (such as fluidity) resulting from this interaction. However, the fact that no DDT-induced changes in membrane conductance (or capacitance) were detected need not mean that structural changes do not occur in natural membranes, since (a) the rate-limiting step in valinomycin-mediated conductance may not be affected by changes in membrane fluidity, and/or (b) hydrocarbon solvent, the major component of most artificial bilayer membranes, itself has a great effect on membrane structure, and may render artificial BLM structure insensitive to DDT. Both of these problems may be eliminated by using a solvent-free membrane system such as liposomes, or BLMs formed from two phospholipid monolayers (Montal and Mueller, 1972); and by the choice of appropriate structural probes, such as absorbing and fluorescent dyes for detecting changes in the lipid polar head groups, spin label probes for detection of conformational changes in the hydrocarbon phase, and interacting pairs of spin label probes for detection of lateral molecular separations (March and Smith, 1973).

~~Should acute DDT intoxication involve direct~~

interaction of DDT with some nonlipid component, that component is probably the ion gating mechanism responsible for nerve excitability. An apparently direct interaction between DDT (but not DDE) and a squid axolemmal suspension has been reported by Barnola et al. (1971), but since the interaction still required the presence of membrane lipids the evidence was not conclusive.

Alternatively, these gating mechanisms may simply be the membrane functions which are most sensitive to changes in their lipid milieu; or if not the most sensitive, they at least cause the most overt symptoms. And nerve hyperexcitability leading to prolonged tremors and convulsions may well be the immediate cause of the eventual prostration and death characteristic of acute lethal DDT intoxication: rapid depletion of energy stores and accumulation of toxic waste products would lead to general metabolic collapse.

If neuromuscular hyperactivity is the immediate cause of death in acute DDT intoxication, it should be noted that there is little evidence to implicate the involvement of the ATPases. Inhibition of the nerve membrane ($\text{Na}^+ + \text{K}^+$)-ATPase, for instance, should cause a decrease in the electrogenic component (Kerkut and York, 1971) of the membrane resting potential, and/or an increase in the K^+ concentration immediately outside the membrane, either of which would cause depolarization (Gardner and Bailey, 1975). No such depolarization has been observed during neurotoxic symptoms (Narahashi, 1971; Wu et al.,

1974). Furthermore, in vivo inhibition of this enzyme, e.g. by ouabain, should cause slow blocking of nerve action, that is, neurotoxic symptoms quite unlike those caused by DDT (Hilton and O'Brien, 1970; Matsumura, 1970).

These remarks also argue against the Mg^{2+} -ATPase being involved in DDT neurotoxicity, since interference with ATP supply should secondarily inhibit the $(Na^{+} + K^{+})$ -ATPase. And while disruption of ATP supply might be expected to have profound physiological consequences, an increase in nerve excitability is not one of them. Nerve excitability is not known to be directly related to ATP supply, and other agents which are known to reduce ATP supply (e.g. electron transport inhibitors, uncouplers) do not cause hyperexcitability. The order in which different components of the CNS fail during histotoxic anoxia (any interference with the tissue's ability to utilize oxygen) is roughly the same order of CNS "descending depression" observed with increasing doses of a narcotic drug (Mountcastle, 1968, p.813) - in other words, depression rather than stimulation is the result.

There is thus no a priori reason to expect the ATPases to be involved in DDT-induced hyperactivity. And until it is known whether DDT reaches in situ mitochondria in quantities sufficient to inhibit the Mg^{2+} -ATPase, and whether in vivo inhibition of this enzyme has any adverse physiological consequences, the role of Mg^{2+} -ATPase in the acute lethality of DDT will remain unresolved. Furthermore, there is no unequivocal evidence that DDT causes any specific biochemical lesions (such

3

OF/DE

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as inhibition of an enzyme). R.D. O'Brien has proposed the term "physiological lesion" to describe the action of a toxicant which interacts with a component whose significance is only apparent in the functioning, fully-integrated cell (O'Brien, 1967, p.4); in the case of DDT, this component appears to be the membrane conductance mechanisms responsible for nerve excitability.

On balance, it seems unlikely that inhibition of ATPases is the basis for the neurotoxic symptoms of acute DDT intoxication. Also, the lack of effect of DDT on the valinomycin-mediated K^+ -conductance of artificial lipid bilayer membranes when both compounds were present in the membrane suggests that the use of valinomycin is unlikely to clarify the effects of DDT on axonal membranes. A more promising line of investigation may be to focus on the axonal sodium inactivation mechanism, which could lead to a better understanding of both this obscure membrane phenomenon and the site of action of DDT.

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Appendix 1

Ancillary reactions

The discovery that the ancillary reactions catalyzed by PK and LDH (Fig. 3) were, as a unit, first-order with respect to ADP concentration, was not expected, but is theoretically possible at sufficiently low concentrations of ADP. Considering just the initial PK-catalyzed phosphorylation of ADP by PEP, the reaction is bisubstrate-biproduct, and involves the formation of a ternary complex (Boyer, 1962). The initial forward reaction rate v_f may then be described by one of the Dalziel equations such as

$$\frac{[E]_0}{v_f} = \phi_0 + \frac{\phi_1}{[A]} + \frac{\phi_2}{[B]} + \frac{\phi_{12}}{[A][B]} \quad (A1)$$

for the ordered "Bi-Bi" mechanism, where $[E]_0$ is the PK concentration, $[A]$ and $[B]$ are the concentrations of PEP and ADP respectively, and the ϕ terms are complex composite rate constants.

Rearranging equation (A1) gives

$$\frac{v_f}{[E]_0} = \frac{[A][B]}{\phi_2[A] + \phi_{12} + [B](\phi_0[A] + \phi_1)} \quad (A2)$$

Under a particular set of circumstances, $v_f/[E]_0$ could be approximately first-order in $[B]$:

(a) $[A]$ must be constant

and (b) $\phi_2[A] + \phi_{12}$ must be much larger than $[B](\phi_0[A] + \phi_1)$.

Condition (a) is approximated during an ADP phosphorylation transient: starting with 2 mM PEP, a 0.3 micromole pulse of ADP causes only $\frac{0.1}{2}$ or 5% change in PEP concentration. Condition (b) may be approximated if [B] is kept sufficiently low, and the [A] term in the constant ($\phi_0 [A] + \phi_1$) is kept reasonably low - a compromise with condition (a).

A further condition is of course that one is measuring only the forward rate v_f : the net equilibrium constant of the three reactions of Fig. 2 will be the product of the individual equilibrium constants

$$\frac{[\text{ADP}][\text{P}_i]}{[\text{ATP}]} \approx 10^7 \quad (\text{Alberty, 1969})$$

$$\frac{[\text{pyruvate}][\text{ATP}]}{[\text{PEP}][\text{ADP}][\text{H}^+]} \approx 1.15 \times 10^8 \quad (\text{Krimsky, 1959})$$

and

$$\frac{[\text{lactate}][\text{NAD}^+]}{[\text{pyruvate}][\text{NADH}][\text{H}^+]} \approx 3.6 \times 10^{11} \quad (\text{Hakala et al., 1956})$$

which is

$$\frac{[\text{lactate}][\text{NAD}^+][\text{P}_i]}{[\text{PEP}][\text{NADH}][\text{H}^+]^2} \approx 4 \times 10^{26}$$

The reverse reaction is obviously thermodynamically unfavourable and the overall reaction effectively irreversible in the range of PEP and NADH concentrations used.

Appendix 2

Substrate concentration

Since the Mg^{2+} -ATPase is generally acknowledged to be the reversal of the terminal transphosphorylation step in oxidative phosphorylation, one supposes that under appropriate conditions - such as an extremely high ratio of [ADP] to [ATP] - it is also reversible in vitro.

Representing this reaction schematically as

where E is ATPase, A is ATP, EA and EB* are two intermediates, and X is ADP, the initial concentration of which is zero, then the rate of (A3) is

$$v_f = \frac{V_A [A]}{K_A + [A]} \quad (A4)$$

where V_A , the maximum rate of substrate turnover, is a direct function of total enzyme concentration $[E]_0$:

$$V_A = \frac{k_2 k_3 [E]_0}{k_2 + k_{-2} + k_3}, \text{ and } K_A = \frac{k_{-1} k_{-2} + k_{-1} k_3 + k_2 k_3}{k_1 (k_2 + k_{-2} + k_3)}$$

Equation (A4) will continue to describe the rate of reaction (A3) as long as the ancillary reactions continue to consume X and regenerate A. Note that equation (A4) allows the determination of V_A and K_A , but not the determination of the individual rate constants (k_1 etc.).

*B may be bound ADP, which apparently requires Mg^{2+} for release (Slater, 1974)

If $[A]$ is set at a value much greater than K_A , equation (A4) simplifies to $v_f \simeq V_A$, that is

$$v_f \simeq k [E]_0 \quad (A5)$$

where

$$k = \frac{k_2 k_3}{k_2 + k_{-2} + k_3} \quad (A6)$$

so that v_f becomes a linear function of $[E]_0$.

Appendix 3

Reaction Temperature

The variation of a reaction rate constant k with temperature is described by the Arrhenius relationship

$$k = Ae^{-E_a/RT} \quad (A7)$$

where A is a constant, the frequency factor

e is the base of the natural logarithms

E_a is the activation energy of the reaction

R is the molar gas constant

and T is the absolute temperature.

Taking the logarithm of both sides of equation (A7) gives a linear relationship

$$\ln k = \ln A - E_a/RT$$

or, using $\log_{10}$,

$$\log k = \log A - E_a/2.303 RT \quad (A8)$$

Plotting experimental values of $\log k$ versus T^{-1} allows calculation of E_a from the slope ($-E_a/2.303 R$) and $\log A$ from the y-intercept. If a particular reaction is found to yield a nonlinear plot (A8), one may conclude that more than one rate constant is involved; the temperature at which a second rate constant becomes rate-limiting normally appears as a break in the plot.

As mentioned in Appendix 2, at high substrate concentration the rate v_f of the ATPase reaction becomes a direct function of ATPase concentration, but the function may contain several rate constants, depending on the complexity of the reaction. Thus while each of the

individual rate constants comprising the composite constant

$$k = \frac{k_2 k_3}{k_2 + k_{-2} + k_3} \quad (A6)$$

will vary independently according to equation (A7), the variation of the composite (A6) will have a more complex dependence on temperature, unless one of the individual rate constants is predominant (Laidler and Bunting, 1973, p.199).

Appendix 4

BLM: analysis of regression.

The linear regression coefficients a and b of the standard equation $y = a + bx$ were analyzed for the least-squares curves fitted to the data of Figs 27, 28, 30, 32 and 33. H_0 and H_A denote the null and alternate hypotheses tested. Three test statistics were used:

$$(i) \quad F_s = \frac{b_1 - b_2}{\frac{\sum x_1^2 + \sum x_2^2}{\sum x_1^2 \sum x_2^2} (\bar{s}_{Y \cdot X}^2)}$$

where b_1 and b_2 are the slopes of the two curves being compared, $\sum x_1^2$ is the sum of squares deviation of the observed X_i values from the sample mean $\bar{X}$ of curve 1, $\sum x_2^2$ is the corresponding value for curve 2, and $\bar{s}_{Y \cdot X}^2$ is the weighted average of the two variances (Sokal and Rohlf, 1969).

$$(ii) \quad t_s = \frac{b - 1}{\hat{\sigma} \sqrt{\sum x^2}}$$

where $\hat{\sigma}$ is the standard error of estimate for the regression (Sokal and Rohlf, 1969).

$$(iii) \quad t_s = \frac{a_2 - a}{\hat{\sigma} \sqrt{\sum x^2} \sqrt{N \sum x^2}}$$

where a_1 , the y-intercept of curve 1, is used for the population parameter a (adapted from Malik and Mullen, 1973).

Test	Fig.	y	x	H ₀	H _A	Statistic (computed)	Statistic (tabulated)	significance
A	27, 28	C	A _m	b ₁ = b ₂	b ₁ ≠ b ₂	F _S = 2.07	F _{.95(1,129)} = 2.75	ns
B	30	ε _m	[VM] _{aq}	b = 1	b ≠ 1	t _S = 0.969	t _{.95,22} = 2.07	ns
C	33	ε _m	[VM] _{lip}	b = 1	b ≠ 1	t _S = 1.96	t _{.975,33} = 2.03	ns
				b > 1	b ≠ 1	t _S = 1.96	t _{.95,33} = 1.69	(s)
D	34	ε _m	[VM] _{lip}	b = 1	b ≠ 1	t _S = 0.872	t _{.80,11} = 0.876	ns
E	33, 34	ε _m	[VM] _{lip}	b ₁ = b ₂	b ₁ ≠ b ₂	F _S ⁻¹ = 3.02	F _{.95(1,44)} = 4.08	ns
F	33, 34	ε _m	[VM] _{lip}	a ₁ = a ₂	a ₁ ≠ a ₂	t _S ['] = -0.0632	t _{.95,11} = 1.80	ns

Appendix 5

Concentrations, activities and partition coefficients

Consider the distribution of DDT between the aqueous and lipid phases of a system such as biological tissue. At equilibrium, the electrochemical potentials of DDT in lipid solution and DDT in aqueous solution are equal. Assuming that DDT remains uncharged in both phases, the $zF\psi$ terms disappear with the standard electrochemical potential terms, leaving an equality of DDT activity terms,

$$a^{\text{lip}} = a^{\text{aq}} \quad (\text{A10})$$

or, substituting concentrations,

$$\frac{c^{\text{aq}}}{c_o^{\text{aq}}} = \frac{c^{\text{lip}}}{c_o^{\text{lip}}} \quad (\text{A11})$$

where c^{aq} and c^{lip} are the concentrations of DDT in the aqueous and lipid phases at equilibrium, and the c_o terms are the corresponding limits of solubility (concentrations at saturation).

While the value of c_o^{aq} is known to be very low - about 1.2 parts per billion or 3.4 nM (Bowman et al, 1960) - one can only guess at the limit of DDT solubility in the lipid phase. DDT is quite soluble in organic solvents - up to 3-4% in saturated hydrocarbons at 27-30°C (Brooks, 1974, p.57). It seems reasonable to use this 3-4% (about 0.1 M) figure as a conservative estimate of the solubility of DDT in biological membranes, since the latter are composed of amphiphilic lipids and lipo-proteins:

DDT is more soluble in cyclic and aromatic hydrocarbons and olefins than in paraffin solvents (Brooks, 1974a, p.55).

Thus a conservative estimate of the partition coefficient (membrane:water)

$$P = \frac{c_o^{lip}}{c_o^{aq}} \quad (A12)$$

of DDT is $0.1/3.4 \times 10^{-9}$ or about 3×10^7 , on the basis of phase volumes. This means that if one were to administer DDT to the membrane phase indirectly, by exposing the membrane to a surrounding aqueous phase which is saturated with DDT, one would require a volume of aqueous phase 3×10^7 -fold larger than the volume of the membrane phase in order to only half-saturate the latter. While this transfer is common in nature (bioaccumulation), it is, to say the least, inconvenient in the laboratory.

In practice, one may attempt to circumvent this problem by

(a) using an aqueous phase "supersaturated" with DDT: thus it was found that inhibition of ATPase occurred only in the presence of precipitated DDT.

or (b) making the aqueous phase more lipophilic; thereby decreasing P : in the preliminary ATPase experiments, this was done with a nonionic surfactant; for the Mg^{2+} -ATPase, the much less surface-active serum albumin was used.

Neither solution alone is ideal: precipitated DDT forms a third phase which may be very slow in reaching equilibrium with the other two phases (Fig. 38), while the surfactant micelles may compete with the ATPase for DDT. The best compromise appears to be the use of an innocuous surface-active material in quantities below its critical micelle concentration, combined with a minimal quantity of finely-dispersed DDT precipitate.

Although OC-treated TBM preparations were not assayed for OC content, a first approximation of the latter may be obtained by assuming

1. DDT uptake by the TBM phase was virtually quantitative. With a maximum aqueous concentration of 1.2 ppb (Bowman et al., 1960), only 0.1% of the I_{50} dose can be in true solution. The presence of 0.02% BSA should increase this very little: even in the presence of 1% BSA, respiratory particles absorb about 80% of DDT doses as high as 0.1 mM (Gonda et al., 1959).
2. The TBM phase contained about 0.4 mg lipid per mg protein (estimated from Lehninger, 1965, p.206). At the I_{50} values (1.4 and 1.8 μ mole DDT/mg TBM protein at 15 and 25°C, respectively; see Fig. 18), the membrane phase should thus contain 1.4 and 1.8 μ mole DDT per 0.4 mg lipid. At a lipid density of 1 g/ml, this corresponds to 3.5 and 4.5 Molar DDT in the membrane lipid at only 50% inhibition, values much higher

than the theoretical limit of 0.1 M used in calculating the partition coefficient P.

Therefore either P was greatly overestimated, or much of the DDT added to the aqueous phase remained there as a separate precipitate (Fig. 38). Alternatively, there may have been a macroscopic association between membrane fragments and precipitated DDT.

END

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