THE EFFECT OF \textit{p,p}^+\text{-DDT} ON THE SHORE CRAB \textit{CARCINUS MAENAS} (L)

A thesis submitted to the Council for National Academic Awards in part fulfilment of the requirements for admittance to the degree of Doctor of Philosophy.

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October, 1979.
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Finally, I owe the successful completion of the practical side to Dr. J.R. Wharfe, who has encouraged me with the work and enabled me to carry it on over the past few months.

The Science Research Council has financed the project.
SUMMARY

Some effects of DDT on the shore crab (*Carcinus maenas*) were investigated. Parameters examined included the activity of gill ATPases and their effects on ion and ammonia levels of the haemolymph. Histological alterations were observed by light microscopy.

In an *in vitro* study, an homogenate of *Carcinus* gills was exposed to concentrations of DDT from $10^{-10}$ M to $10^{-5}$ M. The specific activity of Na$^+$, K$^+$ -ATPase was stimulated at all concentrations. The activity of Mg$^{2+}$ -ATPase was inhibited at all concentrations. Total -ATPase activity represented the combined activity of both.

*In vivo* studies on the effect of DDT on ATPases showed that the activities of both Na$^+$, K$^+$ - and Mg$^{2+}$ -ATPases were inhibited. There was evidence that osmoregulation was affected over the short term, but not over a longer time course. After injection of a solution of ammonium chloride into the haemolymph of *Carcinus*, there was no consistent pattern to the rate of loss of ammonia from the haemolymph in DDT - exposed and control animals.

Histological examination of gill tissue exposed to DDT revealed that the mucopolysaccharides around the gill lamellae were reduced in thickness. Ca$^{2+}$ -ATPase activity and the R.N.A. content of the tissue were reduced.
Estimations of the quantity of DDT and derivatives which had accumulated in some organs after DDT exposure were made. The greatest amounts were found in gills, followed by digestive gland, stomach and gonad.
**THE EFFECT OF \textbf{p,p}'-\textit{DDT} ON THE \textit{SHORE CRAB CARCINUS MAENAS} \textit{(L.)}**

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DISCUSSION
CHAPTER 1
INTRODUCTION - DDT AND ITS EFFECTS ON ORGANISMS

"In the nearly 100 years that have elapsed since DDT was first synthesised, this substance has had an influence on human ecology perhaps unmatched by any other synthetic substance. Through its effectiveness in the conquest of malaria, typhus, and other insect-borne diseases it has played a decisive role in the population explosion. It has also become the classic example of an environmental micropollutant. Significant discoveries about the chemistry of DDT and its analogues are still being made. These have considerable theoretical interest in studies of the enigmatic mode of action of DDT and are of applied interest in control of resistant insects and in environmental quality control."

R.L. METCALF (1973)
1. HISTORY

*p,p'-DDT* is the common name for 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane, a chlorinated hydrocarbon having remarkable insecticidal properties, as well as many disadvantages.

The compound was first synthesised in 1874 by Zeidler (Zeidler, 1874) in Germany, but was rejected and set aside as "of academic interest only". Research began in 1932 which led to the discovery of DDT as an insecticide and the award of the Nobel Prize of 1948 to Dr. Paul Müller of the Basle Laboratories of Geigy (Lauger, Martin and Müller, 1944). The onset of the Second World War gave an opportunity for the insecticide to be used extensively by the allied armies, and in refugee camps for the control of, in particular, body lice carrying typhus, and also mosquitos and flies. Because of difficulties in communications at that time, it was not until 1942-3 that these discoveries became well-known to the public of Britain and the United States.

The first large-scale production of DDT outside Switzerland started at the Manchester branch of Geigy in 1943. By the summer of 1944, production in the United States had reached the rate of 300,000 pounds per month (Brooks 1974a). DDT became a popular household remedy for every insect pest.
By 1946, DDT had shown great promise for the control of a large number of agricultural pests including Colorado beetle, European corn borer, leaf hoppers, flea beetles, alfalfa weevils, pea aphids and fruit pests such as Japanese beetle, codling moth and oriental fruit moth. The Bureau of Entomology and Plant Quarantine considered that at least one more growing season would be needed for full evaluation of the residue hazard and phytotoxic effects. This showed that the introduction of DDT into agriculture was made cautiously and with full regard for environmental information known at the time.

Most of the major uses of DDT had been established by the middle to late 1940's. A more comprehensive account of these can be found in books by Brooks (1974a), West and Campbell (1950) and Brown (1951).

Then came one of the most classic and well-quoted cases in this field - that of Clear Lake in California. In 1949, the problem with gnats (Chaoborus sp.) around the lake became unbearable for the many people using the area for recreation and fishing. After considering all the insecticides available, the authorities decided to use DDD (1,1-dichloro-2,2-bis (p-chlorophenyl)-ethane), a similar compound to DDT and also with insecticidal properties, but less harmful to fish.

\[\text{p,p'-DDD (TDE)}\]
The first application in 1949 was successful, killing off the gnat larvae, and a few other invertebrates, but these latter populations appeared to recover quickly. The fish did not appear to be affected. Over the next three years, the gnats gradually increased again until they reached their original numbers. Further applications of DDD were made and gave reasonable results.

In 1954, side effects were suspected, with large numbers of Western Grebes being killed. These birds nested by the lake shores and it was probable that the first application seriously affected their breeding. Their deaths were most probably caused by the build up of the insecticide through the food chain, as these were fish-eating birds.

This incident started a public outcry, and Rachel Carson (1962), accentuated this with her book 'Silent Spring', a mainly accurate, but one-sided attack on pollutants. Insects which had built up a resistance to DDT were noticed at this time. It was necessary to develop alternative insecticides e.g. organophosphates and carbamates to combat insect resistance.

In Britain, the Peregrine Falcon was being studied and it was found that the number of eggs produced and also shell thickness were decreasing. These falcons ate homing pigeons, which at that time were being dusted with DDT to kill lice in the feathers.

Brown pelicans, a protected species, thrived on Anacapa Island, a nature reserve off the Californian coast. In the late 1960's it was noted that the birds were not reproducing successfully and the eggshells were thin. A normal
shell is about 0.6mm thick around the girth of the egg, while these were only about 0.2mm thick.

All these incidents helped to lead to the reduced usage of DDT, particularly in North America and Europe. In 1968 President Nixon created the Environmental Protection Agency, with the outcome that the United States banned the use of DDT in its own country in 1969. It could still be manufactured, but was 'strictly for export only'. Canada and the Scandinavian countries followed suit.

In Britain, the Advisory Committee on Pesticides and other Toxic Chemicals (1964), concluded that there was insufficient evidence to justify a complete ban on the use of DDT, DDD, \( \gamma \)-BHC (lindane), toxaphene and the cyclodienes, but the use of aldrin and dieldrin was restricted because of bird-poisoning incidents involving seed-dressings.

\[
\text{aldrin} \\
1,2,3,4,10,10\text{-hexachloro-}1,4,4a,5,8,8a\text{-hexahydro-exo-}1,4\text{-endo-}5,8\text{-dimethanonaphthalene}
\]

\[
\text{dieldrin} \\
1,2,3,4,10,10\text{-hexachloro-}6,7\text{-exopxyl-}1,4,4a,5,6,7,8,8a\text{-octachydro-exo-}1,4\text{-endo-}5,8\text{-dimethanonaphthalene}
\]
The Advisory Committee's 1970 review of persistent organochlorine insecticides recommended that the use of DDT on grassland, brassica seed crops, peas, soft fruit and post blossom on top fruit should cease. The consequence of these measures is that pest control in Britain is now more expensive and often less effective than when organochlorines were in common use.
2. PRESENT USE OF DDT

a) IN BRITAIN

In Britain today, DDT is still used in agriculture, although the quantities have decreased. This can be seen in a few crops in Table 1. The acreage covered by the crops remained approximately the same through the years shown.

Table 1 Acreages to which DDT was applied between 1964 and 1967

<table>
<thead>
<tr>
<th>Crop</th>
<th>1964</th>
<th>1965</th>
<th>1966</th>
<th>1967</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foliage and soil treatments to sugar beet</td>
<td>40,000</td>
<td>14,200</td>
<td>4,500</td>
<td>5,000</td>
</tr>
<tr>
<td>Second-early and maincrop potatoes</td>
<td>10,100</td>
<td>5,200</td>
<td>1,600</td>
<td>2,500</td>
</tr>
<tr>
<td>Hops</td>
<td>1,540</td>
<td>750</td>
<td>370</td>
<td>-</td>
</tr>
</tbody>
</table>

(From Department of Education and Science, 1969)

The use of DDT has been limited by agreement under the Pesticides Safety Precautions Scheme. Under this scheme, the manufacturers have undertaken not to:

(i) market a product containing any new chemical for use in agriculture and food storage,

or (ii) introduce a new use of a chemical already on the market,

or (iii) introduce a new formulation which could show an increased hazard,

until recommendations for safe use have been agreed with the Government Departments concerned (Ministry of Agriculture, Fisheries and Food, 1976).

DDT can be used alone or as a mixture with malathion or \( \gamma \)-BHC, in the control of the following: beetles, capsids,
caterpillars, cutworms, leafhoppers, leatherjackets, midges, millepedes, mushroom flies, thrips, sawflies, wasps, weevils, whiteflies and woodlice on agricultural and horticultural crops.

In the M.A.F.F. (1976) publication, it is stated that DDT is harmful to bees, fish and livestock. The minimal interval between the last application and harvesting edible crops must be 2 weeks, and between the former and access of animals and poultry to the treated areas, also 2 weeks. These statements show that it must be used with considerable caution.

The largest non-agricultural use of DDT in Britain is in the paint industry. Anti-fouling paints depend on the slow release of toxic substances for their action, and for this reason DDT and mercuric compounds are used here. The amount of DDT used annually in anti-fouling paints was estimated at 10.1 tonnes (of active chemical) (Dept. of the Environment, 1974). However, experts in this field believe that DDT is not an effective agent, and for this reason, it is unlikely that this pesticide would continue to be used in anti-fouling paints (Dept. of the Environment, 1974).

The amounts of DDT and other pesticides used in Britain by various industries and authorities are shown in Table 2.

b) IN THE UNITED STATES

U.S. domestic use of all organochlorine insecticides is declining. Approximately 70% of DDT produced in 1967 in U.S.A. was exported, mainly to underdeveloped countries e.g. India, Thailand, Brazil, Nepal and Mexico. Domestic usage was reduced nearly 50% between 1958 and 1966 (U.S. Dept. of Health, Education and Welfare, 1969). Fig. 1 shows the quantities of insecticides sold in the United States, persistent types being
<table>
<thead>
<tr>
<th>Area of use</th>
<th>( \gamma )-BHC</th>
<th>DDT</th>
<th>Dichlorvos</th>
<th>Dieldrin</th>
<th>Malathion</th>
<th>Mercury compounds</th>
<th>Penta-/Tetra-chlorophenols</th>
<th>Pyrethrin/Pyrethroid</th>
</tr>
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<tr>
<td>Home</td>
<td>8.1</td>
<td>0.8</td>
<td>34</td>
<td>1.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Woollen Industry</td>
<td></td>
<td></td>
<td>&lt;0.1</td>
<td></td>
<td>2.65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wood preservation (other than in home)</td>
<td>59.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paint Industry</td>
<td>10.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paper Industry</td>
<td></td>
<td></td>
<td>0.4</td>
<td></td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Textiles (other than wool)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Other Industries</td>
<td>0.4</td>
<td>0.2</td>
<td>0.4</td>
<td>0.17</td>
<td>7.6</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local Authorities (^3)</td>
<td>2.9</td>
<td>9.4</td>
<td>0.03</td>
<td>0.02</td>
<td>2.7</td>
<td></td>
<td></td>
<td>0.54</td>
</tr>
<tr>
<td>Central Government</td>
<td>about 2.6</td>
<td>about 2.5</td>
<td>0.01</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>about 73.8</td>
<td>about 23</td>
<td>34.2</td>
<td>22.2</td>
<td>10.3</td>
<td>34.8</td>
<td>&gt;1,250</td>
<td>12.5</td>
</tr>
<tr>
<td>Agriculture (^4)</td>
<td>143</td>
<td>114</td>
<td>0.5</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td>Nil</td>
</tr>
</tbody>
</table>

1. Includes 4.6 tonnes in wood preservatives
2. All in wood preservatives
3. Based on 1968 - 69 usage
compared with other insecticides.

Fig. 1  Sales of insecticides in United States

(Edwards, 1975)

Production of persistent pesticides has also dropped within the United States (Fig. 2), although production of all pesticides in general has increased (Fig. 3).
Fig. 2  Production of persistent pesticides (Edwards, 1975)

Estimated world production of DDT

U.S. production DDT

US production aldrin-toxaphene group

x1000 metric tonnes year⁻¹

years

Fig. 3  U.S. Production of pesticide chemicals (Edwards, 1975)

millions of pounds

years
The summer of 1976 saw the release of DDT in parts of the United States. Bubonic plague was discovered in rodents in six Western States - Colorado, California, New Mexico, Arizona, Wyoming and Nevada. California and Wyoming obtained permission from the Environmental Protection Administration to use DDT to dust burrows where plague-infected rodents lived, in order to kill the plague-carrying fleas found on the animals (The Daily Telegraph, July 19th 1976).

**WORLD-WIDE**

World-wide use of DDT is increasing. For example, in India, the use of 600 tonnes of DDT in 1964-5 has increased to 2,400 tonnes in 1968-9. Corresponding increases in the use of aldrin, dieldrin, heptachlor and chlordane were from 90 tonnes in 1964-5 to 1,050 tonnes in 1968-9. Underdeveloped countries find it difficult to afford DDT, a relatively cheap pesticide, and so it would be impossible to afford one of the less persistent, yet more expensive alternatives, recommended by other governments.

DDT has been used extensively by the World Health Organisation in its vector control programmes (W.H.O., 1971). It has been the main control agent for malaria, Chagas' disease, plague, typhus, yellow fever, haemorrhagic fever, encephalitis, filariasis, African trypanosomiasis, onchocerciasis and leishmaniasis. Suitable alternatives have now been found for all except malaria and trypanosomiasis. In 19 out of 124 countries where it existed, malaria has been eradicated, and another 48 national eradication programmes currently operate. Most of the success, however, has been obtained in the temperate and semi-tropical areas at the edge
of malaria distribution. Indeed, in 1955, W.H.O. said that malaria eradication was feasible, except in Africa South of the Sahara (Dr. M.W. Service, School of Tropical Medicine, Liverpool; Symposium on 'Ecological Effects of Pesticides' held by the Institute of Biology and Linnean Society, London; Sept. 1976).

Malaria control requires residual insecticides with lasting efficiency applied at relatively high levels. DDT is therefore useful in this context. The W.H.O. has tested more than 1,400 new insecticides as vector control agents since 1960, but only malathion (an organophosphate) and propoxur (a carbamate) can be recommended as being safe and efficient in large scale use.
3. CHEMICAL AND PHYSICAL ASPECTS OF DDT

a) MANUFACTURE

*p,p'-DDT* is prepared by the condensation of chlorobenzene and chloral hydrate in the presence of concentrated sulphuric acid or weak oleum at a temperature not greater than 20°C.

Other condensing agents such as hydrofluoric acid, anhydrous aluminium chloride, and fluorosulphonic acid may be employed.

b) PHYSICAL AND CHEMICAL PROPERTIES

Pure *p,p'-DDT* is a white crystalline solid melting at 108.5 - 109°C, above which it undergoes thermal decomposition to DDE and HCl. This is catalysed by FeCl₃, AlCl₃ and ultraviolet light.
Vapour pressure at 20°C is $1.9 \times 10^{-7}$ mm Hg.

DDT is stable in alcoholic permanganate and resistant to acid oxidation. In solution, it is readily dehydrochlorinated by alkalis and organic bases, at temperatures as low as 50°C.

Solubility in water is $1.2 \times 10^{-6} \text{ g.dm}^{-3}$ at 25°C (Bowman, Acree and Corbett, 1960). Its solubility in some solvents is:

- Cyclohexanone: $1160 \text{ g.dm}^{-3}$
- Ethanol: $20 \text{ g.dm}^{-3}$
- Acetone: $580 \text{ g.dm}^{-3}$
- 1,4-dioxan: $920 \text{ g.dm}^{-3}$

(A full list is given in Brooks, 1974a, adapted from West and Campbell, 1950).
4. POLYCHLORINATED BIPHENYLS

Polychlorinated biphenyls (PCBs) are a class of chlorinated aromatic compounds which have some similar properties to DDT.

\[
PCL = \begin{array}{c}
\text{Cl} \\
\text{Cl} \\
\end{array}
\]

\[
X = \text{Cl} \text{ or H}
\]

PCBs are mixtures of isomers prepared by chlorinating biphenyl. Arochlor 1260 (a PCB mixture produced commercially by the Monsanto Co. and containing 60% chlorine by weight) has 11 isomers, 5 of which contain 6 chlorine atoms, 5 containing 7, and 1 containing 8 chlorines (Koeman et al, 1969). Arochlor 1254 consists of 18 isomers (Bagley et al, 1970).

PCBs were first prepared in the 1880's and were used commercially after 1929.

\textbf{a) PHYSICAL AND CHEMICAL PROPERTIES}

PCBs are lipophilic, thermally stable compounds. They are resistant to oxidation, acids, bases and other chemical agents. Excellent dielectric properties result in their being used in dielectric fluids in capacitors and transformers. They
are also used as fire retardants since they are non-flammable. Other uses of PCBs are in industrial fluids (e.g. hydraulic systems, gas turbines and vacuum pumps), as plasticizers (in adhesives, textiles, surface coatings, sealants, printing and copy paper) and in heat-transfer processes. Further details of uses are given by Broadhurst (1972) and Hubbard (1964).

Some chlorobiphenyls were shown to have insecticidal (Deonier et al, 1946) and fungicidal (Bereha and Powell, 1953) properties. Although they were never used as pesticides, there was a recommendation for incorporation into pesticide formulations (Hornstein and Sullivan, 1953; Racine, 1958; and Tsao et al, 1953).

A great deal of detail on the chemical aspects of PCBs - synthesis, chemical reactions etc., is given in the book by Hutzinger et al (1974), and the environmental effects of PCBs, by Peakall (1975b).
5. PESTICIDE CYCLING AND LEVELS OF DDT IN THE ENVIRONMENT

The application of DDT to plants is a highly inefficient method, since most of the pesticide lands on the soil. According to Graham-Bryce of Rothamsted Experimental Station (Conference on 'Ecological Effects of Pesticides' - Institute of Biology and Linnean Society, London; Sept. 1976), the proportion of the applied dose taken up by insects varies from 0.02% (foliar spray) to 6% (aerial spraying of, for example, locust swarms).

a) SOIL

The persistence of organochlorine insecticides in the soil can be summarised in Fig. 4 by Edwards (1966).

Fig. 4 The Breakdown of organochlorine insecticides in soil (Edwards, 1966)
This work was estimated from knowing the half-lives of the various organochlorine compounds. Regression lines were then drawn. Edward's work was confirmed by Nash and Woolson (1967). They treated soils with pesticides and regularly analysed for residues. DDT and dieldrin persist longest in soils, followed by endrin, lindane, chlordane, heptachlor and aldrin in order of decreasing persistence.

The type of soil to which insecticides are applied affects their persistence. Heavier soils and those with much organic matter retain organochlorine insecticides longer than lighter ones. They are much less toxic to insects in heavy and organic soils because they are absorbed and inactivated in these (Edwards, 1975). This is shown in Fig. 5 with aldrin in different soil types.

Fig. 5  Relation between soil organic matter content and aldrin toxicity (Edwards et al, 1957)
Different soil fractions appear to be responsible for binding different insecticides, e.g. heptachlor and DDT appear to be inactivated by the clay fraction, diazinon and parathion by the sand and silt fraction, and dichlorofenthion by both (Harris, 1966). Soil type also influences the rate at which the insecticides are converted into other chemicals.

Organic matter seems to be the most important single soil factor influencing persistence of organochlorines. Also important, are the amount of colloidal material (Abdellatif et al, 1967; Edwards et al, 1957); soil hydrogen ion concentration; temperature; soil moisture; and shading effect and exposure of soil (Lichtenstein et al, 1964).

Most losses of DDT residues from soil can be accounted for by the following: (see also Fig. 6).

(i) volatilisation
(ii) removal by harvesting of organic matter or crops
(iii) water runoff
(iv) chemical degradation
(v) biological degradation (mainly microbial)

However, only a small portion will be volatilised, either through direct volatilisation or codistillation with water. The main portion will remain in the soil and be subject to degradation and to downward and lateral movement with soil water (Gerakis and Sficas, 1974).

Relatively little is known about the way in which organochlorine insecticides break down in soil, although it is known that microorganisms play a major role. Guenzi and Beard have monitored the rate of degradation of DDT in soils under aerobic (1976a) and anaerobic (1976b) conditions. In the
Fig. 6  Pesticide Cycling in the Environment (Fishbein, 1974)
former situation they measured the rate at which DDE was formed, the rates increasing with temperature and in the presence of water. Of the DDT mixed with Raber silty clay loam, 82.1%, 74.5%, 53.2% and 38.3% was recovered as DDT and 6.7%, 12.5%, 21.6% and 34.8% as DDE after 140 days incubation at 30°, 40°, 50° and 60°C, respectively.

They concluded that conversion of DDT to DDE in Raber soil was mainly a chemical process with some microbial activity. 16.4% of the conversion at 30°C was due to microbial degradation and 83.6% due to a chemical mechanism. The microbial portion decreased to 9.2% and the chemical increased to 90.8% at 60°C. The chemical conversion of DDT to DDE was influenced by water, temperature, and the physical and chemical soil properties.

It has been suggested (Downs *et al.*, 1951; Birrell, 1963) that chemical degradation occurs on iron oxides in the soil. Fe and Al oxides may exist as positively charged sites, and the DDT molecule possesses some electro-negative character due to the three Cl⁻ atoms. This could lead to the existence of a site for an adsorbed catalytic reaction.

The same soil type was incubated with DDT under anaerobic conditions (Guenzi and Beard, 1976b). These were created by flooding. The first identifiable intermediate product was DDD (1,1-dichloro-2, 2-bis (p-chlorophenyl) ethane) followed by DDMU (1-chloro-2, 2-bis (p-chlorophenyl) ethylene).
Degradation under anaerobic conditions is mainly as a result of microbial activity. The degradation of DDT and formation of DDD was found to be temperature-sensitive. The optimum temperature for degradation in flooded soil was near $60^\circ$C with no degradation at $2^\circ$C.

b) WATER BODIES

The presence of pesticides in water originates from surface runoff, industrial waste discharges, accidental spills, direct application, sewage effluents, air-drift, dead animals and animal excreta, and through food chains. Organochlorine pesticides are not usually in solution in water, as a result of their very low solubility. Residues are usually carried with particulate matter suspended in the water. Thus sedimentation is an important factor in removing organochlorines from water, high concentrations being found in the mud of lakes and estuaries, in particular (Table 3) (Albone et al, 1972a).

According to Nicholson (1970) and Nicholson and Hill (1970) surface runoff (pesticide adsorbed to soil particles and carried mainly by rain water) is considered the single
most important source. However, Caro and Taylor (1971) using dieldrin showed that this amount was usually very small. Risebrough (1971) suggests that only about 0.1% of the DDT produced is carried by surface runoff and rivers to the sea. Also, he estimated that 25% of DDT produced has been carried via the atmosphere and rain to the oceans. A mathematical model has been developed describing the quantity of runoff water from separate rainfall events on a watershed, and the rate and quantity of sediment and pesticides transported (Bruce et al, 1975).

Harvey and Miklas (1972) studied the levels of DDT residues in phytoplankton of the North and South Atlantic Ocean. They concluded that the atmosphere is the major path for carrying DDT to the oceans. Their reasons were:

(i) there was a uniformity of chlorinated hydrocarbon concentrations in plankton across the North Atlantic consistent with these compounds being added directly from the atmosphere,

(ii) there was little difference in the average concentrations of these pollutants between the North and South Atlantic. Considering the different levels of industrial activity and lack of north to south ocean circulation, atmospheric transport from Europe and N. America to the Southern Hemisphere seems the most likely explanation. Transport of this kind has been shown by atmospheric movement of various tracers, including radioactive fallout,

(iii) the high concentration of PCB's in a plankton sample collected off Iceland in the E. Greenland current bringing water from the Arctic, could only be explained in terms of atmospheric transport, since ocean circulation is generally slow.

Once a pesticide enters a body of water, it may be volatilised to the atmosphere, remain in the water insolution or suspension, or be deposited as sediment. Some pesticides persist longer in water than soils (Schwarz, 1967; Eichelberger and Lichtenberg, 1971).
In contaminated water, much of the pesticide appears to concentrate in the bottom sediments, resulting in much higher levels here than in the water (Tanaka, 1971; Duke et al, 1970; Miles and Harris, 1973; Albone et al, 1972a). Pierce et al (1974) studied the adsorption and desorption of DDT to suspended particulates in aqueous solutions. They investigated the adsorption to a marine sediment, sediment fractions, clay and humic acid suspended in sea water. The latter was found to have a greater adsorbing capacity than clay or sediment. Removal of the humic acid fraction from sediments reduced the adsorption capacity to less than 50% of the original sediment sample.

The suspended humic particulates may be important agents for transporting chlorinated hydrocarbons through the water columns and for concentrating them in sediments and detritus-feeding organisms.

The levels of DDT residues and PCB's in some parts of the environment are summarized in Table 3 (Rhead, 1975).
<table>
<thead>
<tr>
<th>Environment</th>
<th>DDT mass (µg kg(^{-1}))</th>
<th>PCB mass (µg kg(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainwater</td>
<td>0.08 (av. value)</td>
<td>up to 50</td>
<td>Tarrant &amp; Tatton, 1968</td>
</tr>
<tr>
<td>Air-borne dust</td>
<td>up to 50</td>
<td></td>
<td>Risebrough et al, 1968</td>
</tr>
<tr>
<td>Silt-free rivers</td>
<td>0.001 - 0.005</td>
<td></td>
<td>Risebrough et al, 1971</td>
</tr>
<tr>
<td>Silty rivers</td>
<td>0.005 - 0.12</td>
<td></td>
<td></td>
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<tr>
<td>Lake water (Michegan)</td>
<td>0.001 - 0.003</td>
<td></td>
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<tr>
<td>Seawater (incl. particulate matter)</td>
<td>0.0023 - 0.1</td>
<td></td>
<td></td>
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<tr>
<td>Highly polluted harbour (Port of Tagonoura, Japan)</td>
<td>16.8 - 23.6</td>
<td>0.3 - 1.8</td>
<td>Tanaka, 1971</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td></td>
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<tr>
<td>Sediment (off W. USA)</td>
<td>0.04 - 1.5</td>
<td></td>
<td>Claeys, 1972</td>
</tr>
<tr>
<td>Sediment</td>
<td></td>
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</tbody>
</table>
Table 3 (cont'd.)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Range of Values</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plankton (wet wt)</td>
<td>0.0002 - 0.0026</td>
<td>0.007 - 0.64</td>
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<tr>
<td>Plankton (lipids)</td>
<td></td>
<td>100 - 900</td>
</tr>
<tr>
<td>Plankton</td>
<td>0.01 (av. value)</td>
<td>0.02 - 1.2</td>
</tr>
<tr>
<td>Invertebrates</td>
<td></td>
<td>30 - 200</td>
</tr>
<tr>
<td>various (wet wt)</td>
<td></td>
<td>0.5 - 2.0</td>
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<tr>
<td>pink shrimp (wet wt)</td>
<td></td>
<td>0.01 - 0.07</td>
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<tr>
<td>Fish (whole)</td>
<td>0.02 - 14.0</td>
<td>0.02 - 1.2</td>
</tr>
<tr>
<td>Marine mammals (blubber)</td>
<td>0.4 - 800</td>
<td>30 - 200</td>
</tr>
<tr>
<td>seal (fat from tail)</td>
<td></td>
<td>0.35 - 24</td>
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<tr>
<td>Birds</td>
<td></td>
<td>53 - 697</td>
</tr>
<tr>
<td>whole body</td>
<td>3.0 - 66</td>
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<tr>
<td>body fat</td>
<td>41 - 199</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Harvey &amp; Miklas, 1972</td>
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<tr>
<td></td>
<td></td>
<td>Goldberg, 1971</td>
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<tr>
<td></td>
<td></td>
<td>Tatsukawa &amp; Isono, 1971</td>
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<tr>
<td></td>
<td></td>
<td>Claeys, 1972</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jensen et al, 1969</td>
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<tr>
<td></td>
<td></td>
<td>Risebrough, 1971</td>
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<tr>
<td></td>
<td></td>
<td>Wolman &amp; Wilson, 1970</td>
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<td></td>
<td></td>
<td>Butler, 1968</td>
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<tr>
<td></td>
<td></td>
<td>Tatsukawa &amp; Isono, 1971</td>
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<tr>
<td></td>
<td></td>
<td>Risebrough, 1971</td>
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<td>&quot;</td>
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</tbody>
</table>
6. BREAKDOWN OF DDT

a) IN ATMOSPHERE

DDT is persistent in soil and water. In the atmosphere, however, it can become degraded by ultra-violet light. Fleck (1949) showed that when u.v. radiation catalysed the decomposition of DDT, different products were obtained depending on the presence or absence of air. Lindquist et al (1946) decreased the effectiveness of DDT after it was subjected to u.v. radiation. This decrease in activity was shown using a housefly bioassay.

Pokrovskii and Bel'Kevich (1958) reported 40 – 80% DDT losses following exposure to u.v. radiation and sunlight. Dehydrochlorination of DDT to DDE, conversion of DDE to other products, and dehydrochlorination of DDD were demonstrated by Roburn (1963) when DDT was exposed to u.v. radiation of 253.7 nm. However, the ozone layer filters out nearly all the u.v. radiation below 290 nm before it reaches the earth's surface (Platt and Griffiths, 1964). Using u.v. radiation of 300 – 400 nm, Baker and Applegate (1973) demonstrated the degradation of DDT and other pesticides. They did not totally identify and quantify the products, but found that amongst them were DDE and dibenzophenone (DBP).

\[
\begin{align*}
\text{Cl} & \quad \text{C} \quad \text{Cl} \\
\text{O} & \\
\text{DBP}
\end{align*}
\]
A finding of possible environmental significance was made in 1973 by Moilanen and Crosby, when they suggested that DDT could be a source of PCB's by irradiation with naturally-occurring u.v. light (Fig. 7).

Their scheme predicts that DDT vapour is converted to small amounts of DDD, which accumulate in the biosphere, but mostly to DDE. This vapour is then converted to dichloro-benzophenone via DDMU, and then at a very slow rate to dichlorobiphenyl. Some of the DDE is also converted to 3,6-dichlorofluorenone and to small amounts of di, tri-, and tetra-chloro-biphenyls. The fate of 3,6-dichlorofluorenone is unknown.

b) BY MICROORGANISMS

The breakdown of DDT by organisms has been studied in many groups of animals and plants. At the present time there are still various routes which have not yet been worked out. Fries (1972) has presented an informative and critical review of the degradation of chlorinated hydrocarbons, especially DDT.

O'Brien in 1967 suggested there were five principal routes of DDT metabolism, although his views are not now held to be correct:

(i) oxidation to DDA,
(ii) oxidation to kelthane,
(iii) oxidation to DBP,
(iv) dehydrochlorination to DDE,
(v) reductive dechlorination to DDD.

More recent reviews show three or four degradation routes of DDT (Brooks, 1974; Fishbein, 1974) i.e. DDE, DDD, kelthane and DDCN.
Fig. 7 Proposed scheme for degradation of DDT vapour in sunlight (Moilanen and Crosby, 1973)

H - C - C - C - Cl

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H - C - C - C - Cl

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DDD

DDT

Cl

Cl

C = C - C - Cl

C = O

Cl

Cl

DDMU

DBP

PCB

Cl

Cl

Cl

Cl

Cl

Cl

C - C - C - C - Cl

Cl

Cl

3,6-dichlorofluorenone

DDE

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Most work appears to have been carried out on metabolism of DDT by micro-organisms, since these are abundant in soil, sewage, and in the mammalian gut, among other sources. DDD is the most abundant product formed by micro-organisms. Also, DDE, DDMU, DDMS, DDNU, DDA, DPM, DBH, DBP and kelthane have been reported as minor products.
DDMS
1-chloro-2,2-bis(p-chlorophenyl)ethane

DDNU
1,1-bis(p-chlorophenyl)ethylene

DDA
bis(p-chlorophenyl)acetic acid

DPM (DDM)
pp-dichlorodiphenylmethane

DBH
(dichlorobenzohydrol-) conjugate
Albone et al (1972b) and Jensen et al (1972) reported DDCN as the main product of the degradation of DDT in anaerobic sewage sludge. DDT degrades more rapidly in soils under anaerobic conditions than in well-aerated soil, DDD being the major product in the former situation (Parr et al, 1970; Guenzi and Beard, 1967; Burge, 1971).

Patil et al (1970) studied the ability of 20 microbial cultures to degrade endrin, aldrin and DDT. They found that most of the organisms converted DDT to DDD (TDE), and none produced DDE. Some also formed DDA and a dicofol-like compound, showing that the DDT-degradation pathways are complex.

Wedemeyer (1967) proposed a pathway from DDD to DBP (Fig. 8) in *Aerobacter aerogenes* by incubating the intermediates with the micro-organism and examining the products formed. However, beyond DDNU, none of the products could be identified after aerobic or anaerobic incubation of any of the preceding metabolites with *A. aerogenes*. For example, DDA was found only after anaerobic breakdown of DDNU in the presence of glucose as an energy source. DBP was found after anaerobic breakdown of DDA, only when no other breakdown products were present.

The formation of ring-cleavage products from DDT by micro-organisms has been demonstrated by Pfaender and Alexander (1972). *Hydrogenomonas sp.*, a sewage bacterium, was grown in a medium which contained DPM, and *Arthrobacter sp.* (also a sewage bacterium) was cultured in a medium containing PCPA. Extracts of the DPM-grown *Hydrogenomonas sp.* incubated anaerobically with DDT, degraded it to DDD, DBP, DDMS, DDMU and DDE. On addition of oxygen and fresh DPM-grown cells, p-chlorophenyl acetic acid (PCPA), a ring-cleavage product, was formed.
Fig. 8  Degradative pathway of DDT in the environment  
(adapted from Fishbein, 1974 and Wedemeyer, 1967)
*Arthrobacter sp.* was able to form p-chlorophenylglycolaldehyde and other products from PCPA.

\[
\begin{align*}
\text{Cl} & \quad \text{C} & \quad \text{C} & \quad \text{O} \\
\text{H} & \quad \text{I} & \quad \text{C} & \quad \text{O} & \quad \text{H}
\end{align*}
\]

PCPA

The pathway from DDCN to DBP (Fig. 8) was elucidated by anti-oxidation in ethanolic potassium hydroxide. Its tendency to form DBP on treatment with bases is a reaction typical of the dicofol-like substances also formed from DDT in different biological systems. In the studies of Jensen *et al* (1972), it was not certain whether DDCN was formed by direct reaction or via a metabolic pathway.

Micro-organisms are also able to degrade some of the lower chlorinated PCBs. In experiments by Baxter *et al* (1974) on single biphenyl compounds and simple mixtures, many of the lower chlorinated biphenyls (≤ 3 chlorine atoms per molecule) are readily degraded by *Nocardia sp.* and *Pseudomonas sp.* Products were not identified, degradation being measured as the loss of the original compound. Compounds containing up to six Cl⁻ atoms could be degraded under favourable circumstances. In experiments on more complex mixtures such as Aroclor 1242, it was found that some of the lower chlorinated compounds, which were only very slowly degraded alone, degraded quite readily when present in a mixture. This is probably explained by "mutual solubilisation".
c) **BY FISH AND SHELLFISH**

Grzenda *et al* (1970) analysed the DDT residue content of the various parts of the body of goldfish after being fed $^{14}$C-DDT. Since muscle had the lowest mean residue content, the relative distribution of residues among tissues was compared by using the concentration in muscle as a common denominator and the content of each of the other tissues as numerators (Table 4).

No correlation was noted between the per cent lipid content of the tissues and the relative distribution quotients. With the exception of spleen and liver, which also contained DDD, the only metabolite detected at 8 days was DDE. In some individuals, DDD was occasionally noted in blood, kidney, immature ovary and gall bladder. Wedemeyer (1968) also found that DDT was degraded to DDD and DDE in rainbow trout - owing to intrinsic liver dehydrochlorinase activity and intestinal micro-flora.

Work done by Pritchard *et al* (1973) showed that most DDT residue was in the carcass of the winter flounder. This included skin and bone, as well as muscle. They do not state whether subcutaneous fat was included, whereas it was excluded by Wedemeyer (1968). At all times throughout the one-week experiment, DDT accounted for more than 90% of the residue content. Small quantities of DDD and DDE were present. They also identified DDA and possibly DBP. Dvorchik and Maren (1972) worked on dogfish (*Squalus acanthias*) and found that the liver took up the bulk of the DDT injected into the blood stream, and was retained here rather than be distributed around the body to such an extent as in other fish.
Table 4  DDT residue content in parts of goldfish relative to the muscle content after being fed $^{14}$C-DDT
(from Gzenda et al, 1970)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>1.0</td>
</tr>
<tr>
<td>Immature ovary</td>
<td>1.5</td>
</tr>
<tr>
<td>Blood</td>
<td>1.5</td>
</tr>
<tr>
<td>Gill</td>
<td>1.5</td>
</tr>
<tr>
<td>Skin</td>
<td>2.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.5</td>
</tr>
<tr>
<td>Stomach</td>
<td>3.5</td>
</tr>
<tr>
<td>Intestine</td>
<td>3.5</td>
</tr>
<tr>
<td>Gall bladder</td>
<td>3.8</td>
</tr>
<tr>
<td>Kidney</td>
<td>4.0</td>
</tr>
<tr>
<td>Liver</td>
<td>5.0</td>
</tr>
<tr>
<td>Brain</td>
<td>5.0</td>
</tr>
<tr>
<td>Faeces</td>
<td>6.0</td>
</tr>
<tr>
<td>Nerve</td>
<td>12.5</td>
</tr>
<tr>
<td>Testes</td>
<td>13.5</td>
</tr>
<tr>
<td>Mesenteric adipose</td>
<td>23.0</td>
</tr>
</tbody>
</table>

This is because of the large size of the liver (10 - 17% of body weight) and its high lipid content (40 - 60% of wet weight). Therefore, the distribution and toxicity of a pesticide will vary considerably depending upon the type of fish.

When sole (Solea solea) were fed $^{14}$C-DDT, it was found that the concentration in skeletal muscle was the lowest, with brain, liver and gastro-intestinal tract the highest (Ernst and Goerke, 1974). Again, DDE, DDD and a polar compound were formed as metabolites, but in all organs more than 80% of the accumulated DDT remained unchanged.

Using catfish (Heteropneustes fossilis), Agarwal and Gupta (1974) found only DDE as the metabolite, which was found mostly in the kidney, with slightly less in the liver and fat bodies. Their results indicated that signs of poisoning in the fish were strictly related to the concentration of DDT in the brain and spinal cord.
In the lobster (*Homarus americanus*) 90% of the administered $^{14}\text{C-}\text{DDT}$ was found in the hepatopancreas after 7 day's exposure. There was little difference in distribution, whether the pesticide was administered intravascularly, orally or through exposure to the ambient water (Guarino *et al*, 1974) (Table 5 & 6). Like the dogfish liver, the lobster hepatopancreas contains large amounts of fat. The most abundant metabolite seen was DDD, and only about 2% of the radioactivity occurred as DDE and DDA.

**Table 5** TLC assay for DDT and metabolites in Lobster hepatopancreas (Guarino *et al*, 1974)

<table>
<thead>
<tr>
<th>Time after injections (hrs)</th>
<th>% of total radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DDT</td>
</tr>
<tr>
<td>4</td>
<td>92.8 ± 3.3</td>
</tr>
<tr>
<td>24</td>
<td>90.8 ± 1.3</td>
</tr>
<tr>
<td>48</td>
<td>91.0 ± 2.7</td>
</tr>
</tbody>
</table>

**Table 6** Tissue distribution of $^{14}\text{C-}\text{DDT}$ in the lobster 7 days after exposure from water or food (Guarino *et al*, 1974)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ambient Water</th>
<th>Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>90.7</td>
<td>90.5</td>
</tr>
<tr>
<td>Green gland</td>
<td>3.5</td>
<td>4.6</td>
</tr>
<tr>
<td>Heart</td>
<td>0.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Intestine</td>
<td>4.1</td>
<td>6.2</td>
</tr>
<tr>
<td>Male gonad</td>
<td>1.7</td>
<td>2.6</td>
</tr>
<tr>
<td>Egg mass</td>
<td>3.2</td>
<td>5.1</td>
</tr>
<tr>
<td>Brain</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Gill</td>
<td>0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.9</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Tissue values are mean percent of recovered pesticide for 6 animals treated via each route.
Sheridan (1975) working with the blue crab (*Callinectes sapidus*) and Martin *et al* (1973) working with crayfish (*Astacus leptodactylus*) show similar findings in that the hepatopancreas contains the highest levels of DDT. Martin found that decreasing concentrations were found in the ovary, gills, cerebral ganglia, digestive tract, muscles and exoskeleton. They do not note any of the metabolic products of the pesticide, while Sheridan reported a more rapid conversion to DDD and DDE (particularly the former), than was found by Guarino *et al* (1974). Sheridan concluded that the site of metabolism was the hepatopancreas.

d) **BY MAMMALS, AND ITS DISTRIBUTION IN THE BODY**

This aspect has been well-documented in recent years with respect to man in particular, and also to other mammals (Hayes, 1965; Morgan and Roan, 1971; Roan *et al*, 1971; Peterson and Robison, 1964; amongst others).

Initial degradation of DDT is either by dehydrochlorination to give DDE or by substitution of hydrogen to give DDD. DDD readily degrades further through a series of intermediates to give DDA, which is excreted and rarely found as a stored metabolite. If DDE breaks down at all, then it is extremely slowly.

Peterson and Robison (1964) fed rats DDT, DDE and DDD, and then analysed the livers for residues. Those maintained on a diet of 1500 mg. kg$^{-1}$ of DDT, after 6 days, gave a ratio for DDT: DDD:DDE of 3:5:1. Rats which had been administered 1000 mg. kg$^{-1}$ doses for 6 days by stomach tube, contained in the liver a ratio for DDD:DDMU of 1:13, showing the rapid breakdown of the former. Liver and kidney samples from DDE-
treated rats showed only unchanged DDE. Also, urine from rats fed DDE contained no detectable DDA.

White et al (1945) postulated a 2- or 3-step conversion of DDE to DDA; and more recently Datta (1970) has suggested the same. According to Datta, after a one-step conversion to either DDE or DDD, detoxication follows two different pathways in rats:

(i) \[ \text{DDD} \rightarrow \text{DDMU} \rightarrow \text{DDMS} \rightarrow \text{DDNU} \rightarrow \text{DDOH} \rightarrow \text{DDA} \];

(ii) \[ \text{DDE} \rightarrow \text{DDMU} \rightarrow \text{DDNU} \rightarrow \text{DDOH} \rightarrow \text{DDA} \].

He suggests that both pathways operate simultaneously in intact animals, and that the predominance of either pathway depends on the physiological response or the amount of toxicant used.

Storage of metabolites, especially DDE occurs in adipose tissue, since this has a high lipid content. Quantities stored in human adipose tissue are in the order \( p,p'-\text{DDE} > p,p'-\text{DDT} > o,p'-\text{DDT} > p,p'-\text{DDD} \) (Morgan and Roan, 1971). Concentrations in serum are similarly related. Loss of \( p,p'-\text{DDT} \) from adipose storage is very slow, while stored \( p,p'-\text{DDD} \) is excreted much
more rapidly. The fate of much of the stored DDT is unknown (Morgan and Roan, 1972). There seems to be no increase in the amount converted to DDE and the total amount of DDA excreted in the urine does not account for more than one third of the DDT lost in their subjects. Excretion of DDT through the bile into the faeces has been shown in the rat (Jensen et al, 1957; Barnes et al, 1957), but little work has been carried out on faecal excretion in man.

It has been suggested that DDCHO (2,2-bis (p-chlorophenyl) acetaldehyde) is an intermediate between DDOH and DDA in the breakdown of DDT. Suggs et al (1970) were able to synthesise this compound, although it has not yet been found to occur in vivo. DDOH is a substrate of crystalline liver alcohol dehydrogenase, and DDCHO was identified as a product in mass spectrographic and thin-layer chromatographic studies, although it is highly unstable.
7. **THE EFFECTS OF DDT ON ORGANISMS**

a) **INTRODUCTION**

Several authors have summarised the responses shown by organisms that have been used to detect and measure pollution effects of, for example oil, pesticides, PCBs and heavy metals. These have been designed mainly for fish studies, but can be applied to other organisms. (Anderson *et al.*, 1974; Alderdice, 1967; Waldichuk, 1973; Sprague, 1971; and Bayne, 1975).

Possible means of determining sublethal effects of pollutants (Anderson *et al.*, 1974)

Physiological and behavioural aspects of the individual organism may be studied, or factors such as growth and reproduction, which lead to the survival of a whole population. Often it is necessary to correlate an effect in the whole body or in a particular tissue with the pollutant concentration in that area.

(i) **Accumulation and Release**

a) measurement of the extent of accumulation.

b) measurement of the rates of uptake and release.

c) measurement of the specific composition of accumulated and depurated substances.

d) identification of the sites of exchange with the environment and storage within the organism.

e) correlation of tissue content with toxicity data.

f) food web accumulation.

ii) **Physiological Studies**

a) metabolism - whole organism respiration
   - tissue respiration
   - homogenate respiration
   - enzyme activity
b) osmoregulation - decrease in rate and extent of regulation
   - change in ionic composition

c) feeding and nutrition

d) chemical analyses - change in relative composition of carbohydrate, proteins and lipids
   - change in concentration of specific amino acids and metabolic intermediates
   - increase in mucus production
   - alteration in blood proteins

(iii) Behavioural Studies

a) loss of equilibrium

b) modification of locomotor patterns

c) modification of threshold for detection of nutritive or reproductive chemical stimuli

(iv) Reproduction

a) alteration in breeding behaviour

b) interference with spawning

c) decrease in gamete production

d) decrease in success of fertilisation

(e) decrease in survival of larval or juvenile stages

f) decrease in capacity of breeding by second generation

(v) Growth

a) decrease in rate of cell production

b) decrease in rate of growth

c) modification of the number of larval stages or adult molts

d) modification of correlation between various allometric relationships

(vi) Histological Studies

a) development of abnormal growths

b) damage to respiratory or sensory membranes
c) damage to reproductive organs

In its natural habitat where an organism is exposed to a pollutant, the environmental parameters will not remain constant. Changes in temperature or salinity, for example, may lead to synergistic effects with the pollutant, or may reduce the effect of the pollutant.

(vii) Interactions

a) temperature - pollutant combinations
b) salinity - pollutant combinations
c) temperature - salinity - pollutant combinations
d) dissolved oxygen - pollutant combinations
e) multiple pollutant combinations

When an animal is exposed to a change in its environment, its physiological and other processes respond in three stages:

a) immediate response
b) stabilisation of response
c) new steady-state for the particular process. This may be the same as the condition preceding the environmental change, or it may be significantly different (Bayne, 1975)

Therefore, to note any effect of a pollutant, it is necessary to bear these stages in mind and constantly monitor a particular process.

Animals with large amounts of fat appear less susceptible to the effects of DDT, since the pesticide immediately collects in these areas and is not easily dispersed. Those in a fasting state or receiving an inadequate diet, and also young animals, are much more susceptible to the acute toxicity of DDT.

The effects of DDT on organisms that may be measured are numerous. A summary of the more important and well-documented effects will be given here.
b) **EFFECT ON PLANTS**

Relatively little is known about the effects of DDT on plants. Of this, the majority concerns freshwater and marine algae, which are very important food sources for many aquatic animals.

The most significant find is that photosynthesis and growth of algae are inhibited. Wurster (1968) reported photosynthetic inhibition in four species of marine phytoplankton at concentrations as low as a few $\mu g \ dm^{-3}$. Above $100 \mu g \ dm^{-3}$ the inhibition levelled off. These effects depend on the species involved (Menzel et al, 1970). *Dunaliella tertiolecta* from tide-pools and estuaries was unaffected with respect to photosynthetic and growth rates at concentrations of DDT up to $1000 \mu g \ dm^{-3}$; *Cyclotella nana* from the Sargasso Sea was inhibited in $^{14}C$-uptake by concentrations above $1 \mu g \ dm^{-3}$. This probably demonstrates the adaptability of an estuarine species to the more unstable conditions of its environment compared with an open ocean species. It also shows that DDT and other pesticides could alter the species composition of a community.

It is difficult to assess how DDT affects photosynthesis. It is believed that it inhibits electron transport (Bowes and Gee, 1971), or interferes with oxidative phosphorylation or photophosphorylation (Clegg and Koevenig, 1974). The latter found that adenosine triphosphate (ATP) levels were reduced in three species of freshwater algae exposed to $100 \mu g \ dm^{-3}$ of DDT and other pesticides. This is produced in the light reaction of photosynthesis; while in the dark reaction, $CO_2$ is reduced and incorporated into various organic compounds.
using ATP and NADPH$_2$.

A more recent report (Fisher, 1975) shows, using three species of marine algae that the process of photosynthesis is not inhibited by DDT and PCBs. It was found that the $^{14}$C-uptake per cell, which is used as a direct assessment of photosynthesis, was unaffected. So, although there was 72% inhibition of photosynthesis by *Thalassiosira pseudonana* using 50 $\mu$g dm$^{-3}$ of DDT, this was owing to a reduction in cell-division rates.

Inhibition of growth has also been shown in an aquatic angiosperm, *Spirodel*a by a PCB-Aroclor 1242 (Mahanty, 1975). 100 $\mu$g dm$^{-3}$ PCB proved to be lethal, but all concentrations used below that were effective in reducing growth. Colony-growth was affected, as was frond-growth of individuals. Also, chlorophyll-synthesis seemed to be affected, because white stripes alternating with green tissue, a characteristic pattern of chlorosis, developed in treated plants.

Chlorinated insecticides can affect a plant's physiology in such a way as to alter the synthesis of certain amino acids. In maize (*Zea mays*), Thakre and Saxena (1972) found that p,p'-DDT, aldrin, endrin and lindane stimulated the synthesis of arginine and histidine, leucine, lysine, proline and tyrosine, but decreased the content of tryptophan. Synthesis of methionine was stimulated by aldrin, endrin and lindane only. Synthesis of valine was inhibited by aldrin, endrin and DDT only.

c) **EFFECT ON MAN**

There is no evidence that anyone has ever died from accidental, uncomplicated DDT-poisoning. Hayes (1955) has
given a very detailed review of the nervous effects of DDT on man. After a dose of 20g., poisoning is characterised by paralysis of the tongue, lip and part of the face. The patient then suffers from a sense of apprehension, disturbance of equilibrium, dizziness, confusion and tremor. In severe poisoning, convulsions may occur and there may be paralysis of the hands. General symptoms include malaise, headache and fatigue.

Many incidences of dermatitis have been reported (Higgins and Kindel, 1949; Hollander, 1950), mainly associated with direct action of the compound on the skin, although some have been thought to be associated with absorbed DDT.

Some volunteer prisoners in the United States were fed DDT, at the rate of either 3.5 mg. man\(^{-1}\) day\(^{-1}\) or 35 mg. man\(^{-1}\) day\(^{-1}\). During the entire study, which ranged up to 18 months, no volunteer complained of any symptom or showed any sign of illness that did not have an easily recognised cause unrelated to exposure to DDT. The tests carried out were on weight change, haemoglobin level, red blood cell count, white blood cell count, percentage of polymorphonuclear leukocytes, heart rate, systolic blood pressure and pulse pressure, and plasma cholinesterase level (Hayes et al, 1956).

Barnes (1966) considered the possible carcinogenic hazards to man from eating food containing pesticide residues. Experiments on rats with DDT (Fitzhugh and Nelson, 1947), aldrin and dieldrin (Fitzhugh et al, 1964); and also on mice with aldrin and dieldrin (Davis and Fitzhugh, 1962) showed that these pesticides did not behave like typical liver carcinogens (Barnes, 1966). Fitzhugh and Nelson (1947) fed
rats a diet containing respectively 100, 200, 400, 600 and 800 mg. kg\(^{-1}\) of DDT. Of 75 rats surviving 18 months or more, 15 had some liver tumours. There was no reference to the frequency of tumours in the different groups. Other experimental tests (referred to above) show that there was no increase in tumour incidence with increased intake of pesticides.

PCBs have been recorded as causing deaths amongst humans. In fatal cases, they caused yellow atrophy of the liver; and in chronic exposure, dermatitis and fatty degeneration of the liver have been found.

In February 1968, about 700,000 chickens died in Western Japan, the cause attributed to oil manufactured by the Kanemi Oil Company (Tatsukawa and Isono, 1971). Symptoms then began appearing in humans. It was not until November that the cause was traced to PCBs in the rice-bran oil. PCBs were used as a heating medium for removing odour from the oil and a leak had developed in a pipe resulting in 2000-3000 mg dm\(^{-3}\) of PCB becoming mixed with the oil. 14,000 people reported the illness, but only 1000 people were confirmed as victims of the PCB poisoning.

It is impossible to determine many of the sublethal effects on man of both DDT and PCBs. Consequently most work on vertebrates is performed on small mammals, such as mice and rats, or on fish.

d) BEHAVIOUR

Much of the work on DDT and behaviour has been carried out using fish.
(i) Learning

Under favourable experimental conditions, salmonids treated with sublethal doses of DDT are capable of learning a conditioned avoidance response (Jackson et al, 1970). The results indicated that sublethal doses of DDT (20-100 μg dm⁻³ in the water do not actually affect learning, but the effects produced by DDT can, under certain circumstances, alter the ability of the fish to perform the tasks involved. McNicholl and Mackay (1975) showed that DDT at the 96 hr LD 50 dose (0.03 mg DDT g⁻¹ body weight) increased the rate of learning.

(ii) Activity and Schooling

The locomotor activity of the Atlantic salmon acclimated to a particular temperature was found to be increased after exposure to DDT (Javaid, 1972), when compared with untreated animals. The work of Weis and Weis (1974) on goldfish confirms this observation. After three days of exposure to 1 μg dm⁻³ DDT, groups of five fish showed significant increases in activity of individuals, school size and angular deviation. In response to a fright stimulus, treated schools scattered further and did not regroup as soon as the control schools. This type of activity indicates that DDT-treated fish may be more susceptible to predation. The effects of DDT disappeared after the fish had been in clean water for a week.

In a novel environment, goldfish display highly specific non-random exploratory behaviour. Aspects of this behaviour are significantly affected by exposure to 10 μg dm⁻³ DDT for four days; but the effects decreased after maintaining for 20 hours in clean water (Davy et al, 1973).
(iii) Rugophilic responses of barnacles

Barnacle cyprids show a consistent tactile response to surface contour (rugophilic response), preferring holes, grooves and scratches for settlement (Crisp and Barnes, 1954). Meith-Avcin (1974a, b) treated settlement plates with DDT and investigated the rugophilic response pattern. He found that there was generally a lowered settlement density in the presence of DDT, and lowered indices of preference for roughened surfaces. It was concluded that DDT interfered with the tactile discriminatory response, rather than acting as a mechanism which mechanically or physiologically prevented settlement. Exposure of adult barnacles to DDT resulted in anomalous behaviour, the incidence of which increased with increased concentration of DDT (Meith-Avcin, 1974b). Observations included wide gaping of the valves, lateral valve swinging, irregularity of rhythms, trapping of unretracted cirri between closing valves, twisting movements, clumping and curling of cirri, rapid palpitation of cirri, and interruption of normal behavioural patterns.

e) OSMOREGULATION AND ATPASES

(i) Osmoregulation

The body fluids of aquatic organisms can be either hypotonic to the medium (lower ionic concentration than the surrounding water), hypertonic (higher ionic concentration than the water) or isotonic (body fluids are of an equal concentration to the water). Freshwater fish (e.g. goldfish) are generally hypertonic to their environment, while marine fish are generally hypotonic. Fig. 9 shows diagrammatically the mechanisms by which a marine fish maintains a constant
osmotic concentration against a concentration gradient.

**Fig. 9** Water and salt transport in a salt water teleost
(from Prosser and Brown, 1961 and Kinter et al, 1972)

The fish maintains hypotonicity by drinking seawater, absorbing this water plus salts across the intestinal epithelium, and then secreting NaCl across the gill epithelium while retaining free water. The primary mechanism in both gill and intestine is the sodium pump, with which the enzyme Na\(^+\), K\(^+\) – ATPase (Na\(^+\), K\(^+\) – ATP-phospho-hydrolase) is involved.

Crustacea also exhibit osmoregulatory responses in different salinities. *Maia squinado* and *Portunus depurator*, for example, maintain their body fluids isosmotic with the medium over the range of salinity tolerated. In others such as *Carcinus maenas* and *Cancer magister*, the fluids are hyperosmotic to the medium when in dilute, and isosmotic in more
concentrated salinities; while in *Eriocheir sinensis* and *Artemia salina*, the fluids are hyperosmotic in low concentrations (Lockwood, 1962). ATPases and the permeability of the exoskeleton are involved in these mechanisms.

(ii) **Function and characteristics of ATPases**

In maintaining an osmotic balance against an electrochemical gradient, the process of active transport must occur. Ions are transported across cell or plasma membranes using energy, which originates from the breakdown of ATP (adenosine triphosphate) to ADP (adenosine diphosphate). ATP contains a high-energy phosphate link, the breaking of which releases energy and inorganic phosphate.

\[
\text{ATP} \rightarrow \text{ADP} + \text{Pi} + \text{energy}
\]

This reaction is catalysed by ATPases. A number of types are now known, such as Na\(^+\), K\(^+\) - ATPase, Mg\(^{2+}\)-ATPase and Ca\(^{2+}\) - ATPase, all of which are membrane-bound. Most information is known about the former enzyme. Na\(^+\), K\(^+\) - ATPase is associated with the transport of Na\(^+\) and K\(^+\) ions across cell and plasma membranes. The characteristics of this enzyme are enumerated below (Skou, 1965):

1. Located in cell membrane
2. Affinity for Na\(^+\) that is higher than for K\(^+\) at a site located on the inside of the cell membrane.
3. Affinity for K\(^+\) that is higher than for Na\(^+\) on the outside of the membrane.
4. Catalyses the hydrolysis of ATP and thus converts energy from ATP into movement of cations.
5. Capable of hydrolysing ATP at a rate dependent on the concentration of Na\(^+\) outside the cell.
6. Found in all cells in which an active, linked transport of Na\(^+\) and K\(^+\) occurs.
The activity of Na\(^+\), K\(^+\) - ATPase is inhibited by cardiac glycosides e.g. ouabain (Schatzmann, 1953; Glynn, 1957). The latter demonstrated that active transport was prevented by cardiac glycosides at concentrations of \(10^{-5}\) g dm\(^{-3}\).

Since Skou (1957) originally described the activity of a Na\(^+\), K\(^+\) -stimulated, ouabain-sensitive ATPase, its presence has been demonstrated in many organisms and tissues, between which the degree of activity varies considerably (Katz and Epstein, 1968). Activity has been shown to be present in kidney (Wheeler & Whittam, 1964), red blood cells (Wheeler & Whittam, 1964; Post et al, 1960), reticulocytes (Yunis & Arimura, 1966) and platelets (Aledort et al, 1966), brain (Skou, 1962), peripheral nerve (Skou, 1957), liver (Schwartz, 1964), intestinal mucosa (Berg & Szekerczes, 1966), adrenal (Turkington, 1962) and thyroid glands (Wolff & Halmi, 1963), skeletal (Samaha & Gergely, 1966) and cardiac muscle (Auditore & Murray, 1962), parotid gland (Schwartz et al, 1963), ciliary body (Bonting et al, 1961) and retina (Bonting et al, 1961), choroid plexus (Bonting et al, 1964), urinary bladder (Bonting & Canady, 1964), electric organ of eel (Albers & Koval, 1962) and salt gland of birds (Ernst et al, 1967).

Largest amounts have been found in the brain and tissues concerned with secretory functions, e.g. kidney, choroid plexus and ciliary body. The enzymes are absent from non-cellular tissues (e.g. serum, eye lens, vitreous body) or tissues associated with low cell density (e.g. fat and corneal stroma).

Ions necessary for the Na\(^+\), K\(^+\) -ATPase to be active are Mg\(^{2+}\), Na\(^+\) and K\(^+\). The former's presence is obligatory for any
activity when \( \text{Mg}^{2+} \) is present, and \( \text{K}^+ \) increases activity when the system contains \( \text{Mg}^{2+} \) and \( \text{Na}^+ \). An increase in the concentration of \( \text{K}^+ \) ions leads to displacement of \( \text{Na}^+ \) ions from the substrate and accordingly to inhibition of the reaction. The substrate most readily attacked by \( \text{Na}^+ \), \( \text{K}^+ - \text{ATPase} \) is \( \text{Na}^+ - \text{Mg}^{2+} - \text{ATP} \).

A low concentration of \( \text{Ca}^{2+} \) ions inhibits activity. \( \text{Mg}^{2+} - \text{ATPase} \) or mitochondrial ATPase is involved with oxidative phosphorylation and therefore respiration. In mitochondria, this enzyme exists as a complex, one part of which possesses the ATPase activity. Details of this are given by Gomez-Puyon et al (1976). The components of this complex are:

a) a hydrophobic or membrane factor that directs \( \text{H}^+ \) to the catalytic site of ATPase.

b) a protein that confers oligomycin sensitivity to ATPase activity.

c) factor 1 or \( \text{F}_1 \) which possesses the catalytic site for ATP synthesis (\( \text{F}_1 \) is a soluble protein that is characterised by an active oligomycin insensitive ATPase).

d) an ATPase inhibitor whose function is still unknown.

Interference by ion concentration on any one of these components can lead to a modification in the activity of the whole complex.

Mitochondrial ATPase activity can be attributed to a soluble \( \text{F}_1 \) and a particulate \( \text{F}_1 \) (associated with membrane). Binding to the membrane confers different properties on the enzyme (an example of an allosteric property). For example, the activity of soluble \( \text{F}_1 \) ATPase declines very rapidly on exposure to temperatures of 4°C, while at room temperature activity is stable (Pullman et al, 1960). The ATPase activity
of particulate F₁ is very stable at temperatures below 4°C. Lardy et al (1958) showed that soluble F₁ ATPase activity was insensitive to oligomycin (an antibiotic) while ATPase of particulate F₁ is very sensitive.

K⁺ ions stimulate Mg²⁺ -ATPase activity and respiration, while octylguanidine inhibits both activities. The enzyme is Mg²⁺ - dependent and stimulated by the presence of Ca²⁺ ions.

Originally it was thought that Na⁺, K⁺ -ATPase and Mg²⁺ -ATPase were two activities of a single enzyme, as they were always found together in membrane preparations. However, there are many arguments favouring their existence as two different enzymes, for example:

a) widely differing ratios for the two activities in tissues.

b) different pH optima (pH 8.4 for Mg²⁺ -ATPase and 7.3 for Na⁺, K⁺ -ATPase).

c) different effects of various agents e.g. ouabain, urea, NaI, sulphydryl agents etc.

d) different temperature-activity curves for the two activities.

e) different rate and extent of increase for the two activities in the salt gland of ducks given saline to drink.

f) Mg²⁺ -ATPase in erythrocytes hydrolyses only ATP present on the outside, and Na⁺, K⁺ -ATPase hydrolyses only ATP present on the inside of the cell.

Ca²⁺ -ATPase or myosin -ATPase is found in muscle tissue. Its function is the translocation of Ca²⁺ ions against a concentration gradient coupled to the hydrolysis of ATP.

Ca²⁺ -ATPase is associated with both myosin and actomyosin. Its activity in these fractions of skeletal and smooth muscle has been examined under different conditions by Bogach et al (1976).
The mechanism of action, and structure of the ATPase system are vague. Because of its insolubility, it is impossible to apply modern techniques for the determination of protein structure (Bonting, 1970). A number of theories have been advanced regarding the structure of the Na\(^+\), K\(^+\)-ATPase system (Opit & Charnock, 1965; Müller, 1967). The most likely model has been suggested by Albers et al (1968) (Fig. 10).

Fig. 10 Model for the structure of Na\(^+\), K\(^+\)-ATPase system (Albers et al, 1968)
The circle is a cell membrane, on which six membrane sites in different stages of activity have been pictured.

1. $\text{Na}^+$ activated phosphorylation takes place with enzyme in cis-form i.e. with cation binding sites pointing to cytoplasm.

2. Phosphorylated enzyme is rapidly converted into trans-form, with cation binding sites pointing extracellularly.

3&4 In presence of $\text{K}^+$, reaction 3 activates the hydrolytic step 4 which makes the trans-enzyme less stable.

5. It then reverts to the cis-form.

6. The cycle is completed when $\text{Na}^+$ displaces $\text{K}^+$ from the cis-enzyme.

(iii) The effect of DDT on the activity of ATPases

DDT, and several other organochlorine pesticides and PCBs, usually inhibit the action of $\text{Na}^+\text{, K}^+$ -ATPase and $\text{Mg}^{2+}$ -ATPase, although in a few cases, activity is stimulated.

Inhibition has been demonstrated in fish tissues (Price, 1976; Leadem et al., 1974; Davis & Wedemeyer, 1971; Desaih et al., 1975; Desaih et al., 1972; Janicki & Kinter, 1971; Cutkomp et al., 1971; Kinter et al., 1972); in rat and rabbit brain (Koch, 1969; Bratkowski & Matsumura, 1972; Akera et al., 1971); in turtle tissues (Witherspoon & Wells, 1975); in insect tissues (Desaih et al., 1974; Koch et al., 1969); and in lobster nerve (Matsumura & Narahashi, 1971), amongst other animals and tissues.

Desaih et al. (1975) reported a stimulation of $\text{Na}^+\text{, K}^+$ -ATPase activity in tissues of fat-head minnows after in vivo treatment with DDT; and Phillips and Wells (1974) showed stimulation of $\text{Na}^+\text{, K}^+$ -ATPase, and in particular $\text{Mg}^{2+}$ -ATPase in in vitro treatments of tissues of several turtle species.

Associated with the interference of DDT with ATPases,
has been a change in the concentration of ions in body fluids, and the impairment of fluid and amino acid absorption.

After injection of DDT into a marine fish, the Black surfperch (*Embiotoca jacksoni*), Waggoner & Zeeman (1975) found an increase in the plasma osmolality with 10-220 mg. kg\(^{-1}\). This shows a breakdown in the osmoregulatory mechanism but they believed that the deaths caused by high DDT concentrations could not solely be attributed to osmoregulatory failure. An increase in plasma osmolality and Na\(^+\) and K\(^+\) concentrations of seawater-adapted eels occurred after exposure to 1 mg. kg\(^{-1}\) for 6 hours. (Kinter et al, 1972). It was also found that Na\(^+\), K\(^+\)-ATPase activity was inhibited by 38% at 50 mg. kg\(^{-1}\) DDT. The same workers exposed killifish (seawater teleosts) to DDT and a PCB (Aroclor 1221) and the osmolality and Na\(^+\) concentration of the serum increased to near that of the seawater.

When freshwater fish (goldfish) were exposed to 35 \(\mu\)g dm\(^{-3}\) DDT for up to 330 hours, they showed significant but inconsistent decreases in plasma osmotic and sodium concentrations. There was also a significant but inconsistent increase in potassium concentration (Weisbart & Feiner, 1974).

In the hepatopancreas of marine shrimps (*Penaeus aztecus* and *P. duorarum*), the cations were measured after exposure to DDT (Nimmo & Blackman, 1972). With 0.1 \(\mu\)g dm\(^{-3}\) DDT in the water, there was a decrease in Na\(^+\) and K\(^+\) concentrations. The hepatopancreas of these animals is the organ in which the highest amounts of the pesticide have been found (Nimmo et al, 1970). This is comparable to the liver of other groups of animals. In the liver of the marine puffer (*Sphaeroides*
maculatus), Eisler & Edmunds (1966) found that the chlorinated insecticide endrin caused a depression in concentrations of sodium, potassium, calcium, magnesium and zinc ions.

Marine teleosts maintain their body fluid hypotonicity by drinking seawater (Fig. 9). Water, sodium and chloride ions are absorbed by the intestinal epithelium. Water is then retained by the body; while the salts are excreted by the gill epithelium. The addition of 50 mg dm⁻³ of DDT to the medium in which were incubated intestinal sacs of seawater-adapted eels, caused a decrease of 47% in water absorption (Janicki and Kinter, 1971). Intestinal water absorption is believed to be coupled with sodium transport. Janicki and Kinter (1971) suggest that DDT impairs water absorption by inhibiting mucosal ATPase involved in sodium transport.

An in vivo study on intestinal water transport has been carried out by Miller & Kinter (1977) using killifish exposed to DDT. With this water absorbed by the intestine are carried essential nutrients such as amino acids and sugars. They found that as well as an inhibition of water absorption, there was also a decrease in the amino acid transport in the intestine, which is Na⁺-linked. Osmoregulatory disruption was made apparent by an increase in Na⁺ and K⁺ concentrations of the plasma.

f) PROTEIN SYNTHESIS & ENZYME INDUCTION

Animals have the ability to increase the metabolism of many drugs (e.g. barbiturates, antihistamines, anti-inflammatory drugs and analgesics), halogenated hydrocarbon insecticides and other foreign chemicals, by increasing the activity or quantity of hepatic microsomal enzymes (Morello,
Liver weight also increases (Platt & Cockrill, 1969). This effect is blocked by administration of puromycin, an inhibitor of protein synthesis (Conney & Gilman, 1963; Morello, 1965).

Studies with labelled amino acids administered in vivo have shown that phenobarbital causes an increase in the synthesis of certain microsomal membrane-bound proteins, especially cytochrome P-450 (Dehlinger & Schimke, 1972) and NADPH-cytochrome c reductase (Kuriyama et al, 1969). Preparations containing microsomes from liver of rats were treated in vitro with DDT, phenobarbital or methylcholanthrene (Sánchez, 1967). The result was an increase in the incorporation of amino acids into protein.

Cappon & Nicholls (1973; 1974 & 1975) concluded that increased protein synthesis was due to a number of factors and not solely to an increase in endogenous mRNA. A decrease in activity of ribonuclease, and an increase in activity of ribonuclease inhibitor has been considered (Lechner & Pousada, 1971), but Cappon & Nicholls believe that DDT-stimulated liver protein synthesis involves a decrease in some inhibitory factor(s) present in microsome and ribosome preparations. This may act in the control liver by decreasing the initiation steps and the aminoacyl-tRNA binding steps of mRNA translation (Gambino et al, 1973; Adelman & Lovett, 1974).

Phenobarbital decreases the effect of the anticoagulantbishydroxycoumarin and the anticonvulsant effect of diphenylhydantoin (Cucinell et al, 1965). Organohalogenated pesticides and PCBs decrease the effect of the muscle-relaxant,
zoxazolamine by increasing the activity of the microsomal enzyme, zoxazolamine-hydroxylase (Truhaut et al., 1974); and DDT and chlordane protect rats from a lethal dose of the anticoagulant warfarin (Ikeda et al., 1966). The exposure of man to pesticides and other chemicals may consequently alter his response to drugs or steroid hormones.

**g) REPRODUCTION, SURVIVAL & DEVELOPMENT**

(i) Mammalian Reproduction & Hormonal System

Decreased fertility in small mammals as a result of DDT-exposure has been reported in the literature, and these effects may be a result of disturbances in the hormonal system. Mice exposed to levels of 200 mg. kg$^{-1}$ and 300 mg. kg$^{-1}$ of DDT in their diet produced the same number of young per litter as the controls, but the number of females which did not produce any young was significantly higher in those fed 300 mg. kg$^{-1}$ (Bernard & Gaertner, 1964). The number of females dying during the gestation period, and the number of young dying, was much higher in exposed than control animals (Cannon & Holcomb, 1968).

DDT- and PCB-treated male and female mice show an increase in liver weight and an increase in hepatic cytochrome P-450 content (reflecting the drug-metabolising enzyme activity - I.7.f) (Lundberg, 1974; Örberg & Lundberg, 1974). This suggests that the body's natural steroid hormones are being metabolised.

Female mice injected with or fed DDT have shown a decrease in the frequency of implanted ova and a prolongation of the oestrous cycle (Örberg et al., 1972; Lundberg, 1973; Lundberg, 1974). It was initially thought that these effects
were a result of insufficient secretion of oestrogen at the time of implantation, or an alteration in the hormone level because of an increase in drug-metabolising enzyme activity. The results of Lundberg (1974) suggest that decreasing frequencies of implanted ova result from an alteration of the oestrogen/progesterone ratio rather than from a lowering of oestrogen to below a minimum level for implantation.

Castrated male mice fed DDT and PCB showed significant reduction in the weights of seminal vesicles when compared with castrated controls. No significant differences in seminal vesicle weights were found in intact experimental animals when compared with intact controls (Örberg & Lundberg, 1974). In this work, the authors exposed a) castrated, testosterone-treated males, and b) intact males to DDT and PCB (Clophen A60), with the relevant unexposed controls. The growth of seminal vesicles is dependent on androgens, so it was suggested that the reduced weights in castrated, testosterone-treated experimental males were caused by a reduction in the levels of testosterone. No significant weight differences were found in the testes and seminal vesicles of intact experimental and control males.

The results indicate that even though there is an increase in drug-metabolising enzyme activity, the amounts of androgens in DDT-treated and untreated animals are similar. This implies that the male mouse, in this situation, is capable of compensating for the enhanced androgen breakdown, probably by increased synthesis of the hormone via the testis-hypophysis feedback mechanism.
In order to assess the toxicity of new compounds, bio-assays are often carried out to determine how many individuals of a species die over a certain length of time and at a particular concentration, e.g. 96 hr. LC50 is a parameter frequently used, and measures the concentration at which 50% of the test species die after 96 hours' exposure. Pesticide and PCB effects on survival have been carried out using these acute toxicity tests on oyster eggs and larvae (Davis, 1961), Daphnia (Nebeker & Puglisi, 1974), decapod Crustacea (Eisler, 1969; Sandholzer, 1945) and fish (Halter & Johnson, 1974; Nebeker et al., 1974), for example.

In all cases the pesticides killed the test animals before the control animals died. Some examples of 96 hr. LC50s for crustacea exposed to DDT are shown below:

\[
\text{96 hr. LC50 (\mu g \ dm}^{-3} \text{ active ingredient)}
\]

- Ciangon setemspinosa (sand shrimp) 0.6
- Palaeomonetes vulgaris (grass shrimp) 2.0
- Pagurus longicarpus (hermit crab) 6.0

(Eisler, 1969)

Sublethal effects noted in this field have been a decrease in egg hatchability, mean time to hatching, alevin survival, and growth in coho salmon exposed to PCBs (Halter & Johnson, 1974). In the same experiment it was found that DDT produced a shorter median survival time of the fry than did Aroclor 1254, indicating the latter was less toxic to these larvae than DDT. The median survival times were always more than 336 hours for fish exposed to PCB concentrations up
to 32.2 \( \mu g \ dm^{-3} \), compared with 0.8 \( \mu g \ dm^{-3} \) for DDT.

Dethlefsen (1974) exposed embryos of cod, flounder and plaice to DDT and DDE in seawater, ranging from a concentration of 0.005 to 2.5 mg \( dm^{-3} \). It was found that as the DDT concentration increased, so did the number of malformed and dead embryos, and malformed and dead larvae. Mortality of embryos and larvae tended to occur at three susceptible phases - epiboly, beginning of hatching and end of yolk sac phase. These phases coincide with changes in energy supply, and the increasing energy demand of the embryo cannot be satisfied owing to a reduction in activity of ATPases (I.7.e), so resulting in a high mortality rate (Rosenthal & Stelzer, 1971). The length of hatched larvae decreased with increasing concentrations of DDT. The lowest concentrations of DDT still producing harmful effects was 0.05 mg \( dm^{-3} \) for cod and 0.006 mg \( dm^{-3} \) for flounder.

Larvae of fishes are more susceptible to toxic substances than the eggs, and the adults are less susceptible than either eggs or larvae. This is owing to the concentration of lipophilic compounds, such as the organochlorines in the fat of the yolk sac. Once the fish has developed from the egg to the larval stage, this yolk sac is being used as the only food source and so these compounds are released to the young fish.

The same relationship is found between larvae, juveniles and adults of crabs exposed to pollutants. Buchanan et al (1970) found that larvae were more susceptible than juveniles, and juveniles more susceptible than adults of Cancer magister when exposed to different concentrations of the insecticide.
Sevin (carbamate insecticide).

Crab development passes from hatching, through several zoeal stages, to the megalopa and then to the first stage crab. It was found that when two species of crabs — *Menippe mercenaria* and *Rhithropanopeus harrisii*, were exposed to DDT and Mirex (organochlorine insecticide) at concentrations from 0.01 to $10.0 \mu g \ dm^{-3}$ there was a dose-dependent reduction in zoeal survival after the 1st and 2nd stages, and then during the megalopal stage, a very marked reduction in survival (Bookhout *et al.*, 1972). During the transformation of zoeal tissues to those of the megalopa, the insecticide stored in the zoeal fat, is probably released into the haemolymph and so is available to exert its effects (Bookhout & Costlow, 1973).

An increase in the time of development of several crab species was observed with increasing concentrations of Sevin, dieldrin and Mirex (Bookhout & Costlow, 1973). This is a result of a prolongation of the duration of an individual zoeal stage, or the duration of total zoeal and megalopal development. However, several crab species fed DDT or reared in water containing a PCB, revealed no significant changes in the duration of zoeal stages, zoeal development or megalopal development.

(iii) Development and Growth

Effects on the development of several crabs exposed to various concentrations of several pesticides, have already been noted. Abnormalities were noticed in the development of mud-crabs which were fed *Artemia* (brine shrimp) from the Great Salt Lake, Utah when compared with those fed *Artemia* from salt pools in California. Those from Utah contained about three
times as much DDT in their bodies as those from California (Bookhout & Costlow, 1970). Megalopal development was normal in crabs fed California Artemia and 94% - 98% of them developed to the first crab stage. In those fed Utah Artemia, the megalopa were abnormal in that the legs projected backwards in an extended condition, and none of them reached the first crab stage. These effects were attributed to DDT-poisoning.

Weis & Mantel (1976) demonstrated that DDT affected the development of fiddler crabs. Uca pugilator and U. pugnax were exposed to 10 and 25 pp 10^9 of DDT and it was found that the time to molt was shortened; and limb regeneration, especially in crabs stimulated to regenerate by multiple autotomy (removal of limbs), was accelerated. Molting and regeneration are under hormonal control. It was suggested that increased excitation of the central nervous system caused by DDT, could cause the release of some neuroendocrine factor(s) that stimulates the early stages of limb growth.

Valentine et al (1973) examined the possible use of fluctuating asymmetry in the body of an animal, as a measure of environmental stress. This is the random deviation from perfect symmetry of any bilateral anatomical character. They used seven characteristics on three species of marine teleost; barred sand bass (Paralabrax nebulifer), grunion (Leuresthes tenuis) and barred surf perch (Amphistichus argenteus) to test this theory. These characteristics were:

- number of pectoral fin rays
- total number of gill rakers
- scales above the lateral line
- scales below the lateral line
- pored lateral line scales
- length of dentaries
- length of posterior lateral projection of the epiotic
Asymmetry values were shown to increase in highly polluted areas. Grunion exposed to low DDT concentrations of less than 1 \( \mu g \text{ dm}^{-3} \) showed a very significant deterioration in the symmetry of pectoral fin rays (Valentine & Soule, 1973), suggesting that calcium metabolism was affected.

Eggs of the Atlantic silverside (*Menidia menidia*) were exposed to DDT, malathion and Sevin (Weis & Weis, 1976). Development during the egg and larval growth was observed. The main effect was in early stages, preventing successful axis formation. Optic malformations were caused by all three insecticides; the most common being unilateral anophthalmia (absence of eyes) and microphthalmia (reduced size of eyes). Several embryos showed partial convergence of the eye cups, suggesting partial development of cyclopia (a single median eye). These effects were observed at doses of 10 \( \mu g \text{ dm}^{-3} \) of the insecticides.

Growth of the valves of bivalve molluscs has been shown to be affected by pesticides. Yearling oysters (*Crassostrea virginica*) were inhibited in their growth rate after only 24 hour's exposure to aldrin (0.1 mg. dm\(^{-3}\)), chlordane (0.01 mg. dm\(^{-3}\)), o-dichlorobenzene (1.0 mg. dm\(^{-3}\)), DDD (1.0 mg. dm\(^{-3}\)), DDT (0.1 mg. dm\(^{-3}\)), dieldrin (0.1 mg. dm\(^{-3}\)), endrin (0.1 mg. dm\(^{-3}\)), heptachlor (0.01 mg. dm\(^{-3}\)), rotenone (0.01 mg. dm\(^{-3}\)), Sevin (1.0 mg. dm\(^{-3}\)) and toxaphene (0.1 mg. dm\(^{-3}\)). Figures in brackets show the minimum effective concentration (Butler et al, 1962).

(iv) Eggshell-thinning

The phenomenon of eggshell-thinning causing a reduction in the population of many birds, e.g. the brown pelican in USA
and the peregrine falcon in Britain, has been well documented (Ratcliffe, 1967; Hickey & Anderson, 1968; Anderson & Hickey, 1972). This is believed to be attributed to DDE rather than DDT itself.

In a study on 93 nests in a nesting colony of Brown pelicans in S. Carolina, Blus et al (1974) found that nest success was related to residues of DDE and dieldrin in sample eggs. Maximum DDE residues in an egg from a successful nest were 2.37 mg. kg$^{-1}$ and 8.48 mg. kg$^{-1}$ in an egg from an unsuccessful nest. Comparable maximum residues for dieldrin in sample eggs were 0.54 mg. kg$^{-1}$ for successful and 0.99 mg. kg$^{-1}$ for unsuccessful. Residues of DDD, DDT and PCBs in sample eggs were not significantly related to nest success.

Mallard hens (*Anas platyrhynchos*) fed 40 mg. kg$^{-1}$ p,p'-DDE for 96 days, laid eggs with shells which were 15% - 20% thinner than those of control eggs, up to 42 days after exposure (Haegele & Hudson, 1974). These birds were kept for a further year without additional administration of DDE. Eleven months after the feeding of DDE had stopped, the birds laid eggs with shells averaging 7.4% thinner than the controls.

The mechanism of eggshell thinning still remains unclear, but it is now thought that thinning results from a disruption of shell gland (uterus) function. Kolaja & Hinton (1976) demonstrated changes in the morphology of shell gland tissue in all mallards exposed to 75 mg. kg$^{-1}$ DDT. No changes were seen in any birds receiving a normal diet. These changes included oedema of villous projections, pyknosis of glandular epithelium and cytoplasmic vacuolation of living epithelium.

Peakall et al (1975) fed DDE to laying ring doves and
white Pekin ducks, resulting in a 35% and 20% thinning, respectively of the eggshells. The blood Ca\(^{2+}\) levels were unaffected. They concluded that these findings were consistent with inhibition of shell-gland function, rather than decreased Ca\(^{2+}\) supply to the gland.

The shell gland secretes calcium and carbonate during eggshell formation. The calcium is obtained from the blood, and carbonate from metabolic CO\(_2\). These are transported to the calcifying egg. The shell gland mucosa possesses Ca\(^{2+}\) -ATPase and calcium-binding-protein, which are involved with Ca\(^{2+}\) transport; and carbonic anhydrase and possibly, anion (HCO\(_3\)) -ATPase involved with carbonate transport. The inhibition of one or more of these enzymes could account for eggshell-thinning. Cooke (1973) suggested there may be several mechanisms, the relative importance of each being species-dependent. Miller et al (1976) found that DDE-induced eggshell-thinning in ducks is accompanied by decreases in the activities of two shell gland enzymes - Ca\(^{2+}\) -ATPase and carbonic anhydrase, the former being more important.

Quail fed DDE were found to have a reduction in carbonic anhydrase activity of the shell gland of 16 - 19% compared with controls (Bitman et al, 1970); and the ring dove has been reported to have a 60% reduction in shell gland carbonic anhydrase activity during maximal DDE-induced shell-thinning (Peakall, 1975a).

h) OTHER ENZYMES

(i) **Glucose 6-phosphate dehydrogenase**

When rats were fed 5 mg. kg\(^{-1}\) and higher concentrations of DDT, a significant decrease in the activity of glucose 6-
phosphate dehydrogenase in the liver was noted (Tinsley, 1964, 1965). As there was also an increase in liver size, this reduction in enzyme activity could be explained by the dilution effect. This was not found to be the case, as there was a significant depression in the total level of enzyme in the liver. The author suggested that this response to ingestion of DDT is associated with a change in protein metabolism rather than a direct interaction of DDT or its metabolites with the enzyme. The result may be a decrease in the rate of synthesis of glucose 6-phosphate dehydrogenase or the synthesis of a modified enzyme which is less effective.

This is a key enzyme in carbohydrate metabolism, helping to control the relative flow of glucose through the Embden-Meyerhof glycolytic pathway and pentose cycle.

\[
\text{glucose 6-P} \xrightarrow{\text{glucose 6-phosphate dehydrogenase}} \text{NADPH}
\]

A decrease in the enzyme activity will result in decreased production of NADPH. This is utilised in a number of biosynthetic processes, especially the synthesis of fatty acids (Buhler & Benville, 1969).

Ingestion of DDT at a concentration of 100 mg. kg\(^{-1}\) by juvenile coho salmon or adult rainbow trout, had no effect on the activity of this enzyme (Buhler & Benville, 1969). This may be attributed to the very high lipid content of fish which provides for increased storage of DDT and thus prevents the accumulation of the critical concentrations of DDT in the blood or target organs.

(ii) Cholinesterase

Guilbaut et al (1972) studied the activity of cholinesterases in aquatic organisms - brain of trout and crayfish,
and whole body preparations of fiddler crabs. Trout cholinesterase was insensitive to organochlorine and most organophosphorous pesticides (except methyl parathion and DDVP). It was selectively inhibited by Baygon, Matacil, Mesurol and Zectran (carbamates). Fiddler crab cholinesterase was selectively inhibited by Matacil, Mesurol and Zectran, by dieldrin and by paraoxon and methyl parathion (organophosphates). Crayfish cholinesterase was greatly inhibited by paraoxon and Mesurol.

This enzyme was also studied in mammals and birds (sheep, pig, rabbit, pigeon and chicken) by Guilbault et al (1970) using the liver as the source. Chlorinated pesticides did not affect any of the enzyme activities. DDVP, paraoxon, parathion and methyl parathion inhibited activity. Sevin (carbamate) inhibited sheep and pigeon liver cholinesterase.

1) HISTOLOGICAL CHANGES

Studies on ultrastructural changes in tissues caused by DDT have been carried out mainly on small mammals and fish. It has been found that DDT-exposure results in an accumulation of smooth endoplasmic reticulum (ER) in the cell, with which is associated the drug-induced microsomal enzyme system (I.7.f). Ortega (1966) and Kimbrough et al (1971) have demonstrated this in the liver of rats; Weis (1974) in the liver of the guppy; and Couch & Nimmo (1974) in the hepatopancreas of shrimp exposed to PCBs.

It was found that when the relatively DDT-resistant guppy and the DDT-sensitive zebrafish were exposed to this pesticide, the latter showed no proliferation of smooth-ER, a decrease in lipid content and a decrease in stored glycogen.
content of the liver cells (Weis, 1974). There was no change in glycogen and lipid in the guppy liver, and there was an increase in smooth-ER. These three factors could explain the animal's tolerance to DDT.

Other liver structural changes that have been noticed are increased vacuolation of the cytoplasm in rats (Zawistowski & Kechniak, 1973), in certain fishes (Mathur, 1962) and in chicken embryo (Roux et al, 1974); localised necrosis, parenchymatous degeneration of cells and hypertrophy of hepatic cells in fish (Mathur, 1962).

Doses of DDT, dieldrin and lindane lower than 30 mg. kg$^{-1}$ also produced alterations in the lysosomal system of the liver (Roux et al, 1974). There was an increase in the number of lysosomal granules in the cytoplasm and a disappearance of acid phosphatase activity from the Golgi complex.

The effects on the structure of the intestine and kidney of fishes exposed to DDT were less marked than those on the liver (Mathur, 1962). In the former, there was a degeneration of the lining of the epithelium and a few vacuoles were observed in the circular and longitudinal muscles. In the kidney there was a moderate degeneration of the epithelium and a loss of parenchymatous cells in the renal tubules.

j) IMMUNOLOGICAL RESPONSES

Experiments suggest that in animals submitted to DDT ingestion, the defense reactions are moderated.

Wasserman et al (1969) studied the effect of DDT on the immunological response to a foreign protein (ovalbumen). Rats receiving 200 mg. kg$^{-1}$ of DDT in their drinking water for 35 days and 6 mg. of ovalbumen in three injections showed increased
liver weights, decreased spleen weights and a 30% fall in antiovalbumen antibodies, when compared with controls. At the same time there was an increase in the level of the albumen fraction of the serum proteins and a decrease in the level of the γ-globulin fraction.

Similar results were found when rabbits were fed DDT and immunized against Salmonella (Wasserman et al, 1971). The antibody titer against Salmonella was significantly decreased in DDT-Salmonella receiving rabbits compared with rabbits receiving only Salmonella. Total γ-globulins were also significantly decreased in those ingesting DDT.

PCBs have been found to have a similar effect to DDT on the γ-globulin fraction of serum proteins (Wasserman et al, 1973; Vos & De Roij, 1972), but the serum albumen levels fell in rabbits receiving PCBs.

The above authors explain these effects by the action of DDT at one or more stages in the feedback mechanisms of antibody synthesis. This feedback control operates by affecting either the cellular transformation or the synthetic activity of the antibody-forming cells (Dubinski & Fadette, 1966).

k) METABOLIC RATE

Lunn et al (1975) found that there was an increased respiration rate and decreased heart rate, coupled with an increase in 'coughing' rate in rainbow trout exposed to 140 µg dm⁻³ and 350 µg dm⁻³ DDT. This indicated that oxygen uptake may be affected in some way.

Increased oxygen consumption has also been shown in quail fed DDT (Lustick et al, 1971) and the short-tailed shrew
(Braham & Neal, 1974). In the latter case, there was a considerable increase in metabolic rate after one week of feeding, but this became insignificant after the second and third weeks of feeding. The authors suggest this may be due to liver enzyme induction and more efficient removal of DDT from the blood.

In studies on the effect of sublethal doses of DDT on the Black Sea shrimp, Andryushchenko (1972) showed that with 1 mg dm\(^{-3}\) of DDT in the water, there was a marked slowing of overall metabolic rate, and oxygen consumption decreased to less than 25% on the second day. On day three, the rate of oxygen consumption increased, but then decreased until after 15 days, the rate was 40% of the control rate. He suggests that the brief increase in gas exchange was due to the mobilisation of the body's reserves to protect the normal course of its vital processes.

It has been suggested that organochlorines may uncouple oxidative phosphorylation (involving Mg\(^{2+}\)-ATPase - I.7.e), causing an increase in oxygen requirements (Wilber, 1971); or that DDT may interfere with oxygen uptake at the gill-water interface in fish (Rudd, 1966), but there is no definite evidence for either theory.

Increased oxygen consumption may be associated with hyperactivity due to DDT intake. In insects, DDT causes a rapid increase in oxygen consumption which appears to peak during the period of maximal muscular activity, and fall off when paralysis sets in.

1) THYROID GLAND AND ACTIVITY

Jefferies (1973, 1975) has suggested that most of the...
sublethal effects caused by organochlorine insecticides and their metabolites may develop from an initial lesion on the thyroid gland. The thyroids of pigeons showed considerable histological changes after being dosed with DDT, DDE and dieldrin (Jefferies & French, 1969, 1971, 1972). It was found there was a noticeable increase in thyroid size after DDT dosage, and that dosed birds showed a reduction in follicular size and colloid content. (The colloid is a gelatinous material consisting mainly of an iodinated protein, thyroglobulin, which is formed by the secretory epithelium).

These factors are known to be associated with a hyperactive gland which is being stimulated continuously by thyrotrophic hormone (TSH), (Turner, 1966). Jefferies & French (1971) theorised, and Marshall & Tompkins (1968) provided experimental evidence, that this continuous stimulation is produced by the competitive binding of the DDT-group molecules (DDT, DDE, DDD) with the thyroxine-binding serum proteins, thus effectively reducing circulation of the thyroid hormones. Reduced circulation would then lead to continuous release of TSH from the pituitary to stimulate thyroid hyperactivity.

Guillemots given 12 and 25 mg. kg$^{-1}$ day$^{-1}$ of Aroclor 1254 for 45 days showed increased thyroid weight, follicle size and colloid area (Jefferies & Parslow, 1976), slightly different from the effects of DDT; but increasing dose rates produced significant dose-related decreases in these parameters. Birds fed 400 mg. kg$^{-1}$ day$^{-1}$ showed atrophy of the thyroids with 59.6% of the weight, and 27.3% of the colloid area of the controls.

It was hypothesised that the pituitary gland is a target
site for Aroclor 1254 in birds, increased doses causing a decrease in the TSH released, and also a decrease in weight of the pituitary.

Jefferies (1975) details the effects of thyroid hormones on the behaviour and physiology of animals, and it can be seen that DDT- and PCB-affected thyroid activity can cause many of the known sublethal effects of these organochlorines:-

(i) Increase in thyroid hormones in homoiotherms causes an increase in oxygen consumption and energy production in tissues affected.

(ii) There are changes in the movements and behaviour of humans suffering from hypo- or hyper-thyroidism. Excess thyroid hormones in the circulation may react upon the nervous system and may cause emotional instability, nervousness, muscular tremors and even dementia.

(iii) Reduced thyroid activity may cause sterility in stock animals. Semen quality of rams is improved by treating with thyroxine.

(iv) Thyroxine influences the storage of Vitamin A, the rate at which it is used up, and the conversion of carotene to Vitamin A. Hypothyroid rats store very little Vitamin A in the liver, whereas hyperthyroid animals store more than controls.

(v) Thyroid hormones are important in moulting in birds, usually increasing thyroid activity prior to moulting.

(vi) The circulatory system may be affected, the heart weight of growing chicks increasing progressively with dose rate of thyroxine. Hypothyroidism is often accompanied by anaemia.
(vii) The blood volume is much reduced in hypothyroid animals, with extracellular retention of water, Na\(^+\) and Cl\(^-\). Treatment of normal individuals with thyroid hormones causes excessive water and K\(^+\) loss through the kidneys.

(viii) Organ weights have been shown to change with changes in thyroid activity. The increased size of the liver is accompanied by an increase in fat deposition and congested liver sinuses. Hyperthyroid chicks showed a significant decrease in weight of the pituitary; and a decrease in spleen size has been shown in hypothyroid chickens.

(ix) Fat and carbohydrate metabolism may be affected. Fat deposition and liver glycogen concentration are increased in chickens made hypothyroid.

(x) Cockerels made hypothyroid were much more susceptible to common poultry diseases. Chickens having the thyroid gland removed commonly developed respiratory disturbances which were often fatal. These may be connected with Vitamin A levels.

m) NERVOUS SYSTEM

The actual target for DDT in animals is considered to be the central nervous system (C.N.S.). Dale et al (1963) demonstrated that the severity of signs of poisoning in rats after a single dose of DDT was directly proportional to the concentration in the brain. DDT is carried to the CNS in the blood and has also been found in the cerebral spinal fluid, in which the CNS is suspended (Morrison, 1971).

Nervous symptoms for man are described in I.7.c. When DDT is injected into a cockroach, the symptoms of toxicity resulting include increased activity, contractions and tremors.
in the appentages and body, erratic behaviour and loss of 
equilibrium (Yeager & Munson, 1945).

A normal resting polarised nerve fibre has a high $K^+$
and low $Na^+$ concentration internally in the axoplasm (Fig. 11).
To maintain this, active transport takes place by a sodium-
potassium exchange pump. This is where the action of ATPases
come into effect (I.7.e). When an electrical stimulus is
applied to the nerve, depolarisation occurs, during which
there is a rapid diffusion of $Na^+$ down the concentration
gradient into the axoplasm. This reverses the polarity of the
membrane potential. Before $Na^+$ diffusion ceases, that of $K^+$
in the opposite direction begins. This returns the polarity
of the membrane potential to the resting condition, and is
known as repolarisation.

Fig. 11
Polarised Nerve Fibre

\[
\begin{array}{c}
\text{neurilemma} \\
\text{active transport}
\end{array}
\]
It has been reported that ATPases in nerves have been inhibited \textit{in vitro} by DDT (Koch, 1969; Matsumura & Patil, 1969). Also, DDT affects the ionic conductance of the lobster nerve membrane, causing a prolongation of the action potential by slowing the sodium inactivation and suppressing the potassium conductance increase (Fig. 11) (Narahashi & Haas, 1967, 1968). Whether the inhibition of nerve ATPases is causally related to the mechanism of action of DDT on nerves, was discussed by Matsumura & Narahashi (1971). Matsumura (1970) observed that the inhibition of Na$^+$, K$^+$-ATPase by ouabain \textit{in vivo} induces entirely different neurotoxic effects from those caused by DDT, suggesting that the Na$^+$, K$^+$-pump is not the likely DDT target. Matsumura & Narahashi (1971) then demonstrated that there is a Na$^+$, K$^+$ and Mg$^{2+}$-dependent ATPase (in the lobster nerve) that is insensitive to ouabain, and that DDT was found to inhibit both ouabain-sensitive and -insensitive Na$^+$, K$^+$-ATPases.
DDT has been shown to affect the permeability of myelinated nerves of the frog, *Rana pipiens*. Hille (1968) and Narahashi & Haas (1968) showed that DDT had very specific effects on the sodium permeability mechanism, slowing down drastically the turn-off process of sodium permeability. DDA affected the turn-off process of the potassium permeability mechanism (Arhem & Frankenhaeuser, 1974).

In arthropods, evidence suggests that DDT acts on the peripheral nerves rather than the central nerves. For example, legs amputated from poisoned insects continue to tremble, and typical symptoms can be induced by applying DDT to amputated healthy legs. Motor nerves and muscle fibres are only affected by high concentrations of DDT (1000 mg. kg\(^{-1}\)), whereas low concentrations (0.01 mg. kg\(^{-1}\)) affect sensory nerves.

DDT poisoning in insects shows a negative temperature coefficient of intoxication. Insects showing marked signs of poisoning at 15°C can be restored to a normal condition by raising the temperature to 30°C, and this change can repeatedly be reproduced by alternately lowering and raising the temperature. It is known that insects can tolerate larger amounts of DDT at higher temperatures, and this can partly be explained by the fact that the toxicant will be detoxified more rapidly at the site of action at a high rather than a low temperature. Narahashi (1971) suggests that this is not the sole explanation. He concludes that there is an increase in nerve sensitivity at low temperatures and his results support the idea that DDT forms a complex with nerve membranes (I.8.c) which is dissociated with increasing temperature, so that although more DDT may be present at the site of action at
higher temperatures, it is less effective. Eaton & Sternberg (1967 a, b) put forward an alternative explanation for this. They implied that a substance, other than DDT, is produced at the synapses of the C.N.S. as a result of extreme sensory activity, and accumulated there at low but not at high temperatures. This would interfere with synaptic transmission.

It has been reported that DDT poisoning in cockroaches results in an accumulation of acetylcholine in the nervous system (Metcalf, 1955). However, in vitro studies have shown there is no inhibition of cholinesterase in the nerve cord.

The process of the toxic action of a contact insecticide, such as DDT, is schematically summarised in Fig. 12 (from Narahashi, 1964). The insecticide may enter the insect body through the integument, the mouth or the stomata. It may be insecticidally active as its original form (e.g. DDT) or may have to be converted into an active form to exert the toxic action. For example, parathion becomes effective in inhibiting cholinesterase after having been oxidised to paraoxon. The insecticide may be detoxified, and excreted, or may be stored in adipose tissue without exerting any toxic effect. The insecticide or its activated form finally reaches the site of action, in many cases being the nervous system. However, there are generally diffusion barriers surrounding the nerve such as the nerve sheath. After having penetrated the nerve sheath, the process of activation or detoxication may take place.

The insect can now exert its toxic action at the real site of action e.g. at nerve membrane or at synaptic junctions. There are at least two ways by which the insecticide work there: (1) direct physico-chemical action on nerve membrane
Fig. 12  Process of toxic action of contact insecticides

(Narahashi, 1964)

INTEGUMENT

↓

activation

detoxication & excretion

accumulation

NERVE SHEATH

↓

activation

detoxication

accumulation

NEURONE

↓

excitable membrane

enzyme

NERVOUS SYMPTOMS OF POISONING

↓

autotoxin

DEATH
(2) action through inhibition of enzymes. Symptoms of poisoning develop, but these do not necessarily lead the poisoned insect to death. In some cases, the hyperactivity of the nerve caused by insecticides liberates a toxin or toxins which in turn stimulate and paralyse the nerve.

n) **INSECT RESISTANCE**

The reduced use of DDT in agriculture can largely be attributed to the development of resistance amongst both target and non-target pests.

By 1966, there were resistant strains of 194 species of insects and mites to insecticides and acaricides. This was not solely confined to insects, as resistance had been induced in vertebrates such as fish, frogs and mice (White-Stevens, 1971). Populations of the mosquito fish (*Gambusia affinis*) in an area of Mississippi that had been heavily treated with insecticides, had four times the normal tolerance to DDT and DDD (Vinson et al., 1963) and 120 times the resistance to dieldrin and other cyclodienes (Boyd & Ferguson, 1964).

Resistance can be classified as 'behavioural resistance' and 'physiological resistance'. The former can be seen in mosquitoes, when they become so irritated by contact with the surface impregnated with the insecticide, that they are stimulated to fly before they can pick up a lethal dose. It is not known whether this response is a natural avoidance response, present in the species before they ever encountered DDT; or whether it resulted from selection by DDT for individuals carrying preadaptations for this response.

The physiological resistance mechanism is characterised by the ability to detoxify DDT to the relatively nontoxic
metabolite, DDE. The amount of DDE produced proved to be proportional to the DDT-resistance of the strains studied.

The gene conferring DDT-resistance is the allele of the normal gene for DDT-susceptibility. As selection of a normal population proceeds, the frequency of resistant heterozygotes and homozygotes increases, and the susceptible individuals produced by interbreeding of the former are eliminated. If selection pressure increases, the heterozygotes are eventually killed, leaving the fully resistant, homozygous population. If the alleles for resistance are not present in a population, then resistance will not develop because the insecticides will not cause mutations that might produce them (Brooks, 1974b).

Detoxication by dehydrochlorination is the result of the presence of an enzyme, DDT-dehydrochlorinase (DDT-ase) (Sternburg, Kearns & Moorefield, 1954). The amount of enzyme present was found to be proportional to the DDT-resistance level. This enzyme has been reported in DDT-resistant strains of houseflies by Sternburg & Kearns (1950), Perry & Hoskins (1950) and Lipke & Kearns (1960). Non-resistant strains of housefly also produce DDE, but in smaller amounts and at a slower rate.

Sternburg & Kearns (1952) applied DDT to several species of naturally-resistant insects, both orally and topically. They found that the grasshopper (*Melanopus differentialis*) degraded oral and topical doses of DDT to DDE, degradation occurring in the digestive tract and cuticle. Large amounts of DDT appeared unchanged in the excreta, when DDT was fed to the insect. The Mexican bean beetle (*Epilachna varivestis*) degraded orally and topically applied DDT to DDE, and further
converted DDE to an unidentified compound. Neither DDT nor DDE were excreted. The red-banded leaf roller (Argyrotaenia velutinana) degraded topical and oral doses of DDT to DDE. Some DDE and unchanged DDT appeared in the excreta when fed DDT.

DDT-ase is a protein with a molecular weight of 36,000 and an optimum pH of 7.4. It requires glutathione for activation in vitro (Sternburg et al, 1953) and can be inhibited by chlorfenethol (DMC). DMC is often used as a synergist for DDT against resistant houseflies. DDT-ase is synthesised during general protein synthesis as the larva grows, so the last larval stage to pupate has the most enzyme.

Detoxification of DDT by oxidative reactions in insects did not become fully realised until the 1960's, when it was found that a number of drugs in mammals could be metabolised in reactions catalysed by mixed-function oxidases associated with hepatic microsomes (I.7.f). Their presence in insects has been demonstrated by Hodgson (1968), Hodgson & Plapp (1970) and Wilkinson & Brattsten (1972).

The main enzyme that has been studied in this connection is the terminal oxidase of the microsomal system, cytochrome P-450. Resistant strains with high levels of microsomal oxidases usually have high levels of cytochrome P-450 (Philpot & Hodgson, 1971; Hodgson et al, 1974).
8. **THE MODE OF ACTION OF DDT**

The mechanism by which DDT exerts its effects still remains very vague. Several theories have been put forward, from the 1940's up to the present day. The more important ones will be reviewed.

a) **STRUCTURAL TOPOGRAPHY THEORY**

Gunther et al (1954) postulated from their results using mosquito larvae (to minimise transport difficulties), that the DDT-type molecule slips into a cavity in an apoenzyme or other protein involved, and is held there tightly to inhibit the normal subsequent chemistry. Therefore, closely-fitting and tightly-held molecules would be good insecticides, but larger molecules would be sterically hindered and unable to enter the cavity, or if able, with great difficulty, at ordinary temperatures. Both ends of the molecule were considered to be important.

The insecticidal ability of a DDT-type molecule was suggested to be directly correlated with the magnitudes of the van der Waals' attractive forces of the different groups for the protein. Hydrogen-group interaction energies are small, and so when there are many hydrogens in the molecule, the forces would be very weak, and may produce a poor fit. Consequently, when a larger group or atom e.g. methyl or chlorine, is present, there would be a good fit. It has been shown that completely methylated DDT is toxic to mosquito larvae (Pauling & Pressman, 1945).

b) **STRUCTURE-TOXICITY RELATIONSHIP**

Mullins (1955) noticed a difference in the effects of the various isomers of hexachloro-cyclohexane (BHC). For example,
the \( \beta \)-isomer has a relatively plane shape and only very weak physiological activity as a depressant, while the \( \gamma \)-form has a relatively spherical shape and strong insecticidal action (Table 7).

Assuming that both the \( \gamma \)- and \( \beta \)-isomers (these show the greatest difference in physiological activity and molecular structure) act on the cell membrane, and that a simple membrane is composed of cylindrical lipoprotein macromolecules arranged in a regular hexagonal packing, with interspaces or 'pores' between them; Mullins suggested that if the pores were, say, slightly larger than the \( \gamma \)-isomer, the \( \beta \)-isomer would not be able to penetrate in a plane orientation. The \( \beta \)-isomer will, however, be able to penetrate if it were turned 'end-on'. Thus the differences between these two isomers can be expressed in terms of their orientation in the membrane. The \( \gamma \)-form can penetrate in any orientation and is able to rotate to form a tight-fit in the pore in the planar orientation. Strong attractive forces between the \( \text{Cl}^- \) atoms of the isomer and the membrane components would lead to a distortion of the membrane around the interspace, causing ion leakage and resulting excitation. Planer orientations are not possible for the \( \alpha \), \( \beta \), \( \delta \)- and \( \epsilon \)-isomers, although end-on orientations are possible, and for steric reasons, reorientation within the interspace is not possible. This 'end-on' orientation would lead, at the most, to narcotic effects, such as have been postulated when the interspaces become packed with small loosely-fitting molecules with no strong interactions with the membrane around the pore (Mullins, 1954).

The theory proposed can also be applied to DDT and related
Table 7  Configuration, size and action of isomers of BHC
(Mullins, 1955)

<table>
<thead>
<tr>
<th>Isomer</th>
<th>m.pt (°C)</th>
<th>Configuration</th>
<th>Mol. diam. in plane of ring (Å)</th>
<th>Mol. Thickness</th>
<th>Physiological effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>β</td>
<td>297</td>
<td>eeee</td>
<td>9.5 9.5 9.5</td>
<td>5.4</td>
<td>Inert or weak depressant</td>
</tr>
<tr>
<td>δ</td>
<td>130</td>
<td>ppeee</td>
<td>8.5 9.5 9.5</td>
<td>6.3</td>
<td>Strong depressant</td>
</tr>
<tr>
<td>α</td>
<td>157</td>
<td>ppee</td>
<td>8.5 8.5 8.5</td>
<td>7.2</td>
<td>Weak excitant</td>
</tr>
<tr>
<td>γ</td>
<td>112</td>
<td>peppee</td>
<td>7.5 9.5 9.5</td>
<td>7.2</td>
<td>Strong excitant</td>
</tr>
<tr>
<td>ε</td>
<td>219</td>
<td>peppee</td>
<td>7.5 9.5 8.5</td>
<td>7.2</td>
<td>Non-insecticidal</td>
</tr>
<tr>
<td>η</td>
<td>90</td>
<td>peppee</td>
<td>7.5 9.5 8.5</td>
<td>7.2</td>
<td>Non-insecticidal</td>
</tr>
<tr>
<td>θ</td>
<td>124°</td>
<td>peppee</td>
<td>8.5 9.5 8.5</td>
<td>6.3</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

e (equatorial) represents a Cl⁻ atom located approximately in the plane of the cyclohexane ring.

p (polar) represents Cl⁻ atoms distributed alternately above and below the plane of the ring.

a, b, c - three values for diameters in the plane of the ring at intervals of 60° each.

substances. For example, diphenyltrichloroethane can occupy the interspace in several orientations. One is with one benzene ring parallel to the surface (the attractive forces of the halogens are not fully effective in this position), and another is with both benzene rings in an 'end-on' position (the attractive forces of the two benzene rings and the halogens are made use of in this position). To direct the orientation of diphenyltrichloroethane to the latter position,
or to a lesser extent, m,m'-substitution of an atom such as chlorine, would be necessary. Substitution by a larger group would result in delayed or inhibited penetration.

c) FORMATION OF CHARGE-TRANSFER COMPLEX

It was suggested by O'Brien and Matsumura (1964) that DDT and other chlorinated hydrocarbons formed a complex, possibly of the charge-transfer type, with a component of the nerve axon, thereby exerting their effects. Two characteristics which suggested this idea were a) the high electron affinity of these compounds, and b) their extreme persistence in the environment, indicating low biological reactivity and the fact that their interaction with nerves depends on a physical rather than chemical reaction.

Membranes formed of alternate layers of two compounds selected with appropriate electron affinity and ionization potential, such that they can be acceptor and donor in a charge-transfer complex, are known to display semiconductivity (Brophy & Buttrey, 1962). This suggested that DDT and related compounds act by forming a charge-transfer complex with a component of the axon, thus destabilising it, perhaps by inducing local semi-conductivity.

O'Brien & Matsumura (1964) were able to show that DDT does form complexes with components of insect nerve. The only evidence that it was of a charge-transfer type was an observation that on incubation of $10^{-5}$ M DDT with whole nerve cord homogenates, or the appropriate Sephadex fraction, or the combined diethylaminoethyl cellulose fractions, a new shoulder of absorption was produced in the ultra-violet spectrum in the 245 m\(\mu\) to 270 m\(\mu\) range. Later, Matsumura &
O'Brien (1966) carried out further experiments using ultra-violet and fluorescent data to try to verify the formation of a charge-transfer complex.

d) MOLECULAR WEDGE

This model proposed by Holen (1969) also involves binding of DDT to the cell membrane. He concluded that the aliphatic part of the DDT molecule has a critical diameter for toxicity of 6.1 to 6.3 \( \text{Å} \) and an area of 50 - 60 \( \text{Å}^2 \). The limiting distance between \( p,p' \)-negative atom dipoles was found to be 11.5 \( \text{Å} \) with a higher limit of 14.0 \( \text{Å} \) when alkyl groups are present. Holen proposed that DDT interacts with the unit membrane (a protein layer and a lipid layer separated by about half the height of the DDT molecule) in such a way that the aromatic rings form a complex with the protein layer, and the aliphatic portion fits into a recess in the lipid layer, having the dimensions of the hydrated Na\(^+\) ion. The DDT molecule could thus be regarded as a wedge between the coils of a phospholipoprotein spring. This spring would be selectively permeable to non-solvated K\(^+\) ions when compressed and to hydrated Na\(^+\) ions when expanded. Therefore, when DDT, acting as a wedge, holds the spring in the expanded Na\(^+\)-permeable state, there will be a delay in the falling phase of the action potential, characteristic of DDT-poisoning in the nerve.

The negative temperature coefficient of intoxication evidence supports this, for an increase in temperature would result in dissociation of the aromatic rings from the protein layer so that the molecules are no longer held in position, and the spring is free to revert to its compressed state.
ACID PHOSPHOLIPID DESTABILISATION

Sharp et al (1974) provided evidence for the theory that chlorinated aromatic hydrocarbons e.g. DDT and Aroclor 1254 inactivated membrane Na\(^+\), K\(^+\) -ATPases by interfering with the stabilising function of acid phospholipids rather than the protein itself. They suggested that chlorinated hydrocarbons, when administered in experiments in the form of oil-in-water dispersions, inactivated Na\(^+\), K\(^+\) -ATPases as a result of forming mixed micelles of the enzymes and the inhibitor. This enzyme inactivation is unrelated to the hydrogen bonding of chlorinated hydrocarbons to phospholipid phosphate groups, as DDE and PCBs are incapable of such interactions (they do not possess benzhydryl hydrogens).
BIBLIOGRAPHY


BAYNE, B.L. (1975) Physiological Stress. 4th FAO/SIDA training course on aquatic pollution in relation to protection of living resources.


COX, J.L. (1971) DDT residues in seawater and particulate matter in the California current system. Fish Bull. 69 (2) 443-450.


DEPT. OF EDUCATION & SCIENCE (1969) Further review of certain persistent organochlorine pesticides used in Great Britain. Report by the Advisory Committee on Pesticides and Other Toxic Chemicals.

DEPT. OF THE ENVIRONMENT (1974) A report by the Central Unit of Environmental Pollution - The Non-Agricultural Uses of Pesticides in Great Britain. Pollution Paper No. 3.


JEFFERIES, D.J. & PARSLOW, J.L.F. (1976) Thyroid changes in PCB-dosed guillemots and their indication of one of the mechanisms of action of these materials. Environ. Pollut. 10 293-311.


PLATT, R.B. & GRIFFITHS, J.F. (1964) Environmental mea-

POKROVSKII, E.A. & BEL'KEVICH, V.I. (1958) The losses of
toxicity of preparations of DDT and gannexane under the

(1960) Membrane adenosine triphosphatase as participant
in active transport of sodium and potassium in human

PRICE, N.R. (1976) The effect of two insecticides on the
Ca\(^{2+}\) & Mg\(^{2+}\) — activated ATPase of the sarcoplasmic
reticulum of the flounder, Platichthys flesus. Comp.
Biochem. Physiol. 55C 91-94.

Physiology (2nd ed.), Philadelphia, W.B. Saunders.

Partial resolution of the enzymes catalyzing oxidative

RACINE, J. (1958) French Patent 1,206,352. Insecticidal and

RATCLIFFE, D.A. (1967) Decrease in eggshell weight in certain

RHEAD, M.M. (1975) The fate of DDT and PCBs in the marine
environment. In Chemical Society Specialist Periodical
Reports — Environmental Chemistry Vol. 1 137-159.


RISEBROUGH, R.W.; HUGGETT, R.J.; GRIFFIN, J.J. & GOLDBERG, E.D.
(1968) Pesticides: Transatlantic movements in the north-
est trades. Science 159 1233-1235.

excretion of DDA following ingestion of DDT and DDT

ROBURN, J. (1963) Effect of sunlight and ultraviolet
radiation on chlorinated pesticide residues. Chem. &
Ind. 38 1555-1556.

— Dinitrophenol auf die Embryonalentwicklung des Herings

de la zone de golgi et du systéme lysosomial dans les
hépatocytes en culture sous l'effet des pesticides


WEDEMEYER, G. (1968) Role of intestinal microflora in the degradation of DDT by rainbow trout (Salmo gairdneri). Life Sci. 7 219-223.


ADDITION

CHAPTER II
THE EFFECT OF DDT ON ION LEVELS IN THE HAEMOLYMPH OF CARCINUS MAENAS.

1. INTRODUCTION - THE BIOLOGY OF THE SHORE CRAB

The shore crab, *Carcinus maenas* (L) was chosen for the experimental work on the effects of DDT, as it is a representative species of the estuarine and littoral habitat in Great Britain. In inhabiting these areas, it would be exposed to many pollutants, especially insecticides used in agricultural areas which run off the land carried by rain water, into the rivers and the sea.

This crustacean is also easily obtained and kept under laboratory conditions. All specimens used in this work were obtained from the Marine Biological Association Laboratories, Plymouth, who caught the crabs in the estuary of the River Tamar.

a) EXTERNAL FEATURES

As in all crustaceans, the body consists of head, thorax and abdomen, the first two, in the case of crabs, being fused to form the cephalothorax. The greatly reduced abdomen is tucked underneath the cephalothorax. The main features are shown in Figs. 1 and 2. The adults are easily sexed by a number of features, the main one being the shape of the abdomen. In males, it is triangular, while in females it is broad and rounded (Fig. 2). Throughout the work, males only were used, as these seemed to be the more abundant of the individuals caught, and this excluded any sexual variation in physiological and biochemical parameters. Individuals of approximately the same size were used in each set of experiments, minimising any variation caused by age.

b) INTERNAL ANATOMY

The body fluid or haemolymph is contained in an 'open'
Fig. 1

Dorsal Surface of Male Carcinus maenas (L) (xl) (Crothers, 1967)

- 2nd antenna
- chela
- antero-lateral teeth
- 2nd walking leg
- 3rd walking leg
- 4th walking leg
- 5th walking leg
- carpus
- propus
- dactylus
- merus
- abdomen
Fig. 2

a) Ventral Surface of Female Carcinus maenas (xl)

(Crothers, 1967)

b) Ventral Surface of Male Carcinus maenas (xl)

(Crothers, 1967)
circulatory system and performs most of the functions of vertebrate blood, carrying nutrients, dissolved gasses and waste products. The haemolymph also contains haemocytes of two morphologically distinct types, which may be important in carbohydrate metabolism (Johnston et al., 1973; Johnston & Spencer Davies, 1972).

The respiratory pigment is haemocyanin, a copper-containing protein. Electrophoretic studies have revealed a number of distinct proteins, including two haemocyanins and an apohaemocyanin (Uglow, 1969, a, b).

Haemolymph is isosmotic with 100% seawater but is hyperosmotic with lower salinities (Fig. 3). Although the total ion concentration is approximately the same as seawater, the composition is not, and so some active transport occurs even in 100% seawater. For example, Webb (1940) states the composition of ions in the haemolymph of individuals maintained in normal seawater as:

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>110%</td>
</tr>
<tr>
<td>K⁺</td>
<td>118%</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>108%</td>
</tr>
<tr>
<td>Mg²⁻</td>
<td>34%</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>104%</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>61%</td>
</tr>
</tbody>
</table>

The concentration of each ion is expressed as a percentage of the concentration that would be expected if the blood were in dialysis equilibrium with the external medium.

Processes responsible for osmotic and ionic balance are (Webb, 1940):-

a) active absorption by the gills of Na⁺, K⁺, Ca²⁺ and Cl⁻ at a rate greater than that at which they are lost by diffusion,

b) differential excretion by the antennary gland, which tends to conserve K⁺ and eliminate Mg²⁺ and SO₄²⁻.
Fig. 3  Change of blood concentration with seawater concentration in *Carcinus maenas*
Fig. 4  Diagrams to Show Approximate Position of Viscera in Body of Carcinus maenas (Crothers, 1967)

a) Dorsal View

b) Lateral View
c) inward diffusion across the gills of Mg$^{2+}$ and SO$_4^{2-}$ in accordance with concentration gradient.

*Carcinus* has nine pairs of gills through which gaseous and ionic exchange take place. These are enclosed in two gill chambers, each having six openings - a small slit at the base of each walking leg, a large hole at the base of the chela and the largest in front of the mouth. Water-flow through the gills has been estimated at $1 \text{ cm}^3 (\text{g.wt. crab})^{-1} \text{ min.}^{-1}$ for large animals and $1.5 \text{ cm}^3 (\text{g.wt. crab})^{-1} \text{ min.}^{-1}$ for small ones (Arudpragasam & Naylor, 1964).

The relative positions of the organs of the body are shown in Fig. 4.

2. OSMOREGULATION

a) **INTRODUCTION**

Initially just the ion levels of the haemolymph of *Carcinus maenas* were measured under exposure to DDT, but later this was combined with assays on the activity of gill ATPases (Chapter III).

The haemolymph can be easily sampled from the animal and does not undergo clotting as readily as vertebrate blood.

Changes in the ion levels and total osmolality of the haemolymph were estimated as a measurement of the possible effects of DDT.
In 100% seawater, the body fluids of *Carcinus* are nearly isosmotic with the water. In this situation some active transport of ions with regard to osmoregulation must be taking place although at a slower rate than in low salinities. Preliminary experiments were performed in 100% seawater, but later this was changed to 50% seawater, in order to increase the difference in concentration between water and haemolymph leading to a higher degree of active transport and enzyme activity.

The levels of DDT in the environment are shown in Table 3 (I.5.b). Cox (1971) estimated that levels of DDT in the open ocean were between 2.3 and 5.6 x 10^{-9} g. dm^{-3}. In order to have some relevance to these environmental levels, low concentrations of DDT were used initially. These were later raised to higher levels of 100 - 200 x 10^{-9} g. dm^{-3}. These would represent concentrations occurring in the water in the event of accidental spillages or levels in localised areas resulting from run-off from agricultural land.

It has been estimated that the levels of organochlorine pesticides being transported by Britain's rivers to the sea average 180 x 10^{-6} g. dm^{-3} (Agricultural Research Council, 1970).

To facilitate monitoring of levels in the experimental tank, $^{14}$C-DDT was used.

b) **INSTRUMENTATION AND METHODS**

(i) **Preparation and extraction of $^{14}$C-DDT solutions in Seawater**

Uniformly ring-labelled $^{14}$C-DDT in benzene solution was obtained from The Radiochemical Centre, Amersham, Bucks., having a specific activity of 15 - 30 mCi mmol^{-1}. 
Appropriate amounts were redissolved in 95% ethanol ('AnalaR' grade - B.D.H. Chemicals Ltd., Poole, Dorset) and applied to the experimental tanks with thorough mixing.

At regular intervals, water samples (100cm$^3$) were removed for extraction and quantification of $^{14}$C-DDT. The samples were shaken with three aliquots (25cm$^3$) of toluene, the solvent fractions being pooled in a round-bottomed flask (250 cm$^3$) and evaporated to near-dryness using a Büchi rotary-evaporator.

(ii) Liquid-Scintillation Counting

The organic scintillant used was a 0.5% solution of Butyl-PBD ($\text{C}_8\text{H}_{12}$ - butylphenyl $\mathcal{J}$ - 5-$\mathcal{J}$ - biphenyl $\mathcal{J}$ - 1,3,4 - oxadiazole) (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) in toluene. Aliquots (10 cm$^3$) were added to the DDT extract in toluene, and the solution transferred to a scintillation vial. Radioactive assay was carried out in a Phillips Liquid Scintillation Analyser.

(iii) Production of a Quench Curve

In order to produce accurate results from radioactive counting in disintegrations per minute (d.p.m.), a quench curve must first be drawn. This shows the efficiency of counting $^{14}$C-compounds in the presence of the scintillant butyl-PBD by this particular Liquid Scintillation Analyser.

Identical aliquots of a labelled standard, $^{14}$C-n-hexadecane (1g = 2.26 x 10$^6$dpm) were radioactively counted in the presence of various quantities of carbon tetrachloride (to cause quenching, and thereby alter the external standard ratio). The external standard ratio is that between the quenched sample and a radioactive standard which is outside the vial but within
the liquid scintillation analyser. The efficiency of counting under these conditions is shown below.

<table>
<thead>
<tr>
<th>Volume $\text{CCl}_4$ (mm$^3$)</th>
<th>External Standard Ratio</th>
<th>Counts per minute (cpm)</th>
<th>% efficiency of counting</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.225</td>
<td>19839.8</td>
<td>87.6</td>
</tr>
<tr>
<td>10</td>
<td>1.182</td>
<td>19532.0</td>
<td>86.4</td>
</tr>
<tr>
<td>20</td>
<td>0.927</td>
<td>19083.4</td>
<td>84.4</td>
</tr>
<tr>
<td>30</td>
<td>0.779</td>
<td>18585.6</td>
<td>82.2</td>
</tr>
<tr>
<td>50</td>
<td>0.600</td>
<td>17030.5</td>
<td>75.3</td>
</tr>
<tr>
<td>70</td>
<td>0.514</td>
<td>15941.1</td>
<td>70.5</td>
</tr>
<tr>
<td>90</td>
<td>0.410</td>
<td>14247.3</td>
<td>63.0</td>
</tr>
<tr>
<td>100</td>
<td>0.388</td>
<td>13642.5</td>
<td>60.3</td>
</tr>
<tr>
<td>120</td>
<td>0.350</td>
<td>12917.3</td>
<td>57.1</td>
</tr>
<tr>
<td>150</td>
<td>0.231</td>
<td>9649.8</td>
<td>42.7</td>
</tr>
</tbody>
</table>

Counting efficiency never reaches 100%, even in the complete absence of carbon tetrachloride.

A quench curve of efficiency (%) against external standard ratio can now be drawn (Fig. 5). Counts per minute (c.p.m.) subsequently obtained in results can therefore be converted to d.p.m. (counts at 100% counting efficiency).

(iv) **Sampling of Haemolymph**

Haemolymph was taken by piercing the basal arthrodial membrane at the junction between the walking legs and the body of *Carcinus*. Sterile disposable syringes (1 cm$^3$) and needles (19G) were used (Gillette Industries Ltd., Middlesex).

a) **Measurement of chloride ions**

The concentration of Cl$^-$ ions was determined by the coulometric method using a Buchler-Cotlove Direct Reading Chloridometer (Model 4-2008). Haemolymph samples were diluted with distilled water prior to estimation by this method. Readings were in mEq. dm$^{-3}$. 
Fig. 5  Quench curve for $^{14}$C-compounds in Butyl-PBD

% efficiency of counting vs. External Standard Ratio
\textbf{Measurement of sodium and potassium ions}

The concentration of $\text{Na}^+$ and $\text{K}^+$ ions was estimated using an IL 343 Flame Photometer (Instrumentation Laboratories). The samples were diluted and readings were in mEq. dm$^{-3}$.

\textbf{Measurement of osmolality}

The total ion concentration or osmolality was determined using an Osmette semi-automatic cryoscopic osmometer (Osmette A-Precision Systems Inc.). The haemolymph samples ($0.2cm^3$) were not diluted for this. Readings were in milliosmoles per Kg (mOsm.kg$^{-1}$).

\textbf{Static Water System}

The initial experiments were carried out using transparent plastic tanks ($32 \times 22 \times 20$ cm) each containing water ($10dm^3$) and five animals. The water was aerated by means of a small air-pump and air-stones. As well as the experimental tank ($^{14}$C-DDT in ethanol), two controls (seawater and ethanol, seawater only) were also set up.

Ethanol caused the seawater to cloud very quickly, resulting in the necessity to change the water frequently. To minimize this factor and make the conditions more 'comfortable' for the animals, it was decided to set up a circulating, filtered system.

\textbf{Circulating Water System}

Three separate systems were built up, each consisting of two tanks and a filter, the water being pumped through a cooler. One of these systems is depicted in Fig. 6. The tanks ($30dm^3$) were made from glass-reinforced polyester (Osmaglass Cisterns-Osma Plastics Ltd., Hayes, Middlesex). The filter pump (Eheim, W. Germany) contained glass wool as filter medium.
Fig. 6 Circulating seawater system

overflow

water

pump and filter

cooler
The cooler ('maxi-cool' 14-Marston Paxman Ltd., Brighouse, Yorks.) kept the water at 10 - 11°C. Each system contained water (40dm³).

A holding tank containing water (28dm³) supplied each two-tank system. To the holding tank was added the DDT solution in ethanol (10cm³), ethanol only (10cm³) or nothing, depending on the system.

10cm³ of ethanol in 28dm³ of water resulted in a solution of 0.036% ethanol in water. As this water was dripped into the two-tank system, a very much lower ethanol concentration would finally result.

c) EXPERIMENTATION

(i) Experiment 1

The normal daily variation in the ion levels of the haemolymph of Carcinus maenas

Two individual crabs of the same sex (males) and approximately the same weight (35 - 40g) were kept in a tank of aerated circulating 100% seawater kept at 10 - 11°C. Haemolymph (0.5cm³) and seawater (0.5cm³) samples were taken over a period of 19 days and analysed for osmolality, Na⁺, K⁺ and Cl⁻ ions. These figures are shown in Table 1, and in graph form in Figs. 7: a, b, c, d.

The ion levels varied considerably from day to day, even when the seawater concentration of a particular ion was approximately constant.
| Day | CRAB A | | | CRAB B | | | | SEAWATER | | | |
|-----|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|     | Osm. 1 | mEq dm⁻³ | mEq dm⁻³ | Osm. 1 | mEq dm⁻³ | mEq dm⁻³ | Osm. 1 | mEq dm⁻³ | mEq dm⁻³ | Osm. 1 | mEq dm⁻³ | mEq dm⁻³ | mEq dm⁻³ |
|     | mOsm kg⁻¹ | Na⁺ | K⁺ | Cl⁻ | mOsm kg⁻¹ | Na⁺ | K⁺ | Cl⁻ | mOsm kg⁻¹ | Na⁺ | K⁺ | Cl⁻ | mOsm kg⁻¹ | Na⁺ | K⁺ | Cl⁻ |
| 1   | 1041   | 475   | 9.5 | 483.8 | 1043   | 465   | 8.5 | 461.3 | 1031   | 505   | 11.0 | 576.3 |        |
| 3   | 1055   | 515   | 11.5 | 512.5 | 1069   | 515   | 10.5 | 545.0 | 1045   | 495   | 11.0 | 565.0 |        |
| 5   | 1040   | 510   | 11.0 | 510.0 | 1062   | 515   | 9.5  | 532.5 | 1041   | 485   | 10.5 | 568.8 |        |
| 8   | 1060   | 500   | 9.0  | 517.5 | 1080   | 515   | 7.0  | 532.5 | 1037   | 515   | 11.5 | 561.3 |        |
| 10  | 1039   | 430   | 9.0  | 431.3 | 1051   | 505   | 9.0  | 506.3 | 1032   | 465   | 10.5 | 561.3 |        |
| 12  | 1045   | 460   | 9.5  | 518.8 | 1054   | 445   | 8.5  | 510.0 | 1034   | 410   | 9.5  | 578.8 |        |
| 15  | 1058   | 500   | 9.0  | 500.0 | 1074   | 560   | 10.0 | 563.8 | 1049   | 520   | 10.5 | 575.5 |        |
| 17  | 1063   | 430   | 8.5  | 443.8 | 1059   | 535   | 9.5  | 560.0 | 1042   | 500   | 12.0 | 583.8 |        |
| 19  | 1065   | 515   | 9.0  | 532.5 | 1067   | 425   | 7.5  | 442.5 | 1034   | 520   | 11.0 | 561.3 |        |

Table 1: Ion levels of haemolymph of *C. maenas* showing normal variation over a period of 19 days.
Fig. 7  Normal variation in ion levels of haemolymph of *Carcinus maenas* in 100% seawater

a) Sodium ions

b) Chloride ions
Fig. 7  Normal variation in ion levels of haemolymph of
Carcinus maenas in 100% seawater

Potassium ions

Osmolality

mEq dm$^{-3}$

mOsm kg$^{-1}$

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 days

-- crab A
--- crab B
-- -- seawater
(ii) Experiment 2

The effect of applying $2 \times 10^{-9} \text{g.dm}^{-3}$ of $^{14}$C-DDT to a static 100% seawater system on the ion levels of haemolymph of *Carcinus maenas*.

This was a short-term experiment lasting 10 days and employing the static water system. $^{14}$C-DDT solution in ethanol was added intermittently to the experimental tank (Tank A) to give a mean concentration during the experiment of $2.98 \times 10^{-9} \text{g.dm}^{-3}$. Ethanol alone, to act as a control, was added to Tank B. Tank C was the seawater control. Each tank held water (10dm$^3$) and five crabs of similar weight (36-44g). Haemolymph (0.5 cm$^3$) and water (0.5 cm$^3$) samples were taken for analysis of ion levels.

The crabs were identified by painting markings on the dorsal surface of the carapace with enamel paint.

The results are shown in Table 2: a, b, c.

There was no significant effect on ion levels and osmolality under these conditions, with the possible exception of an increased level of haemolymph sodium after 1 day of exposure to DDT (crab : seawater ratio = 1.24, compared with 0.98 for control animals). It was then thought that by using 50% seawater, a more noticeable effect on these parameters may be observed. In this situation a greater distinction would exist between ion and osmolality concentrations of water and haemolymph. Thus, if the osmoregulatory mechanism were affected, any change in haemolymph ion concentration would be more noticeable.
TABLE 2a - Ion levels of crabs exposed to 2-3x10^{-9} g.dm^{-3} {^{14}C-}DDT for 1 to 10 days. (TANK A)

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Sodium ions (Na^{+}) mEq.dm^{-3}</th>
<th>Potassium ions (K^{+}) mEq.dm^{-3}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>634 ± 9</td>
<td>510</td>
</tr>
<tr>
<td>3</td>
<td>479 ± 18</td>
<td>470</td>
</tr>
<tr>
<td>6</td>
<td>501 ± 14</td>
<td>510</td>
</tr>
<tr>
<td>8</td>
<td>502 ± 24</td>
<td>515</td>
</tr>
<tr>
<td>10</td>
<td>535 ± 11</td>
<td>540</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Chloride ions (Cl^{-}) mEq.dm^{-3}</th>
<th>Osmolality (mOsm.kg^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>532 ± 11</td>
<td>556</td>
</tr>
<tr>
<td>3</td>
<td>492 ± 20</td>
<td>533</td>
</tr>
<tr>
<td>6</td>
<td>502 ± 2</td>
<td>558</td>
</tr>
<tr>
<td>8</td>
<td>512 ± 29</td>
<td>560</td>
</tr>
<tr>
<td>10</td>
<td>525 ± 16</td>
<td>573</td>
</tr>
</tbody>
</table>

Values shown for crab haemolymph are mean and standard deviation of five individuals.
TABLE 2b - Ion levels of crabs exposed to ethanol only for 1 to 10 days. (TANK B)

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Sodium ions (Na⁺) mEq.dm⁻³</th>
<th>Potassium ions (K⁺) mEq.dm⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>574 ± 46</td>
<td>645</td>
</tr>
<tr>
<td>3</td>
<td>485 ± 21</td>
<td>475</td>
</tr>
<tr>
<td>6</td>
<td>527 ± 25</td>
<td>510</td>
</tr>
<tr>
<td>8</td>
<td>530 ± 14</td>
<td>520</td>
</tr>
<tr>
<td>10</td>
<td>536 ± 14</td>
<td>490</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Chloride ions (Cl⁻) mEq.dm⁻³</th>
<th>Osmolality (mOsm.kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>489 ± 12</td>
<td>553</td>
</tr>
<tr>
<td>3</td>
<td>489 ± 24</td>
<td>548</td>
</tr>
<tr>
<td>6</td>
<td>504 ± 27</td>
<td>558</td>
</tr>
<tr>
<td>8</td>
<td>511 ± 19</td>
<td>560</td>
</tr>
<tr>
<td>10</td>
<td>546 ± 18</td>
<td>545</td>
</tr>
</tbody>
</table>

Values shown for crab haemolymph are mean and standard deviation of five individuals.
<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Sodium ions (Na⁺) mEq.dm⁻³</th>
<th>Potassium ions (K⁺) mEq.dm⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>586 ± 40</td>
<td>600</td>
</tr>
<tr>
<td>3</td>
<td>514 ± 14</td>
<td>485</td>
</tr>
<tr>
<td>6</td>
<td>515 ± 30</td>
<td>510</td>
</tr>
<tr>
<td>8</td>
<td>492 ± 40</td>
<td>510</td>
</tr>
<tr>
<td>10</td>
<td>510 ± 29</td>
<td>515</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Chloride ions (Cl⁻¹) mEq.dm⁻³</th>
<th>Osmolality (mOsm.kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>476 ± 12</td>
<td>982 ± 15</td>
</tr>
<tr>
<td>3</td>
<td>496 ± 31</td>
<td>1007 ± 9</td>
</tr>
<tr>
<td>6</td>
<td>477 ± 25</td>
<td>992 ± 31</td>
</tr>
<tr>
<td>8</td>
<td>465 ± 16</td>
<td>1013 ± 21</td>
</tr>
<tr>
<td>10</td>
<td>493 ± 12</td>
<td>1010 ± 7</td>
</tr>
</tbody>
</table>

Values shown for crab haemolymph are mean and standard deviation of five individuals.
(iii) Experiment 3

The effect of applying \(3 \times 10^{-9} \text{g dm}^{-3}\) \(^{14}\text{C}\)-DDT in acetone to a static 50% seawater system on the ion levels of *Carcinus maenas* haemolymph.

Crabs of similar weights were kept in 50% seawater for about two weeks prior to experimentation to allow time for adaptation to this dilution. As ethanol caused the water to turn cloudy, it was decided to use acetone ("Aristar" grade) as the DDT-carrier.

The experiment was carried out over 19 days, but at the end, only one animal from each of Tanks A and B was surviving. Even though used at the same concentration as ethanol in the previous experiments, acetone proved to be more toxic to *Carcinus* (see Fig. 8).

For this reason, this particular experiment was abandoned and it was decided to use ethanol for future use, despite clouding and therefore the necessity to frequently change the water.

Using 50% seawater resulted in a marked difference between ion levels of the water and haemolymph. However, up to the time of most of the deaths (12 - 14 days), no significant changes in the ion levels of the haemolymph were observed when comparisons were made between experimental and control organisms.

(iv) Experiment 4

The effect of applying \(50 \times 10^{-9} \text{g dm}^{-3}\) \(^{14}\text{C}\)-DDT to a static 50% seawater system on the ion levels of *Carcinus maenas* haemolymph.

\(^{14}\text{C}\)-DDT in ethanol was added to 50% seawater in a holding tank, and this was dripped into Tank A at such a rate as to
The survival rate of *Carcinus maenas* in 50% seawater, when using acetone as the DDT-carrier.
result in an average concentration of $50 \times 10^{-9}\text{g.dm}^{-3}$ in the water. A similar system was arranged for Tank B with ethanol in the holding tank.

Five animals of the same sex and similar weights were kept in each tank and the experiment lasted 36 days.

The results of analysis of ions in the haemolymph and seawater are shown in Table 3: a, b, c.

There is a noticeable difference between ion and osmolality levels of the haemolymph and seawater under these conditions. However, there are no signs that osmoregulatory ability in these animals has been impaired by the presence of DDT.
<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Sodium ions (Na(^+)) mEq.dm(^{-3})</th>
<th>Potassium ions (K(^+)) mEq.dm(^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>339 ± 20</td>
<td>225</td>
</tr>
<tr>
<td>8</td>
<td>355 ± 9</td>
<td>275</td>
</tr>
<tr>
<td>11</td>
<td>352 ± 23</td>
<td>240</td>
</tr>
<tr>
<td>15</td>
<td>368 ± 26</td>
<td>220</td>
</tr>
<tr>
<td>18</td>
<td>364 ± 35</td>
<td>230</td>
</tr>
<tr>
<td>22</td>
<td>349 ± 20</td>
<td>240</td>
</tr>
<tr>
<td>25</td>
<td>361 ± 14</td>
<td>240</td>
</tr>
<tr>
<td>30</td>
<td>355 ± 15</td>
<td>230</td>
</tr>
<tr>
<td>36</td>
<td>352 ± 24</td>
<td>235</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Chloride ions (Cl(^-)) mEq.dm(^{-3})</th>
<th>Osmolality (mOsm.kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>324 ± 19</td>
<td>706 ± 32</td>
</tr>
<tr>
<td>8</td>
<td>356 ± 32</td>
<td>727 ± 13</td>
</tr>
<tr>
<td>11</td>
<td>354 ± 24</td>
<td>721 ± 19</td>
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<td>15</td>
<td>360 ± 29</td>
<td>742 ± 14</td>
</tr>
<tr>
<td>18</td>
<td>351 ± 19</td>
<td>733 ± 35</td>
</tr>
<tr>
<td>22</td>
<td>332 ± 16</td>
<td>709 ± 12</td>
</tr>
<tr>
<td>25</td>
<td>360 ± 16</td>
<td>723 ± 7</td>
</tr>
<tr>
<td>30</td>
<td>333 ± 18</td>
<td>672 ± 90</td>
</tr>
<tr>
<td>36</td>
<td>329 ± 15</td>
<td>695 ± 13</td>
</tr>
</tbody>
</table>

Values shown for crab haemolymph are mean and standard deviation of five individuals.
### Table 3a: Ion Levels of Crabs Exposed to Control Only, in Seawater (TANK B)

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Sodium ions (Na⁺) mEq.dm⁻³</th>
<th>Potassium ions (K⁺) mEq.dm⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>372 ± 15</td>
<td>270</td>
</tr>
<tr>
<td>8</td>
<td>357 ± 25</td>
<td>255</td>
</tr>
<tr>
<td>11</td>
<td>325 ± 27</td>
<td>240</td>
</tr>
<tr>
<td>15</td>
<td>346 ± 20</td>
<td>225</td>
</tr>
<tr>
<td>18</td>
<td>340 ± 21</td>
<td>230</td>
</tr>
<tr>
<td>22</td>
<td>356 ± 14</td>
<td>245</td>
</tr>
<tr>
<td>25</td>
<td>353 ± 16</td>
<td>245</td>
</tr>
<tr>
<td>30</td>
<td>343 ± 16</td>
<td>230</td>
</tr>
<tr>
<td>36</td>
<td>335 ± 22</td>
<td>210</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Chloride ions (Cl⁻) mEq.dm⁻³</th>
<th>Osmolality (mOsm.kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>355 ± 18</td>
<td>308</td>
</tr>
<tr>
<td>8</td>
<td>347 ± 14</td>
<td>258</td>
</tr>
<tr>
<td>11</td>
<td>327 ± 21</td>
<td>258</td>
</tr>
<tr>
<td>15</td>
<td>350 ± 14</td>
<td>278</td>
</tr>
<tr>
<td>18</td>
<td>328 ± 18</td>
<td>258</td>
</tr>
<tr>
<td>22</td>
<td>337 ± 18</td>
<td>278</td>
</tr>
<tr>
<td>25</td>
<td>338 ± 17</td>
<td>278</td>
</tr>
<tr>
<td>30</td>
<td>318 ± 22</td>
<td>260</td>
</tr>
<tr>
<td>36</td>
<td>319 ± 19</td>
<td>238</td>
</tr>
</tbody>
</table>

Values shown for crab haemolymph are mean and standard deviation of five individuals.
<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Sodium ions (Na(^+)) mEq.dm(^{-3})</th>
<th>Potassium ions (K(^+)) mEq.dm(^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>350 ± 27</td>
<td>225</td>
</tr>
<tr>
<td>8</td>
<td>388 ± 26</td>
<td>240</td>
</tr>
<tr>
<td>11</td>
<td>360 ± 14</td>
<td>235</td>
</tr>
<tr>
<td>15</td>
<td>354 ± 13</td>
<td>235</td>
</tr>
<tr>
<td>18</td>
<td>350 ± 17</td>
<td>240</td>
</tr>
<tr>
<td>22</td>
<td>371 ± 32</td>
<td>255</td>
</tr>
<tr>
<td>25</td>
<td>366 ± 23</td>
<td>255</td>
</tr>
<tr>
<td>30</td>
<td>363 ± 13</td>
<td>245</td>
</tr>
<tr>
<td>36</td>
<td>350 ± 11</td>
<td>225</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Chloride ions (Cl(^-)) mEq.dm(^{-3})</th>
<th>Osmolality (mOsm.kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>331 ± 24</td>
<td>233</td>
</tr>
<tr>
<td>8</td>
<td>328 ± 14</td>
<td>243</td>
</tr>
<tr>
<td>11</td>
<td>363 ± 15</td>
<td>243</td>
</tr>
<tr>
<td>15</td>
<td>353 ± 14</td>
<td>268</td>
</tr>
<tr>
<td>18</td>
<td>336 ± 17</td>
<td>270</td>
</tr>
<tr>
<td>22</td>
<td>339 ± 25</td>
<td>280</td>
</tr>
<tr>
<td>25</td>
<td>366 ± 14</td>
<td>280</td>
</tr>
<tr>
<td>30</td>
<td>346 ± 28</td>
<td>268</td>
</tr>
<tr>
<td>36</td>
<td>337 ± 13</td>
<td>238</td>
</tr>
</tbody>
</table>

Values shown for crab haemolymph are mean and standard deviation of five individuals.
So far, no striking effect of DDT on the ability of *Carcinus maenas* to osmoregulate had been observed. It was decided that in further experiments, the concentration of DDT used would be raised, and to combine a study on ion levels of the haemolymph with studies on the activity of the enzymes involved in active transport and osmoregulation — the ATPases.
BIBLIOGRAPHY


CHAPTER III
THE EFFECT OF DDT ON THE ACTIVITY OF ATP-ASES IN CARCINUS MAENAS.

1) INTRODUCTION

The characteristics of these membrane-bound enzymes, particularly Na\(^+\), K\(^+\)-ATPase and Mg\(^{2+}\)-ATPase have already been discussed in detail (I.7.2). In this section, several references have been given to work which has shown the effect of DDT on the activity of ATPases - both inhibitory and stimulatory.

No changes were shown in haemolymph ion levels as a result of DDT exposure. Since ATPases are known to be involved in active transport and osmoregulation, it was decided to investigate the effect of DDT on their activity.

Following this, further studies could be carried out on osmoregulatory ability, or on another aspect of metabolism associated with active transport.

2) ENZYME ASSAY

The activities of Na\(^+\), K\(^+\)-ATPase, Mg\(^{2+}\)-ATPase and total (Na\(^+\), K\(^+\), Mg\(^{2+}\))-ATPase were assayed in homogenates of Carcinus gill tissue. The activity was determined by measuring the amount of inorganic phosphate (P\(_i\)) produced when adenosine triphosphate (ATP) is converted to adenosine diphosphate (ADP). This was expressed as specific activity i.e. inorganic phosphate produced per unit of protein.

The crabs were killed by destruction of the thoracic ganglion, by piercing the exoskeleton in the region of a small depression in the ventral surface of the cephalothorax under the abdominal flap (see II.1 Fig. 2). The dorsal surface of the cephalothorax was then cut away to expose the viscera. The gills were rapidly removed and pooled in unbuffered ice-cold sucrose solution (0.25M).
(a) **PREPARATION OF HOMOGENATE**

The gill tissue was homogenised in a Potter-Elvehjem homogeniser with Teflon pestle connected to a motor. The homogeniser was held in an ice-bucket. This homogenate was then centrifuged at 4°C in an M.S.E. High Speed 18 centrifuge.

Preliminary investigations were carried out to determine which cellular fraction exhibited the highest enzyme activity. This fraction was then to be used in future studies.

Koch (1969) estimated that the highest ATPase activity was either in the nuclear fraction (600 - 1000g) containing large fragments of cell membranes and other cell debris, or in microsomes (30,000 - 100,000g). In some studies the highest activity has been distributed between the two.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Conditions</th>
<th>Ultrastructure</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>900g x 10 mins</td>
<td>nuclei, cell fragments, red blood cells</td>
</tr>
<tr>
<td>B</td>
<td>13,000g x 20 mins</td>
<td>mitochondria, nerve endings</td>
</tr>
<tr>
<td>S</td>
<td>supernatant from 13,000g</td>
<td>microsomes, solubles</td>
</tr>
</tbody>
</table>

(From Koch (1969))

Mitochondria have little or no Na\(^+\), K\(^+\)-ATPase activity but show Mg\(^{2+}\)-ATPase activity. Microsomes are not organelles themselves, but are thought to be largely derived from endoplasmic reticulum, which in some cells is continuous with the cell membrane. During homogenisation the endoplasmic reticulum is broken up and bits form themselves into vesicles which float in the fluid and are centrifuged down last.
In this preliminary work, the homogenate was centrifuged at 1000g, the supernatant then being centrifuged at 13,000g, and then at 35,000g. The pellet obtained at each stage was resuspended in ice-cold sucrose solution (0.25M) and these, together with the final supernatant obtained at 35,000g, were assayed for total ATPase activity. The specific activity of each fraction is shown below:

Activity of Total-ATPase at various stages of differential centrifugation

<table>
<thead>
<tr>
<th>Force</th>
<th>Time</th>
<th>μgP₁ (mg protein)⁻¹ hr⁻¹</th>
<th>μMP₁ (mg protein)⁻¹ hr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000g</td>
<td>20 mins</td>
<td>47.0</td>
<td>1.5</td>
</tr>
<tr>
<td>13,000g</td>
<td>30 mins</td>
<td>93.0</td>
<td>3.0</td>
</tr>
<tr>
<td>35,000g</td>
<td>30 mins</td>
<td>54.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Supernatant</td>
<td></td>
<td>20.7</td>
<td>0.67</td>
</tr>
</tbody>
</table>

On the basis of these results, it was decided to use the middle two fractions in future estimations.

The homogenate in sucrose solution was centrifuged at 1000g for 30 minutes, the supernatant removed and centrifuged at 35,000g. The pellet obtained from this was then re-homogenised in ice-cold sucrose solution, and this homogenate used in the enzyme assays. This contained microsomes and mitochondria.

(b) PROTEIN ESTIMATION

Both protein and phosphate were estimated colorimetrically, and therefore for each of these a calibration graph had to be constructed using standard solutions.

Protein was estimated by the method of Lowry et al (1951) modified by Leggett Bailey (1962). The standard used was bovine serum albumen (Cohn fraction V), which was made to different concentrations with distilled water. This contained
96% protein (Koch Light Laboratories, Colnbrook, Bucks.).

Reagents:

A. 2% Na$_2$CO$_3$ in 0.1N NaOH
B. 0.5% CuSO$_4$·5H$_2$O in 1% sodium citrate
C. 1cm$^3$ reagent B mixed with 50cm$^3$ reagent A
D. Folin-Ciocalteu reagent diluted to 1N in acid.

An aliquot (0.2cm$^3$) of the protein solution was mixed with reagent C (3cm$^3$) and the solution allowed to stand for 10 minutes at room temperature. Reagent D (0.3cm$^3$) was added and mixed thoroughly. This was allowed to stand for colour-development for 70 minutes, and the absorbance read at 750 µm on a Unicam SP 600 spectrophotometer.

The range of standard solutions used and their optical densities are shown below. A calibration graph was drawn.

<table>
<thead>
<tr>
<th>Albumen in 100cm$^3$ water</th>
<th>Protein in 0.2cm$^3$</th>
<th>Optical density (absorbance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10$^{-2}$</td>
<td>19.20 x 10$^{-6}$g</td>
<td>0.21</td>
</tr>
<tr>
<td>1.51 x 10$^{-2}$g</td>
<td>28.99 x 10$^{-6}$g</td>
<td>0.39</td>
</tr>
<tr>
<td>2.08 x 10$^{-2}$g</td>
<td>39.94 x 10$^{-6}$g</td>
<td>0.47</td>
</tr>
<tr>
<td>4.01 x 10$^{-2}$g</td>
<td>76.99 x 10$^{-6}$g</td>
<td>0.84</td>
</tr>
<tr>
<td>4.98 x 10$^{-2}$g</td>
<td>95.60 x 10$^{-6}$g</td>
<td>1.00</td>
</tr>
<tr>
<td>6.02 x 10$^{-2}$g</td>
<td>115.58 x 10$^{-6}$g</td>
<td>1.20</td>
</tr>
<tr>
<td>7.14 x 10$^{-2}$g</td>
<td>137.09 x 10$^{-6}$g</td>
<td>1.40</td>
</tr>
<tr>
<td>7.70 x 10$^{-2}$g</td>
<td>147.84 x 10$^{-6}$g</td>
<td>1.45</td>
</tr>
</tbody>
</table>

To estimate the protein content of the gill homogenate, an aliquot of tissue (0.2cm$^3$) was used in the method (as above). This was carried out in triplicate in each case, and from the mean optical density value, the protein content could thus be determined.
CONDITIONS FOR INCUBATION

Total (Na\(^+\), K\(^+\), Mg\(^{2+}\))-ATPase and Mg\(^{2+}\)-ATPase were measured directly, and from these the Na\(^+\), K\(^+\)-ATPase activity could be determined by difference. The only difference in medium composition for the two incubations, was the inclusion of the cardiac glycoside, ouabain in that for the Mg\(^{2+}\)-ATPase assay. This specifically inhibits Na\(^+\), K\(^+\)-ATPase.

It was essential to determine the optimum conditions for the enzymatic reaction prior to carrying out the experimental tests. These included incubation temperature, pH and composition of the medium and substrate concentration.

1) **Medium**

According to Skou (1965), if the medium contains Na\(^+\) as well as Mg\(^{2+}\) (III.2.c.ii), the addition of one of the cations: K\(^+\), Rb\(^+\), Cs\(^+\), NH\(_4\)^+ or Li\(^+\) leads to a considerable increase in activity. The highest activity is obtained with NH\(_4\)^+, followed by K\(^+\), Rb\(^+\), Cs\(^+\) and the lowest with Li\(^+\). The enzyme system, however, has the highest affinity for K\(^+\), followed by Rb\(^+\), NH\(_4\)^+, Cs\(^+\) and Li\(^+\) in that order.

Therefore, it was decided to compare the activity of the enzymes in the presence of K\(^+\) and NH\(_4\)^+ ions. The composition of the media are shown below (modified from Phillips & Wells, 1974).

<table>
<thead>
<tr>
<th>Medium for Total-ATPase</th>
<th>Medium for Mg(^{2+})-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td></td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>100 mM NaCl</td>
</tr>
<tr>
<td>20 mM KCl</td>
<td>20 mM KCl</td>
</tr>
<tr>
<td>50 mM Tris buffer (pH 7.5)</td>
<td>50 mM Tris buffer (pH 7.5)</td>
</tr>
<tr>
<td></td>
<td>1 mM ouabain</td>
</tr>
<tr>
<td>b)</td>
<td></td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>100 mM NaCl</td>
</tr>
<tr>
<td>20 mM NH(_4)Cl</td>
<td>20 mM NH(_4)Cl</td>
</tr>
<tr>
<td>50 mM Tris buffer (pH 7.5)</td>
<td>50 mM Tris buffer (pH 7.5)</td>
</tr>
<tr>
<td></td>
<td>1 mM ouabain</td>
</tr>
</tbody>
</table>
In comparing the enzyme activities in the two media (a and b), it was found that there was little difference between the activities (Table 1). It was decided to use $K^+$ ions in the media for future assays.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Total-ATPase $\mu$gP$_i$ (mg protein)$^{-1}$ hr$^{-1}$</th>
<th>Mg$^{2+}$-ATPase $\mu$gP$_i$ (mg protein)$^{-1}$ hr$^{-1}$</th>
<th>Na$^+$, K$^+$-ATPase $\mu$gP$_i$ (mg protein)$^{-1}$ hr$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>a : $K^+$</td>
<td>115.00</td>
<td>57.69</td>
<td>57.31</td>
</tr>
<tr>
<td>b : $NH_4^+$</td>
<td>100.00</td>
<td>51.28</td>
<td>48.72</td>
</tr>
</tbody>
</table>

Many workers have included the chelating agent, ethylene diamine tetra acetic acid (EDTA) in the medium to bind traces of heavy metal ions which may be inhibitory to ATPases. However, when used in the sucrose solution, EDTA (0.005M) disodium salt appeared to significantly inhibit the enzyme activity. It was decided to exclude this compound. Akera et al (1971) and Jackson & Gardner (1973), amongst others, do not include EDTA in their enzyme assays.

ii) **Substrate**

The enzyme reaction was initiated by the addition of the substrate to the medium and gill homogenate. This consisted of the disodium salt of adenosine triphosphate (Na$_2$ATP) (100mM) and $MgCl_2$ (100mM). Mg$^{2+}$ ions are essential for the activity of ATPases.

The actual concentrations of these two compounds were kept constant, while the volume added to the reaction mixture was varied to determine greatest activity (Table 2).
Table 2 - Activity of ATPase in the presence of various volumes of substrate.

<table>
<thead>
<tr>
<th>Volume of substrate (cm$^3$)</th>
<th>Total ATPase activity $\mu$gP$_i$ (mg protein)$^{-1}$hr$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.03</td>
<td>156.5</td>
</tr>
<tr>
<td>0.05</td>
<td>174.4</td>
</tr>
<tr>
<td>0.1</td>
<td>167.9</td>
</tr>
<tr>
<td>0.2</td>
<td>34.3</td>
</tr>
<tr>
<td>0.3</td>
<td>6.5</td>
</tr>
</tbody>
</table>

A graph could be drawn from these figures showing that the greatest activity was obtained when a volume of about 50 mm$^3$ was added (Fig. 1). Either side of this, the activity was reduced.

Fig. 1: Total ATPase activity in the presence of different volumes of substrate (Na$_2$ATP + MgCl$_2$)
iii) Physical Conditions

Investigations on the effect of temperature on enzyme activity were initially limited by the availability of constant temperature rooms in which a mechanical shaker could be kept. At a later stage, a Psychrotherm Incubation Shaker was made available, which could be set at any temperature.

The temperatures at which the reaction was carried out were $21^\circ$, $35^\circ$, $40^\circ$C. Activity of Total-ATPase was greatest at $35^\circ$C.

The pH of the reaction medium was also varied, by altering the amount of hydrochloric acid in which the Tris-buffer was made up. The activity of Total-ATPase under these conditions is shown below.

<table>
<thead>
<tr>
<th>pH</th>
<th>Total ATPase activity $\mu$gP$_i$(mg protein)$^{-1}$ hr$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>103.5</td>
</tr>
<tr>
<td>6.4</td>
<td>117.6</td>
</tr>
<tr>
<td>7.2</td>
<td>112.7</td>
</tr>
<tr>
<td>7.5</td>
<td>145.2</td>
</tr>
<tr>
<td>8.0</td>
<td>120.5</td>
</tr>
</tbody>
</table>

A pH of 7.5 was intended in the enzyme assays. However, Na$_2$ATP in solution was very acidic, and addition of 50 mm$^3$ of this to the enzyme preparation in sucrose solution, resulted in a lowering of the pH by 0.5. A final pH of 7.0 was therefore attained.

These preliminary runs resulted in values being obtained which provided optimum conditions for the enzyme reactions described in the following section on incubation procedure.
(d) INCUBATION PROCEDURE

To an aliquot (0.2 cm$^3$) of the enzyme preparation in polythene-stoppered glass vials, was added the medium (4 cm$^3$), the composition of which has been described earlier (III.2.c.i.), at pH 7.0. In the case of in vitro studies, a DDT solution was added at this point (III.3.a.). The vials were shaken at 120 r.p.m. at 15°C for 30 minutes in a Psychrotherm Incubation Shaker. This procedure allowed the medium (and DDT) to pervade the tissues before assaying ATPase activity.

The enzyme reaction was initiated by addition of the substrate (50 mm$^3$), containing MgCl$_2$ (100 mM) and Na$_2$ATP (100 mM) in distilled water. Incubation was continued at 35°C for one hour with constant shaking at 120 r.p.m.

The reaction was terminated by the addition of ice-cold 30% trichloroacetic acid (1 cm$^3$), which precipitates the protein. The vials were kept at 3°C for 30 minutes in order to assist this, before estimation of phosphate.

Incubations were carried out in triplicate and with a 'control' reaction for each particular set of conditions. The control acted as a 'blank' for colorimetric phosphate estimation, and was identical to the others in composition, except that the enzyme preparation was added at the end of the incubation period.

(e) PHOSPHATE ESTIMATION

Inorganic phosphate was measured colorimetrically by the method of Fiske and Subbarow (1925) and Oser (1965).
Reagents

A Acid molybdate solution (2.5% ammonium molybdate in 5N H₂SO₄).

B Reducing solution (27.4g sodium metabisulphite + 10g metal = p-methyl-aminophenol sulphate, made up to 500cm³ with distilled water).

After allowing the protein precipitate to settle, an aliquot (1cm³) of the clear supernatant was removed, and diluted with distilled water (2cm³). To this was added Reagent A (0.5cm³), followed by Reagent B (0.4cm³). This was shaken well and allowed to stand at room temperature for 30 minutes for colour development. The optical density was read at 750 μm.

As for measurement of protein, a calibration graph must be constructed for phosphate. Standards of potassium hydrogen orthophosphate (K₂HPO₄) were made up in distilled water, and the optical density (absorbance) noted for different dilutions. When estimating phosphate in the reaction mixtures, trichloroacetic acid would be present in the 1cm³ of supernatant removed. Therefore 0.8cm³ of standard and 0.2cm³ of 30% trichloroacetic acid were used in the production of the calibration graph.

Optical density of standards

<table>
<thead>
<tr>
<th>P₁ in 0.8cm³ (x 10⁻³ g)</th>
<th>Optical density (absorbance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0031</td>
<td>0.082</td>
</tr>
<tr>
<td>0.0062</td>
<td>0.157</td>
</tr>
<tr>
<td>0.0124</td>
<td>0.310</td>
</tr>
<tr>
<td>0.0186</td>
<td>0.470</td>
</tr>
<tr>
<td>0.0248</td>
<td>0.625</td>
</tr>
</tbody>
</table>

The activity of the enzymes are expressed as mgP₁ produced (mg. protein)⁻¹ hr⁻¹.
3) EXPERIMENTATION

(a) EXPERIMENT 1

In vitro changes in the activity of ATPases in the gills of Carcinus maenas exposed to various concentrations of DDT (Jowett et al., 1978)

Crabs were maintained for ten days in 50% seawater (15%S) in the circulating system (II.2.b.vi) at 10° - 11°C. At this seawater concentration, the ATPases were considered to be more active. The gills were removed and the enzyme preparation obtained by the procedure already discussed (III.2.a).

p,p'-DDT (99+% pure : Ralph N. Emanuel Ltd., Wembley) solutions were made up in ethanol (95% w.w.) to give concentrations of $10^{-5}$ M, $10^{-6}$ M, $10^{-8}$ M, $10^{-9}$ M and $10^{-10}$ M in the final mixture when 10mm$^3$ of the solution were added. This would give equivalent concentrations of:

- $10^{-5}$ M $\equiv 354.5 \times 10^{-5}$ g. dm$^{-3}$
- $10^{-6}$ M $\equiv 354.5 \times 10^{-6}$ g. dm$^{-3}$
- $10^{-8}$ M $\equiv 354.5 \times 10^{-8}$ g. dm$^{-3}$
- $10^{-9}$ M $\equiv 354.5 \times 10^{-9}$ g. dm$^{-3}$
- $10^{-10}$ M $\equiv 354.5 \times 10^{-10}$ g. dm$^{-3}$

DDT solution (10mm$^3$) was added to the medium (4cm$^3$) and enzyme preparation (0.2cm$^3$) and the vials shaken at 120 r.p.m. at 15°C for 30 minutes to allow the medium and DDT to pervade the tissues. Controls containing no additives, and solely ethanol, were also set up. The incubation was carried out in triplicate. Incubation, estimation of protein and phosphate are as described earlier (III.2).
Table 3 shows the activities of the enzymes in the presence of different DDT concentrations, and in the control reactions. These data are graphed in Fig. 2. The figures in brackets show the range of values and these are depicted as bars on the graph.

Table 4 shows the percentage change in activity of the enzyme systems from the controls in the presence of the various concentrations of DDT.

The data were assessed statistically by first carrying out an analysis of variance of the measured specific activities. The least significant differences (L.S.D.) were then calculated according to Snedecor and Cochran (1972) for probability values of $P = 0.05$ and $P = 0.01$.

The L.S.D.'s are:

<table>
<thead>
<tr>
<th></th>
<th>$P = 0.05$</th>
<th>$P = 0.01$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total $-\text{ATPase}$</td>
<td>12.1</td>
<td>16.5</td>
</tr>
<tr>
<td>Mg$^{2+}$ $-\text{ATPase}$</td>
<td>19.8</td>
<td>27.1</td>
</tr>
<tr>
<td>Na$^+$, K$^+$ $-\text{ATPase}$</td>
<td>11.5</td>
<td>15.7</td>
</tr>
</tbody>
</table>

Where two means differ by more than the L.S.D., they can be considered to be significantly different at the respective probability value.

The significance of inhibition or stimulation of enzyme activity by DDT is shown on Table 4.

This statistical work was carried out by Dr. B.L. Bayne of the Institute for Marine Environmental Research.
Table 3 - Activity of ATPases in the presence of \( p,p'\)-DDT

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total -ATPase</th>
<th>( \mu g \text{Pi} (\text{mg protein})^{-1} \text{ hr}^{-1} )</th>
<th>( \text{Mg}^{2+} ) -ATPase</th>
<th>( \text{Na}^+, \text{K}^+ ) -ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>152.34</td>
<td>135.68</td>
<td>16.67</td>
<td>(9.65 - 29.83)</td>
</tr>
<tr>
<td></td>
<td>(145.61 - 159.65)</td>
<td>(129.83 - 141.23)</td>
<td>(96.49 - 114.04)</td>
<td>(42.11 - 56.14)</td>
</tr>
<tr>
<td>ETHANOL</td>
<td>152.05</td>
<td>135.09</td>
<td>16.96</td>
<td>(9.65 - 27.19)</td>
</tr>
<tr>
<td></td>
<td>(139.47 - 159.65)</td>
<td>(129.83 - 145.61)</td>
<td>(98.25 - 102.63)</td>
<td>(43.86 - 64.91)</td>
</tr>
<tr>
<td>( 10^{-10} ) M DDT</td>
<td>154.97</td>
<td>107.90</td>
<td>52.92</td>
<td>(42.11 - 49.12)</td>
</tr>
<tr>
<td></td>
<td>(152.63 - 156.14)</td>
<td>(96.49 - 114.04)</td>
<td>(75.81 - 97.63)</td>
<td>(27.19 - 43.86)</td>
</tr>
<tr>
<td>( 10^{-9} ) M DDT</td>
<td>153.80</td>
<td>100.88</td>
<td>46.78</td>
<td>(42.11 - 49.12)</td>
</tr>
<tr>
<td></td>
<td>(145.61 - 167.54)</td>
<td>(86.84 - 95.61)</td>
<td>(86.84 - 95.61)</td>
<td>(27.19 - 43.86)</td>
</tr>
<tr>
<td>( 10^{-8} ) M DDT</td>
<td>138.60</td>
<td>91.81</td>
<td>40.35</td>
<td>(34.21 - 46.49)</td>
</tr>
<tr>
<td></td>
<td>(135.09 - 144.74)</td>
<td>(75.81 - 97.63)</td>
<td>(58.77 - 69.30)</td>
<td>(34.21 - 46.49)</td>
</tr>
<tr>
<td>( 10^{-6} ) M DDT</td>
<td>125.75</td>
<td>89.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(119.30 - 136.84)</td>
<td>(75.81 - 97.63)</td>
<td></td>
<td>(34.21 - 46.49)</td>
</tr>
<tr>
<td>( 10^{-5} ) M DDT</td>
<td>104.68</td>
<td>64.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(99.12 - 111.40)</td>
<td>(58.77 - 69.30)</td>
<td></td>
<td>(34.21 - 46.49)</td>
</tr>
</tbody>
</table>

Activities show the mean and range of triplicate samples.
The activity of Total-, Mg$^{2+}$- and Na$^+$, K$^+$-ATPases of *Carcinus maenas* gill homogenate in the presence of various concentrations of p,p'$'$-DDT.

All activities show the mean and range of three measurements.
Fig. 2

The diagram illustrates the effect of different concentrations of DDT on enzyme activity measured in µgP_i (mg protein)^{-1} hour^{-1}.

- The highest enzyme activity is observed at concentrations of 10^{-10} M to 10^{-11} M DDT.
- As the concentration of DDT increases from 10^{-11} M to 10^{-5} M, enzyme activity decreases significantly.
- The concentration of DDT is indicated on the x-axis, while enzyme activity is shown on the y-axis.

The error bars indicate the variability of the data points.
The percentage change in activity of gill ATPases of *Carcinus maenas* in the presence of various concentrations of p,p'-DDT

<table>
<thead>
<tr>
<th>Conc. of p,p'-DDT</th>
<th>Total -ATPase activity (%)</th>
<th>Mg$^{2+}$-ATPase activity (%)</th>
<th>Na$^+, K^+$-ATPase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-5}$ M</td>
<td>-13.18**</td>
<td>-52.50**</td>
<td>+141.76**</td>
</tr>
<tr>
<td>$10^{-6}$ M</td>
<td>-17.33**</td>
<td>-34.18**</td>
<td>+120.73**</td>
</tr>
<tr>
<td>$10^{-8}$ M</td>
<td>-8.69*</td>
<td>-32.20**</td>
<td>+180.29**</td>
</tr>
<tr>
<td>$10^{-9}$ M</td>
<td>+1.14</td>
<td>-25.51**</td>
<td>+217.08**</td>
</tr>
<tr>
<td>$10^{-10}$ M</td>
<td>+1.89</td>
<td>-20.32*</td>
<td>+182.09**</td>
</tr>
<tr>
<td>Ethanol</td>
<td>-0.10</td>
<td>-0.24</td>
<td>+1.62</td>
</tr>
</tbody>
</table>

- denotes inhibition of activity  
+ denotes stimulation of activity  
* denotes values different from control at $P = 0.05$  
** denotes values different from control at $P = 0.01$.

Mg$^{2+}$-ATPase was shown (Table 3 & Fig. 2) to be inhibited at all of the concentrations of DDT used, ranging from 20.32% at $10^{-10}$ M to 52.50% inhibition at $10^{-5}$ M DDT (Table 4). There was no stimulation of activity. Na$^+, K^+$-ATPase, however, showed a considerable increase in activity (Table 3 & Fig. 2) at all concentrations of DDT. In the lower concentrations ($10^{-9}$ M DDT) stimulation of activity reached a maximum of 217.08% (Table 4). As the concentration was increased further, the activity decreased, but even at $10^{-5}$ M DDT, activity was 141.76% of the control. Total -ATPase activity is a sum of the two previous enzyme systems. This total activity was not significantly altered at the lower p,p'-DDT concentrations tested, and an inhibition of activity occurred at higher concentrations (Fig. 2). Under all conditions, Na$^+, K^+$-ATPase
activity was lower than Mg$^{2+}$-ATPase activity.

(b) EXPERIMENT 2

To show the normal variation in activity of ATPases between individuals maintained in 50% seawater

The use of pooled tissues from several crabs for the *in vitro* experiment, removed any possibility of individual variation in enzyme activity. The *in vivo* experiments, however, involved the use of tissues from 5 individual crabs, taking the mean value of these.

In an effort to determine the degree of individual variation in gill Total- $\text{Mg}^{2+}$, and $\text{Na}^+$, $\text{K}^+$-ATPase activities, assays were carried out according to III.2. Animals of similar sizes and the same sex (male) were used. The weights of crabs and gills are shown in Table 5, and the activities of ATPases in Table 6.

**Table 5**  Weight of gills and protein content of gill homogenate of 5 individual crabs

<table>
<thead>
<tr>
<th>Individual</th>
<th>Wt. of crab (g)</th>
<th>Wt. of gills (g)</th>
<th>Protein per 0.2 cm$^3$ homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Absorbance</td>
</tr>
<tr>
<td>A</td>
<td>75.41</td>
<td>2.341</td>
<td>0.705</td>
</tr>
<tr>
<td>B</td>
<td>71.07</td>
<td>2.382</td>
<td>1.018</td>
</tr>
<tr>
<td>C</td>
<td>71.33</td>
<td>2.318</td>
<td>0.749</td>
</tr>
<tr>
<td>D</td>
<td>75.06</td>
<td>2.286</td>
<td>0.695</td>
</tr>
<tr>
<td>E</td>
<td>72.71</td>
<td>3.010</td>
<td>0.795</td>
</tr>
<tr>
<td></td>
<td>Total - ATPase (μg Pi/(mg protein)⁻¹ hr⁻¹)</td>
<td>Mg²⁺ -ATPase (μg Pi/(mg protein)⁻¹ hr⁻¹)</td>
<td>Na⁺; K⁺ -ATPase (μg Pi/(mg protein)⁻¹ hr⁻¹)</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------------------------</td>
<td>---------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>A</td>
<td>164.7 (156.7 - 179.1)</td>
<td>81.1 (79.1 - 82.1)</td>
<td>83.6 (74.6 - 100.0)</td>
</tr>
<tr>
<td>B</td>
<td>180.4 (166.0 - 201.0)</td>
<td>93.8 (90.7 - 99.0)</td>
<td>86.6 (67.0 - 109.2)</td>
</tr>
<tr>
<td>C</td>
<td>270.4 (238.0 - 301.4)</td>
<td>126.8 (118.3 - 132.4)</td>
<td>143.6 (108.4 - 183.1)</td>
</tr>
<tr>
<td>D</td>
<td>217.7 (200.0 - 243.9)</td>
<td>96.5 (95.5 - 97.0)</td>
<td>121.2 (103.0 - 146.9)</td>
</tr>
<tr>
<td>E</td>
<td>272.4 (264.5 - 288.2)</td>
<td>144.3 (138.2 - 150.0)</td>
<td>218.1 (114.5 - 150.0)</td>
</tr>
<tr>
<td></td>
<td>Mean (± SD) activity for 5 individuals =</td>
<td>Mean (± SD) activity for 5 individuals =</td>
<td>Mean (± SD) activity for 5 individuals =</td>
</tr>
<tr>
<td></td>
<td>221.1 ± 49.8</td>
<td>108.5 ± 26.1</td>
<td>112.6 ± 26.4</td>
</tr>
</tbody>
</table>

Activities show the mean and range of triplicate samples.
As with ion levels in the haemolymph, there is a large variation in ATPase activities in gill homogenates of individuals (Table 6), despite sex and size variation being minimized.

Maintenance of the crabs in 50% seawater could accentuate individual variation from that which may be expected in 100% seawater. The former concentration was selected, since the necessity for the animals to osmoregulate, maintaining the body fluids hyperosmotic to the water, would be greater. Consequently, it would be expected that ATPase activities would be greater, and therefore easier to measure.

(c) EXPERIMENT 3

_in vivo_ changes in the activity of ATPases in the gills, and ion levels in the haemolymph of _Carcinus maenas_ exposed to 200 × 10^{-9} \text{gdm}^{-3} \text{14}^{\text{C}}\text{-DDT}

This study was an extension of Experiment 2, taking the previous results as activities at zero time.

The concentration of DDT used was higher than used previously. A solution containing \text{14}^{\text{C}}\text{-DDT} and unlabelled DDT was made up, thus resulting in a greater mass to radioactivity ratio.

50% seawater in the circulating system was kept at 10°-11°C and filtered through glass wool.

After 8, 17 and 29 days, 5 animals from each tank were sacrificed for gill ATPase assays. The activities of Mg^{2+}, Na^{+}, K^{+} and Total-ATPases are summarised in Table 7.
### Summary of results of ATPase activities in gill homogenates with exposure to 200 × 10⁻⁹ gdm⁻³ ¹⁴C-DDT, and relevant controls

<table>
<thead>
<tr>
<th></th>
<th>Total-ATPase</th>
<th>Mg²⁺-ATPase</th>
<th>Na⁺, K⁺-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0 days</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SW</td>
<td>221.12 ± 49.8</td>
<td>108.50 ± 26.1</td>
<td>112.62 ± 26.4</td>
</tr>
<tr>
<td>DDT ETOH</td>
<td>180.31 ± 78.8</td>
<td>103.77 ± 38.2</td>
<td>76.54 ± 46.1</td>
</tr>
<tr>
<td>SW</td>
<td>282.48 ± 54.6</td>
<td>163.77 ± 36.9</td>
<td>118.71 ± 20.3</td>
</tr>
<tr>
<td>SW</td>
<td>240.22 ± 38.6</td>
<td>144.05 ± 13.5</td>
<td>96.18 ± 26.0</td>
</tr>
<tr>
<td><strong>17 days</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDT ETOH</td>
<td>155.60 ± 33.9</td>
<td>93.76 ± 13.4</td>
<td>61.84 ± 24.9</td>
</tr>
<tr>
<td>SW</td>
<td>272.82 ± 43.3</td>
<td>151.48 ± 25.5</td>
<td>121.35 ± 19.5</td>
</tr>
<tr>
<td>SW</td>
<td>305.10 ± 16.8</td>
<td>142.39 ± 6.8</td>
<td>162.71 ± 18.9</td>
</tr>
<tr>
<td><strong>29 days</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDT ETOH</td>
<td>135.58 ± 8.6</td>
<td>71.28 ± 13.3</td>
<td>64.30 ± 10.5</td>
</tr>
<tr>
<td>SW</td>
<td>179.12 ± 4.5</td>
<td>94.54 ± 12.1</td>
<td>85.18 ± 13.0</td>
</tr>
<tr>
<td>SW</td>
<td>182.41 ± 10.4</td>
<td>91.19 ± 26.7</td>
<td>91.23 ± 18.2</td>
</tr>
</tbody>
</table>

Figures show the mean and one standard deviation of 5 individuals under each condition.

The activities shown at the start are taken from Table 6. Activities are measured as μg Pi (mg protein)⁻¹ hr⁻¹.
TABLE 8a - Ion levels of crabs exposed to $200 \times 10^{-9}$ g.dm$^{-3}$ $^{14}$C-DDT in 50% seawater. (TANK A)

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Sodium ions (Na$^+$) mEq.dm$^{-3}$</th>
<th>Potassium ions (K$^+$) mEq.dm$^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>$366 \pm 47$</td>
<td>230</td>
</tr>
<tr>
<td>8</td>
<td>$365 \pm 17$</td>
<td>220</td>
</tr>
<tr>
<td>15</td>
<td>$346 \pm 21$</td>
<td>225</td>
</tr>
<tr>
<td>20</td>
<td>$355 \pm 17$</td>
<td>235</td>
</tr>
<tr>
<td>28</td>
<td>$355 \pm 19$</td>
<td>290</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Chloride ions (Cl$^-$) mEq.dm$^{-3}$</th>
<th>Osmolality (mOsm.kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>$369 \pm 29$</td>
<td>258</td>
</tr>
<tr>
<td>8</td>
<td>$349 \pm 21$</td>
<td>223</td>
</tr>
<tr>
<td>15</td>
<td>$333 \pm 22$</td>
<td>240</td>
</tr>
<tr>
<td>20</td>
<td>$342 \pm 14$</td>
<td>258</td>
</tr>
<tr>
<td>28</td>
<td>$340 \pm 14$</td>
<td>268</td>
</tr>
</tbody>
</table>

Values shown for crab haemolymph are mean and standard deviation of five individuals.
### TABLE 8b - Ion levels of crabs exposed to ethanol only, in 50% seawater. (TANK B)

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Sodium ions (Na⁺) mEq.dm⁻³</th>
<th>Potassium ions (K⁺) mEq.dm⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>359 ± 31</td>
<td>215</td>
</tr>
<tr>
<td>8</td>
<td>354 ± 24</td>
<td>240</td>
</tr>
<tr>
<td>15</td>
<td>359 ± 22</td>
<td>225</td>
</tr>
<tr>
<td>20</td>
<td>368 ± 21</td>
<td>230</td>
</tr>
<tr>
<td>28</td>
<td>367 ± 30</td>
<td>240</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Chloride ions (Cl⁻) mEq.dm⁻³</th>
<th>Osmolality (mOsm.kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>366 ± 34</td>
<td>265</td>
</tr>
<tr>
<td>8</td>
<td>331 ± 28</td>
<td>268</td>
</tr>
<tr>
<td>15</td>
<td>328 ± 21</td>
<td>253</td>
</tr>
<tr>
<td>20</td>
<td>349 ± 24</td>
<td>258</td>
</tr>
<tr>
<td>28</td>
<td>348 ± 25</td>
<td>263</td>
</tr>
</tbody>
</table>

Values shown for crab haemolymph are mean and standard deviation of five individuals.
TABLE 8c - Ion levels of crabs in control 50% seawater. (TANK C)

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Sodium ions (Na⁺) mEq.dm⁻³</th>
<th>Potassium ions (K⁺) mEq.dm⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>374 ± 18</td>
<td>215</td>
</tr>
<tr>
<td>8</td>
<td>389 ± 14</td>
<td>235</td>
</tr>
<tr>
<td>15</td>
<td>367 ± 24</td>
<td>230</td>
</tr>
<tr>
<td>20</td>
<td>367 ± 15</td>
<td>225</td>
</tr>
<tr>
<td>28</td>
<td>373 ± 12</td>
<td>240</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Chloride ions (Cl⁻) mEq.dm⁻³</th>
<th>Osmolality (mOsm.kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>1</td>
<td>384 ± 20</td>
<td>268</td>
</tr>
<tr>
<td>8</td>
<td>367 ± 17</td>
<td>253</td>
</tr>
<tr>
<td>15</td>
<td>353 ± 26</td>
<td>258</td>
</tr>
<tr>
<td>20</td>
<td>349 ± 14</td>
<td>250</td>
</tr>
<tr>
<td>28</td>
<td>358 ± 8</td>
<td>258</td>
</tr>
</tbody>
</table>

Values shown for crab haemolymph are mean and standard deviation of five individuals.
Haemolymph samples were taken after 1, 8, 15, 20 and 28 day's exposure. Ion levels and osmolality are shown in Table 8 a,b,c.

ATPase activities

Although the standard deviations shown in Table 7 are high, the majority come within those of the normal variation between individuals (III.3.b). Throughout, the activities of enzymes when exposed to DDT are lower than in the respective controls. Although the activities of DDT-exposed ATPases decrease with time, suggesting an increased degree of inhibition with time, the activities of the controls also do similarly. The animals were not fed during the experiment, therefore, this general decrease in activity may be due to a deterioration of the tissues and enzymes concerned.

Ethanol appeared to affect the activity, whereas this had no effect during the in vitro experiment, in which the ethanol was at a lower concentration. The ATPase activity was both inhibited and stimulated under these conditions.

The concentration of $200 \times 10^{-9} \text{g.d.m}^{-3}$ DDT was selected, because at a similar concentration ($354.5 \times 10^{-9} \text{g.d.m}^{-3} \equiv 10^{-9} \text{M DDT}$) in Experiment 1, the ATPase activities were significantly affected. Mg$^{2+}$-ATPase activity had been inhibited 25%, and Na$^+$, K$^+$-ATPase activity was stimulated 217% from the controls.

Haemolymph ion levels

As in the previous experiments using lower concentrations of DDT, there is no significant change in ion levels or osmolality of the haemolymph, during exposure to about $200 \times 10^{-9} \text{g.d.m}^{-3}$ DDT. The most obvious difference between the ion levels
in this experiment when compared to Experiments 3 and 4
(II.2.c.iii,iv) is the reduction in variation between concentra-
tions shown by each individual at the different time intervals.
This can be attributed to the use of the circulating system, in
which the water is filtered and at a constant controlled
temperature. There is also a larger volume of water than in
the static system used in Experiments 3 and 4 (II.2.c.iii,iv).

This system, therefore, provides much more suitable con-
ditions for the animals themselves, and for monitoring any
haemolymph ion change as a result of stress.

(d) EXPERIMENT 4

The \textit{in vivo} effect of simultaneously applying
200 \times 10^{-9} \text{g.dm}^{-3} of \textsuperscript{14}C-DDT and gradually lowering
the salinity (from 50\% through 30\% to 15\%), on the
activity of ATPases in a gill homogenate, and ion
levels in the haemolymph of \textit{Carcinus maenas}

ATPases in a gill homogenate of \textit{Carcinus maenas} have been
shown to be inhibited at about 200 \times 10^{-9} \text{g.dm}^{-3} in 50\% seawater
after 8 days (Experiment 3). With this inhibition there is no
change in the haemolymph ion level, since the animals may be
able to withstand exposure to DDT at such a stress level of
50\% seawater. This dilution may frequently be encountered by
the crabs in their natural habitat.

The aim of this experiment was to add further stress by
diluting the seawater in stages, while continuously exposing
the crabs to DDT. Under these conditions the osmoregulatory
mechanism may be unable to cope with both burdens, and some
change in ion levels may become evident.

Ethanol was shown to have little effect on ion levels, and
ATPase activities in the in vitro study (III.3.a.) and only slight effects on the in vivo ATPase activities (III.3.c.). It was decided to omit the ethanol control tanks in this experiment, although still retaining the seawater control.

Crabs acclimated to 50% seawater were maintained in this for 8 days while being exposed to about 200 x 10^{-9} g.dm^{-3}^{14}\text{C-DDT}. At this point a gill ATPase enzyme assay was carried out. The water was then changed to 30% and after 10 days in this, changed to 15% for a further 7 days. After this an ATPase assay was carried out. During the experiment, haemolymph samples were taken for ion analysis.

The results of this are shown in Tables 9: a,b and in graph form in Figs. 3, 4, 5 and 6.

The activities of Mg^{2+}, Na^{+}, K^{+} and Total-ATPases after 8 days and 25 days are summarised in Table 10.
TABLE 9a - Ion levels of crabs exposed to $200 \times 10^{-9}$ g.dm$^{-3}$ $^{14}$C-DDT in decreasing salinities. (TANK A)

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Sodium ions (Na$^+$) mEq.dm$^{-3}$</th>
<th>Chloride ions (Cl$^-$) mEq.dm$^{-3}$</th>
<th>Potassium ions (K$^+$) mEq.dm$^{-3}$</th>
<th>Osmolality (mOsm.kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>50% seawater</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>$413 \pm 20$</td>
<td>235</td>
<td>$7.6 \pm 0.9$</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>$389 \pm 19$</td>
<td>230</td>
<td>$7.7 \pm 0.6$</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>$374 \pm 25$</td>
<td>230</td>
<td>$8.5 \pm 0.6$</td>
<td>5.5</td>
</tr>
<tr>
<td>30% seawater</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>$314 \pm 16$</td>
<td>140</td>
<td>$7.3 \pm 0.6$</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>$320 \pm 29$</td>
<td>145</td>
<td>$7.1 \pm 0.7$</td>
<td>3.5</td>
</tr>
<tr>
<td>15% seawater</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>$259 \pm 51$</td>
<td>65</td>
<td>$4.4 \pm 0.9$</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>$259 \pm 16$</td>
<td>75</td>
<td>$4.7 \pm 0.7$</td>
<td>2.0</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% seawater</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>$404 \pm 24$</td>
<td>291</td>
<td>$830 \pm 16$</td>
<td>508</td>
</tr>
<tr>
<td></td>
<td>$396 \pm 11$</td>
<td>289</td>
<td>$782 \pm 19$</td>
<td>515</td>
</tr>
<tr>
<td></td>
<td>$400 \pm 19$</td>
<td>288</td>
<td>$772 \pm 20$</td>
<td>522</td>
</tr>
<tr>
<td>30% seawater</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>$340 \pm 11$</td>
<td>253</td>
<td>$670 \pm 37$</td>
<td>322</td>
</tr>
<tr>
<td></td>
<td>$336 \pm 10$</td>
<td>249</td>
<td>$648 \pm 47$</td>
<td>323</td>
</tr>
<tr>
<td>15% seawater</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>$305 \pm 7$</td>
<td>115</td>
<td>$543 \pm 45$</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>$297 \pm 9$</td>
<td>110</td>
<td>$556 \pm 48$</td>
<td>169</td>
</tr>
</tbody>
</table>

Values shown for crab haemolymph are mean and standard deviation of five individuals.
<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Sodium ions ($\text{Na}^+$) mEq.dm$^{-3}$</th>
<th>Potassium ions ($\text{K}^+$) mEq.dm$^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>50% seawater</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>407 ± 14</td>
<td>255</td>
</tr>
<tr>
<td>4</td>
<td>395 ± 10</td>
<td>235</td>
</tr>
<tr>
<td>7</td>
<td>407 ± 8</td>
<td>250</td>
</tr>
<tr>
<td>30% seawater</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>330 ± 28</td>
<td>140</td>
</tr>
<tr>
<td>18</td>
<td>333 ± 21</td>
<td>150</td>
</tr>
<tr>
<td>15% seawater</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>286 ± 61</td>
<td>75</td>
</tr>
<tr>
<td>25</td>
<td>261 ± 85</td>
<td>70</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Chloride ions ($\text{Cl}^-$) mEq.dm$^{-3}$</th>
<th>Osmolality (mOsm.kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crabs</td>
<td>Seawater</td>
</tr>
<tr>
<td>50% seawater</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>407 ± 19</td>
<td>294</td>
</tr>
<tr>
<td>4</td>
<td>408 ± 19</td>
<td>281</td>
</tr>
<tr>
<td>7</td>
<td>409 ± 23</td>
<td>287</td>
</tr>
<tr>
<td>30% seawater</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>344 ± 14</td>
<td>250</td>
</tr>
<tr>
<td>18</td>
<td>340 ± 18</td>
<td>244</td>
</tr>
<tr>
<td>15% seawater</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>304 ± 12</td>
<td>105</td>
</tr>
<tr>
<td>25</td>
<td>305 ± 13</td>
<td>117</td>
</tr>
</tbody>
</table>

Values shown for crab haemolymph are mean and standard deviation of five individuals.
Fig. 3 - Sodium ion concentration in haemolymph of five individuals of Carcinus maenas in decreasing salinities.

TANK A

TANK B
Fig. 4 - Potassium ion concentration in haemolymph of five individuals of *Carinus maenas* in decreasing salinities.

**TANK A**

**TANK B**
Fig. 5 - Chloride ion concentration in haemolymph of five individuals of *Carinus maenas* in decreasing salinities.

**TANK A**

**TANK B**
Fig. 6 - Osmolality of haemolymph of five individuals of Carcinus maenas in decreasing salinities.

TANK A

- haemolymph
- seawater

TANK B
### Table 10
Summary of results of ATPase activities in gill homogenates with exposure to $200 \times 10^{-9}$ g.dm$^{-3}$ $^{14}$C-DDT, and relevant controls; after 8 days in 50% seawater and 25 days in 50%, 30% and 15% seawater

<table>
<thead>
<tr>
<th></th>
<th>Total-ATPase</th>
<th>$\text{Mg}^{2+}$-ATPase</th>
<th>$\text{Na}^+, \text{K}^+$-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 days SW (50%)</td>
<td>221.12 ± 49.8</td>
<td>108.50 ± 26.1</td>
<td>112.62 ± 26.4</td>
</tr>
<tr>
<td>8 days DDT SW (50%)</td>
<td>157.83 ± 35.6</td>
<td>100.57 ± 25.3</td>
<td>57.25 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>185.36 ± 23.9</td>
<td>124.37 ± 28.7</td>
<td>61.00 ± 14.2</td>
</tr>
<tr>
<td></td>
<td>(14.9% inhibition)</td>
<td>(19.1% inhibition)</td>
<td>(6.2% inhibition)</td>
</tr>
<tr>
<td>25 days DDT SW (15%)</td>
<td>291.93 ± 23.1</td>
<td>176.90 ± 5.4</td>
<td>115.03 ± 20.6</td>
</tr>
<tr>
<td></td>
<td>434.85 ± 33.3</td>
<td>231.34 ± 17.2</td>
<td>203.51 ± 27.4</td>
</tr>
<tr>
<td></td>
<td>(32.9% inhibition)</td>
<td>(23.5% inhibition)</td>
<td>(43.5% inhibition)</td>
</tr>
</tbody>
</table>

Figures show the mean and one standard deviation of 5 individuals under each condition.

The activities shown at the start are taken from Table 6.

**Haemolymph ion levels**

There is no evidence of osmoregulatory break-down shown by the levels of ions and osmolality of haemolymph.

The graphs (Figs. 3 to 6) need an explanation on the way they show the changes in seawater concentration. Between 7 and 16 days, and 18 and 23 days, the slopes suggest a gradual decrease in levels, although this is not so. These periods represented the time allowed for acclimation after changing the water, and before haemolymph sampling on days 16 and 23.

The most noticeable phenomenon as the concentration of seawater decreases, is the increase in variability between ion levels of individuals at a particular point in time. This is present in both treated and control animals, but more exaggerated in the latter situation in the case of osmolality.
As *Carcinus* experiences lower seawater concentrations, the ability to cope with this increased stress varies between individuals. However, the presence of DDT in the water seems to reduce this variation.

**ATPase activity**

After 8 days' exposure to $200 \times 10^{-9}\text{g.dm}^{-3}$ DDT, a slight inhibition of activity of Total-, Mg$^{2+}$- and Na$^+$, K$^+$-ATPases is observed (Table 10) when compared with controls. This is 14.9%, 19.1% and 6.2% respectively. After 25 days' exposure, this degree of inhibition has increased to 32.9%, 23.5% and 43.5% respectively. The activities themselves have also increased after 25 days. This is because the animals are in 15% seawater, resulting in an increase in the rate of active transport to maintain osmotic balance.

(e) **EXPERIMENT 5**

The *in vivo* effect of simultaneously applying $200 \times 10^{-9}\text{g.dm}^{-3}$ of $^{14}$C-DDT and lowering the seawater concentration at a greater rate (100% through 50% to 15%), on the activity of ATPases in a gill homogenate, and ion levels in the haemolymph of *Carcinus maenas*

This can be regarded as a slight modification of Experiment 4 (III.3.d). The aim is to add further stress to that with which the animals were able to cope in Experiment 4. The salinity was lowered in larger steps, from 100% seawater in which the crabs were not exposed to DDT, to 50%, in which they were divided into DDT-exposed and controls. Then finally to 15% seawater, in which the controls were divided into two groups - treated and untreated crabs. This plan is shown overleaf-
The final groups are, therefore, made up of:

A) animals exposed to DDT for 25 days, passing through 50% seawater (15 days) and 15% seawater (10 days).

B) animals exposed to DDT for 10 days in 15% seawater. Prior to this they were not exposed to DDT.

C) animals which have not been exposed to any DDT, but have been through 50% and 15% dilutions. These acted as controls throughout.

The presence of ethanol controls would have greatly complicated the experiment and would have led to difficulties in accommodating the animals. Therefore, these controls were omitted.

The seawater was kept at 100% concentration for 5 days, after which it was changed to 50% for 15 days, and 15% for a further 10 days. The DDT was added in ethanol (95%), being made up of $^{14}$C-DDT and unlabelled DDT, averaging $200 \times 10^{-9} \text{g.dm}^{-3}$.
throughout the experiment.

Haemolymph samples were taken throughout (Tables 11 to 14, and Figs. 7 to 10). ATPase activities in gill homogenates were assayed in 100% seawater, and after 1½ and 9 days in each of the lower salinities. These are summarised in Table 15.
**TABLE 11** - Sodium ion concentration in haemolymph of *Carcinus maenas*.

(mEq.dm$^{-3}$)

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>no DDT</th>
<th>DDT</th>
<th>no DDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% SW(485)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>504 ± 11*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>504 ± 7*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% SW(250)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>375 ± 36+</td>
<td>366 ± 24†</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>356 ± 41+</td>
<td>353 ± 16†</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>367 ± 33+</td>
<td>365 ± 15†</td>
<td></td>
</tr>
<tr>
<td>15% SW(80)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>210 ± 69+</td>
<td>224 ± 34+</td>
<td>187 ± 17†</td>
</tr>
<tr>
<td>30</td>
<td>193 ± 44+</td>
<td>222 ± 40†</td>
<td></td>
</tr>
</tbody>
</table>

* mean and standard deviation of five individuals.
† mean and standard deviation of ten individuals.
* mean and standard deviation of fifteen individuals.
TABLE 12 - Potassium ion concentration in haemolymph of Carcinus maenas.
(mEq dm$^{-3}$)

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>100% SW (10.0)</th>
<th>50% SW (5.5)</th>
<th>15% SW (2.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no DDT</td>
<td>DDT</td>
<td>no DDT</td>
</tr>
<tr>
<td></td>
<td>mean ± standard deviation of five individuals.</td>
<td>mean ± standard deviation of ten individuals.</td>
<td>mean ± standard deviation of fifteen individuals.</td>
</tr>
<tr>
<td>1</td>
<td>9.8 ± 0.8*</td>
<td>7.9 ± 0.6+</td>
<td>4.1 ± 0.7+</td>
</tr>
<tr>
<td>5</td>
<td>10.3 ± 0.8*</td>
<td>8.6 ± 0.9+</td>
<td>4.9 ± 0.4+</td>
</tr>
<tr>
<td>9</td>
<td>8.6 ± 0.9+</td>
<td>8.8 ± 0.5+</td>
<td>5.3 ± 0.6+</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>9.3 ± 0.9†</td>
<td>3.6 ± 0.4†</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>8.6 ± 0.8†</td>
<td>9.3 ± 0.9†</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>8.8 ± 0.8†</td>
<td>9.3 ± 0.9†</td>
<td></td>
</tr>
</tbody>
</table>

*mean and standard deviation of fifteen individuals.
†mean and standard deviation of ten individuals.
<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>Chloride ion concentration in haemolymph of <em>Carcinus maenas</em> (mEq. dm$^{-3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>no DDT</strong></td>
</tr>
<tr>
<td>100% SW(538)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>491 ± 15*</td>
</tr>
<tr>
<td>5</td>
<td>509 ± 14*</td>
</tr>
<tr>
<td>50% SW(259)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>342 ± 37†</td>
</tr>
<tr>
<td>12</td>
<td>334 ± 52†</td>
</tr>
<tr>
<td>20</td>
<td>333 ± 28†</td>
</tr>
<tr>
<td>15% SW(73)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>171 ± 63†</td>
</tr>
<tr>
<td>30</td>
<td>156 ± 48†</td>
</tr>
<tr>
<td></td>
<td><strong>DDT</strong></td>
</tr>
<tr>
<td>100% SW(538)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>332 ± 25†</td>
</tr>
<tr>
<td>5</td>
<td>320 ± 19†</td>
</tr>
<tr>
<td>50% SW(259)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>324 ± 16†</td>
</tr>
<tr>
<td>12</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
</tr>
<tr>
<td>15% SW(73)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>143 ± 15†</td>
</tr>
<tr>
<td>30</td>
<td>173 ± 42†</td>
</tr>
</tbody>
</table>

* mean and standard deviation of five individuals.
† mean and standard deviation of ten individuals.
+ mean and standard deviation of fifteen individuals.
TABLE 14 - Osmolality of haemolymph of Carcinus maenas.

(mOsm.kg$^{-1}$)

<table>
<thead>
<tr>
<th>No. of days' exposure</th>
<th>100% SW (1011)</th>
<th>50% SW (509)</th>
<th>15% SW (161)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no DDT</td>
<td>DDT</td>
<td>no DDT</td>
</tr>
<tr>
<td>1</td>
<td>1033 ± 14*</td>
<td>753 ± 50+</td>
<td>467 ± 100+</td>
</tr>
<tr>
<td>5</td>
<td>1023 ± 6*</td>
<td>725 ± 65+</td>
<td>414 ± 91+</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>713 ± 52+</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>483 ± 37+</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td>451 ± 37+</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td>432 ± 23+</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* mean and standard deviation of fifteen individuals.
† mean and standard deviation of ten individuals.
Fig. 7 - Sodium ion concentration in haemolymph of five individuals of Carinus maenas in decreasing salinities.

TANK A
100% 50% 15%
no DDT DDT DDT

TANK B
100% 50% 15%
no DDT no DDT DDT

TANK C
100% 50% 15%
no DDT no DDT DDT
Fig. 9 - Chloride ion concentration in haemolymph of five individuals of *Carcinus maenas* in decreasing salinities.

**TANK A**
- 100% no DDT
- 50% DDT
- 15% DDT

**TANK B**
- 100% no DDT
- 50% no DDT
- 15% DDT

**TANK C**
- 100% no DDT
- 50% no DDT
- 15% no DDT

---

mEq L⁻¹
days
Fig. 10 - Osmolality of haemolymph of five individuals of Carcinus maenas in decreasing salinities.

TANK A

100% no DDT

50% DDT

15% DDT

--- haemolymph
--- seawater

TANK B

100% no DDT

50% no DDT

15% no DDT

--- haemolymph
--- seawater

TANK C

100% no DDT

50% no DDT

15% no DDT

--- haemolymph
--- seawater
<table>
<thead>
<tr>
<th></th>
<th>Total-ATPase</th>
<th>Mg(^{2+})-ATPase</th>
<th>Na(^+),K(^+)-ATPase</th>
<th>Total-ATPase</th>
<th>Mg(^{2+})-ATPase</th>
<th>Na(^+),K(^+)-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% SW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>119.86 ± 39.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>72.31 ± 40.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48.44 ± 18.5</td>
</tr>
<tr>
<td>50% SW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1(^\frac{1}{2}) days</td>
<td>151.46 ± 34.7</td>
<td>105.90 ± 26.6</td>
<td>45.55 ± 14.7</td>
<td>149.50 ± 20.4</td>
<td>114.28 ± 14.2</td>
<td>35.62 ± 20.0</td>
</tr>
<tr>
<td>9 days</td>
<td>153.95 ± 23.9</td>
<td>100.43 ± 8.2</td>
<td>53.63 ± 22.1</td>
<td>211.39 ± 21.5</td>
<td>117.04 ± 9.0</td>
<td>97.15 ± 25.9</td>
</tr>
<tr>
<td>15% SW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1(^\frac{1}{2}) days</td>
<td>203.38 ± 8.5</td>
<td>103.73 ± 21.3</td>
<td>100.06 ± 17.5</td>
<td>179.00 ± 31.8</td>
<td>106.53 ± 17.9</td>
<td>72.47 ± 23.4</td>
</tr>
<tr>
<td>9 days</td>
<td>233.46 ± 15.4</td>
<td>148.29 ± 14.8</td>
<td>85.30 ± 12.3</td>
<td>231.66 ± 10.1</td>
<td>120.73 ± 14.5</td>
<td>110.93 ± 22.1</td>
</tr>
</tbody>
</table>
All crabs survived until the end, except for the five from which haemolymph samples were taken in the Group C controls. These died between days 24 and 30.

**Haemolymph ion levels**

Examination of the graphs of Cl$^-$ ion concentrations (Fig. 9: a, b, c) suggests there may have been some disruption of the osmoregulatory mechanism in *Carcinus* with respect to chloride ions. Although there is some variation in levels between individuals, it is apparent that the levels of Cl$^-$ ions in some individuals are very close to those of the seawater, having decreased under the influence of DDT. This is more noticeable in Fig. 9a, showing animals exposed to DDT for 25 days.

However, this is only a tentative suggestion, as no evidence for osmoregulatory disruption can be seen from the other graphs.

The change of water concentration from 100% to 50%, and especially from 50% to 15%, together with the addition of DDT, acted as a "shock" mechanism to the crab's osmoregulatory system. However, the ion levels were generally unaffected in the lower seawater concentrations, whether DDT was present from day 5 or from day 20.

In 100% seawater, *Carcinus* needs to osmoregulate to only a very small extent. In this situation the ion levels of all five individuals in each group are very close to each other. When the necessity to osmoregulate increases, the variation in ability of individuals to osmoregulate becomes more evident. This is shown by the wider spacing of the lines on the graphs.

This phenomenon was also seen in Experiment 4, but in this
case, the presence of DDT appeared to reduce this variability with respect to Na\(^+\) ion concentration and osmolality. K\(^+\) and Cl\(^-\) ion concentrations showed little difference. The results of Experiment 5 show the reverse, although this is slightly difficult to compare in Group C because of the premature deaths. Taking Figs. 7, 9 and 10, for example, ion levels in the total absence of DDT are very close (Figs. 7c, 9c, 10c). The addition of DDT on day 20 caused a slight variation between individuals after this date (Figs. 7b, 9b, 10b). The addition of DDT on day 5 caused variations to be more evident immediately afterwards (Figs. 7a, 9a, 10a).

This suggests that DDT is affecting the ability of *Carcinus* to osmoregulate in low salinities. The results of Experiment 4 contradict this, however.

**ATPase activity**

There appears to be no consistant relationship between activities of Total-, Mg\(^{2+}\)- and Na\(^+\),K\(^+\)-ATPases in the presence or absence of DDT, unlike previous readings. The relative values of Mg\(^{2+}\)-ATPase and Na\(^+\),K\(^+\)-ATPase are consistent with previous activities, in that the latter is always smaller than the former.

It is not possible to detect any inhibition which may have increased with duration of DDT exposure, as the enzyme activities changed with seawater concentration. ATPase activities increased as seawater concentrations decreased.
It has now been demonstrated that DDT affects the activity of ATPases, both *in vivo* and *in vitro*. The fact that this effect does not manifest itself in the ion levels of the haemolymph, must mean that some other physiological mechanism is being affected. For this reason, it was decided to investigate ammonia excretion from the haemolymph, as ATPases may be involved in this process.
BIBLIOGRAPHY


Amino acids in the internal body fluids play an important role in establishing equilibrium between the internal and external fluids. During initial acclimation to a new salinity, changes in the size of the free amino acid pool occur by oxidative catabolism. Ammonia is produced as a result of this, which leads to an increase in haemolymph pH with dilution of the medium (Weiland & Mangum, 1975). Gerard & Gilles (1972) found that in Callinectes sapidus, the concentration of amino acids in muscle decreases to 32% of the initial value when crabs are moved from 32-34% to 16 - 17%. At the same time blood ammonia rises by about 0.1mM.dm$^{-3}$. 

Ammonia is the main nitrogenous waste product, being produced mainly from amino acid breakdown. Loss is mainly through the gills, but it is unlikely that this is the main site of deamination in the body. Ammonia diffuses from the haemolymph into the gill cells, but diffusion into the water does not occur, except at very low salinities. This takes place by active transport. Mangum (1976) discusses in great detail the relationship between osmotic and excretory responses. She suggests that NH$_3$ is eliminated by a process of exchanging Na$^+$ ions with NH$_4^+$ ions at the gill membrane surface, catalysed by Na$^+$.K$^+$-ATPase. Osmotic balance is normally maintained by an exchange between Na$^+$ and K$^+$ ions. 

When a crab moves from high to low salinity, it is moving from a medium which is alkaline to the body fluids and would therefore block NH$_3$ output by diffusion, to an acid medium
in which NH$_3$ diffusion would occur. In low salinities, therefore, there must be some passive loss of ammonia. Mangum believes this is not very great, though, because in low salinity, ammonia output can be greatly reduced by addition of ouabain, which specifically inhibits Na$^+$.K$^+$-ATPase.

Towle & Taylor (1976) and Towle et al (1976) studied the activity of Na$^+$.K$^+$-ATPase in the microsomal fraction of the gill of blue crabs. They found that there was very little difference in enzyme activity when NH$_4^+$ was substituted for K$^+$ in the incubation medium. The concentration of Na$^+$ remained the same. Work described earlier in this thesis (III.2.c.i) also supports this. The data of Towle et al (1976) are shown (Fig. 1), taken from Mangum (1976).

**Fig. 1** ATPase activities in gills of *Callinectes sapidus* acclimated to 5% salinity (Towle et al, 1976)
When the level of NaCl in mammalian blood is changed, the concentration of H\(^+\) changes in the opposite direction. However, there is lack of evidence for this happening in Crustacea. The work of Towle et al (1976) shown in Fig. 1 demonstrates that ATPase activity is greatly reduced when H\(^+\) is present with Na\(^+\), but K\(^+\) or NH\(_4^+\) are absent. There is also no evidence of an enzyme which is capable of carrying out a Na\(^+\)-H\(^+\) exchange.

In low salinities, *Carcinus maenas* and the blue crab, *Callinectes sapidus* (Towle et al, 1976) maintain their haemolymph ion and osmolality levels higher than the surrounding water. This results from actively transporting Na\(^+\) ions from the water into the haemolymph. In low salinities, the rate of NH\(_4^+\) loss from the haemolymph increases. Therefore it is feasible that an NH\(_4^+\)-Na\(^+\) exchange could exist, which may be catalysed by Na\(^+\),K\(^+\)-ATPase.

Since p,p'-DDT inhibits Na\(^+\),K\(^+\)-ATPase in some instances and stimulates activity in others (III.3.a), changes in the levels of ammonia in the haemolymph, or the rate of loss of ammonia from the haemolymph might occur as a result.

(2) ESTIMATION OF AMMONIA

The conventional method for determination of ammonia in solution is using Nessler's reagent, giving an orange-red colour which can be measured colorimetrically. However, it was not possible to use this reagent with *Carcinus* haemolymph as a cloudy suspension formed. Estimation of ammonia in this case was carried out using the microdiffusion method of Conway & Byrne (1933) and Conway (1962).
a) PRINCIPLE OF ANALYSIS

The apparatus is known as a "Conway unit", consisting of a small glass dish with thick walls, within which is a smaller chamber formed by a circular wall of glass arising from the floor (Fig. 2). During determination the dish is covered by a square glass lid having one roughened surface. A small amount of vaseline was smeared on the lid to create air-tight conditions within.

Hydrochloric acid is introduced to the inner chamber, and then the haemolymph sample into the outer chamber. The addition of alkali to the sample, causes the liberation of ammonia, which is then absorbed by the acid. The air-tight seal with the lid minimises any loss of ammonia from the apparatus, and inclusion of any ammonia that may be in the surrounding atmosphere. After a suitable time for absorption, the acid is titrated against barium hydroxide. This determines the amount of acid remaining in the inner chamber, from which can be estimated the quantity of ammonia present.

b) REAGENTS

N/1000 hydrochloric acid and indicator

5cm³ Tashiro's reagent (200cm³ of 0.1% alcoholic solution of methyl red + 50cm³ 0.1% alcoholic solution of methylene blue) were run into a volumetric flask (500cm³) and absolute alcohol (100cm³) added. Distilled water was run in to about ¾ the volume of the flask, and the indicator brought to the neutral point by adding a little dilute alkali until the red colour just disappeared. N/10 hydrochloric acid (5cm³) was added and the solution made up to 500cm³. This gave an N/1000 acid solution.
Fig. 2  Standard Conway Unit

Top View

Vertical Section
Saturated potassium carbonate

After dissolving potassium carbonate (ca. 110g) in water (100cm$^3$), the solution was boiled for 10 minutes, at first vigorously and then gently. This is necessary to free it of any ammonia present.

Barium hydroxide

This was prepared from stock barium hydroxide solution (N/10). The Conway method suggests using N/10 barium hydroxide (21.4cm$^3$) diluted to 1000cm$^3$. However, the use of a more dilute alkali solution proved to give more accurate readings. A solution of barium hydroxide (10.7cm$^3$) made up to 1000cm$^3$ was used.

c) METHOD

Hydrochloric acid (1cm$^3$) was placed in the central area of the dish. Haemolymph (0.2cm$^3$), taken using a hypodermic syringe, was introduced into the outer chamber. The vaselined lid was placed on top and then potassium carbonate (1cm$^3$) pipetted into the outer chamber, as the lid was pushed slightly to one side. The dish was gently rotated to mix the two outer liquids, but not so violently that there was mixing between the inner and outer chambers.

Release of ammonia was facilitated by standing at 38$^\circ$C, and the dishes were left for 2 hours. The contents of the central chamber were then titrated against the barium hydroxide solution until the pink colour just disappeared.

It was essential that the dishes were thoroughly cleaned after use - with hot soapy water followed by a rinse with clean water and then distilled water. When not in use the dishes were kept in a dilute acid solution. It was also important that the determinations were carried out in an ammonia-free
3. EXPERIMENTATION

a) EXPERIMENT 1

The effect of $200 \times 10^{-9}$ g dm$^{-3}$ $^{14}$C-DDT on the level of ammonia in the haemolymph of *Carcinus maenas*.

Crabs were kept in seawater (100%) at $10^\circ-11^\circ$C in the circulating system. DDT was introduced to give an average concentration of $200 \times 10^{-9}$ g dm$^{-3}$ throughout the experiment in Tank A. An ethanol control (Tank B) and seawater control (Tank C) were also set up.

Haemolymph samples (0.5 cm$^3$) were taken over a period of 49 days, and the ammonia content determined (IV.2). As the ammonia level in the body fluid varies in relation to food intake, the animals were fed on one day, the water changed the following day, and haemolymph samples taken on the third day. This routine was followed prior to each sampling.

For each set of samples, a 'blank' determination was also carried out, in which haemolymph was replaced by an equal volume of distilled water.

Ammonia levels in the haemolymph are shown in Table 1. The method by which the ammonia content can be determined from the volume of barium hydroxide used in titration is shown.
### TABLE 1 - Ammonia levels in haemolymph of *Carcinus maenas* exposed to \(^{14}\text{C}-\text{DDT}\), expressed as \(10^{-6}\) g NH\(_3\) cm\(^{-3}\) and mg N 100 cm\(^{-3}\).

#### TANK A - DDT exposed

<table>
<thead>
<tr>
<th>DAY</th>
<th>(x10^{-6}) g NH(_3) cm(^{-3})</th>
<th>mg N 100 cm(^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.0 ± 1.9</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>15.5 ± 2.2</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>14</td>
<td>14.2 ± 2.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>22</td>
<td>15.5 ± 1.7</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>36</td>
<td>18.2 ± 2.8</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>43</td>
<td>12.2 ± 0.9</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>49</td>
<td>11.6 ± 1.9</td>
<td>1.0 ± 0.2</td>
</tr>
</tbody>
</table>

#### TANK B - Ethanol control

<table>
<thead>
<tr>
<th>DAY</th>
<th>(x10^{-6}) g NH(_3) cm(^{-3})</th>
<th>mg N 100 cm(^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17.5 ± 1.9</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>14.8 ± 1.6</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>14</td>
<td>14.4 ± 1.1</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>22</td>
<td>14.8 ± 3.3</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>36</td>
<td>15.8 ± 4.2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>43</td>
<td>18.1 ± 2.0</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>49</td>
<td>14.2 ± 2.2</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

#### TANK C - Seawater control

<table>
<thead>
<tr>
<th>DAY</th>
<th>(x10^{-6}) g NH(_3) cm(^{-3})</th>
<th>mg N 100 cm(^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.8 ± 1.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>18.0 ± 2.5</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>14</td>
<td>15.8 ± 2.0</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>22</td>
<td>19.6 ± 1.8</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>36</td>
<td>13.4 ± 1.9</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>43</td>
<td>16.3 ± 1.8</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>49</td>
<td>15.0 ± 2.6</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>
Calculation to find the amount of ammonia present in haemolymph samples

Equations:

\[ \text{Ba(OH)}_2 + 2\text{HCl} \rightarrow \text{BaCl}_2 + 2\text{H}_2\text{O} \]  

(1)

\[ \text{HCl} + \text{NH}_3 \rightarrow \text{NH}_4\text{Cl} \]  

(2)

As the hydrochloric acid solution is made up, 5cm\(^3\) HCl are present in 500cm\(^3\) solution.

\[ \therefore \text{In 1cm}^3\text{ solution (in central chamber) are 0.01cm}^3\text{HCl} \]

The standard hydrochloric acid (0.1N) consists of:-

\[ 3.65\text{g HCl in 1000cm}^3 \]

\[ \therefore \text{In 5cm}^3\text{ HCl are } 3.65 \times 5 \text{ g. HCl} \]

\[ = \frac{18.25 \times 10^{-3} \text{ g. HCl}}{1000} \]

This is made up to 500cm\(^3\).

\[ \therefore \text{In 500cm}^3\text{ solution are } 18.25 \times 10^{-3} \text{ g. HCl} \]

\[ \therefore \text{In 1cm}^3\text{ solution (or 0.01 cm}^3\text{ HCl) are} \]

\[ \frac{18.25 \times 10^{-3} \text{ g. HCl}}{500} = 36.5 \times 10^{-6} \text{ g. HCl} \]

Taking the blank reading as 0.85cm\(^3\) Ba(OH)\(_2\), when no ammonia is present,

\[ 36.5 \times 10^{-6} \text{g. HCl} \equiv 0.85\text{cm}^3 \text{ Ba(OH)}_2 \]

\[ 36.5\text{g. HCl} \equiv 17\text{g. NH}_3 \text{ (from Equation 2)} \]

\[ \therefore 36.5 \times 10^{-6} \text{g. HCl} \equiv 17 \times 10^{-6} \text{g. NH}_3 \]

Assuming that the reaction went to completion, and no Ba(OH)\(_2\) was used in the titration (i.e. 36.5 \times 10^{-6} \text{g. HCl in the 1cm}^3\text{ of solution was completely neutralised by the } 17 \times 10^{-6} \text{g. NH}_3), then
0.00\text{cm}^3 \text{Ba(OH)}_2 \equiv 17 \times 10^{-6} \text{g. NH}_3

Also, it is known that

0.85\text{cm}^3 \text{Ba(OH)}_2 \equiv 0.00\text{g. NH}_3

A calibration graph can be drawn from these figures, as there is a linear relationship between \text{Ba(OH)}_2 used and ammonia content.

From this graph the amount of ammonia present per unit volume of haemolymph can be determined.

It is apparent that no significant change has occurred in the level of ammonia in the haemolymph of crabs exposed to DDT when compared with the controls.

The paper by Mangum (1976) was acquired after this experiment had commenced. Consequently it was realised that more meaningful results would have been obtained if the work had been carried out at a lower salinity, or if the experiment had involved a salinity change. In this situation, the haemolymph ammonia level would have risen and would then have been eliminated via the gills by active transport involving Na^+\text{-},K^+\text{-ATPase.}

Ammonia elimination is still occurring in 100\% seawater, being a product of other processes besides amino acid breakdown. More than 80\% of the total nitrogen excreted by \textit{Carcinus maenas} is in the form of ammonia, and this is lost at a rate of 1.1 mg.N (25g. crab)^{-1} day ^{-1} under normal seawater conditions (Needham, 1955). At lower salinities, however, the rate of loss would probably have been greater and more easily detected.
The ammonia values have been included in the two forms (Table 1), in order that comparisons can be made with the results of other workers. It can be seen that comparable results have been obtained with those of Binns (1969) and Delaunay (1931).

Haemolymph ammonia levels of *Carcinus maenas* obtained by Delaunay and Binns

<table>
<thead>
<tr>
<th></th>
<th>Conc. as mg. NH$_3$ (Mean ± S.E.)</th>
<th>Conc. as mg. N%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delaunay (1931)</td>
<td>-</td>
<td>2.10</td>
</tr>
<tr>
<td>Binns (1969)</td>
<td>1.61 ± 0.29</td>
<td>1.32</td>
</tr>
</tbody>
</table>
b) EXPERIMENT 2

The effect of $200 \times 10^{-9}$ g. dm$^{-3}$ $^{14}$C-DDT on the rate of loss of ammonia from the haemolymph of *Carcinus maenas* after injection of ammonium chloride solution.

Injection of an ammonium solution into the haemolymph of *Carcinus maenas* would produce the same effect as moving the animal to a lower salinity, with respect to haemolymph ammonia levels. Higher levels than normally found in the body can be introduced this way, and the rate of loss from the haemolymph at such levels will be much easier to monitor.

Various strength solutions of $\text{NH}_4\text{Cl}$ were made up - 8.8%, 3.0%, 0.88% and 0.18%. Assuming that the haemolymph content of a crab is about $4\text{cm}^3$, injection of the solution ($0.2\text{cm}^3$) would result in concentrations 85, 29, 8.5 and 1.7 times greater respectively, than the normal values shown in Table 1.

**Preliminary Study**

This was carried out on a single animal to determine the approximate rate of loss of ammonia from the haemolymph, and whether the animal could survive the injection of this quantity of ammonium chloride.

As sampling was to be more frequent than in previous experiments, $0.2\text{cm}^3$ rather than $0.5\text{cm}^3$ haemolymph samples were taken.

The most concentrated solution (8.8% $\text{NH}_4\text{Cl}$) was used. An aliquot ($0.2\text{cm}^3$) was injected using a fine 25G hypodermic needle, through the basal arthrodial membrane of a walking leg. The animal was left out of water for 10 minutes to allow for faster healing of the wound, and thus minimise the immediate loss of solution from the body. It was then placed
into a tank of seawater at 10°C for 10 minutes before the first sample was taken. This period of time would probably allow most of the ammonia to pass around the body. Samples were also taken after 40, 80, 180 and 240 minutes. The rate of ammonia loss is shown in Fig. 3.

Almost immediately after injection of the 8.8% NH₄Cl solution, severe paralysis was noticed in the crab. This diminished as the ammonia level dropped in the haemolymph.

**Experiment**

Crabs were kept in the circulating seawater (100%) system. Those in Tank A were exposed to approximately 200×10⁻⁹ g.dm⁻³¹⁴C-DDT for 10 days before injection of ammonium chloride (0.2cm³) solution. Ethanol controls (Tank B) and seawater controls (Tank C) were also maintained for 10 days prior to injection. The former controls were not used after the first experiment.

Three individuals were employed in each situation, a haemolymph sample (0.2cm³) being taken at various intervals after injection. The animals were left out of water for 10 minutes and then in water for a further 10 minutes before the first sample was taken.

The mean values and ranges of ammonia levels for three individuals are shown in Tables 2 and 3, and Figs. 4 and 5.
To show the rate of loss of NH$_3$ from haemolymph of *Carcinus maenas* after injection of NH$_4$Cl solution.
TABLE 2 — Ammonia levels in haemolymph of *Carcinus maenas* after exposure to 14C-DDT (and two controls), and injection of 8.8% ammonium chloride solution.

<table>
<thead>
<tr>
<th>Time from injection (mins)</th>
<th>Ammonia levels (x10^-6 g NH_3 cm^-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DDT - exposed</strong></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>67.7 (60.0 - 73.0)</td>
</tr>
<tr>
<td>40</td>
<td>39.5 (36.0 - 43.0)</td>
</tr>
<tr>
<td>135</td>
<td>13.5 (11.0 - 16.0)</td>
</tr>
<tr>
<td>165</td>
<td>12.0 (8.0 - 15.5)</td>
</tr>
<tr>
<td><strong>Ethanol control</strong></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>52.8 (51.0 - 55.0)</td>
</tr>
<tr>
<td>40</td>
<td>10.7 (9.5 - 12.0)</td>
</tr>
<tr>
<td>135</td>
<td>10.5 (9.0 - 11.5)</td>
</tr>
<tr>
<td>165</td>
<td>8.0 (5.5 - 9.5)</td>
</tr>
<tr>
<td><strong>Seawater control</strong></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>33.3 (31.5 - 35.0)</td>
</tr>
<tr>
<td>40</td>
<td>15.2 (10.5 - 21.0)</td>
</tr>
<tr>
<td>135</td>
<td>11.2 (7.5 - 15.0)</td>
</tr>
<tr>
<td>165</td>
<td>11.5 (9.5 - 13.5)</td>
</tr>
</tbody>
</table>
### TABLE 3 - Ammonia levels \((\times 10^{-6} \text{ g NH}_3 \text{ cm}^{-3})\) in haemolymph of Carcinus maenas after exposure to \(^{14}\text{C-}\text{DDT}\) (and one control), and injection of various concentrations of ammonium chloride solution.

<table>
<thead>
<tr>
<th>Time from injection (mins.)</th>
<th>(8.8% \text{ NH}_4\text{Cl}) (55.9x10^{-4} \text{g NH}_3 injected)</th>
<th>(3.0% \text{ NH}_4\text{Cl}) (19.1x10^{-4} \text{g NH}_3 injected)</th>
<th>(0.88% \text{ NH}_4\text{Cl}) (5.6x10^{-5} \text{g NH}_3 injected)</th>
<th>(0.18% \text{ NH}_4\text{Cl}) (1.1x10^{-5} \text{g NH}_3 injected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDT-exposed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>46.2 (32.5-63.5)</td>
<td>58.0 (57.0-59.0)</td>
<td>34.2 (24.5-46.0)</td>
<td>38.3 (33.0-44.0)</td>
</tr>
<tr>
<td>40</td>
<td>14.7 (11.5-18.5)</td>
<td>17.3 (15.0-19.5)</td>
<td>31.0 (26.5-39.0)</td>
<td>25.7 (22.5-32.0)</td>
</tr>
<tr>
<td>80</td>
<td>11.0 (9.5-13.0)</td>
<td>20.3 (13.0-31.5)</td>
<td>29.8 (25.5-32.0)</td>
<td>22.2 (19.5-23.5)</td>
</tr>
<tr>
<td>140</td>
<td>10.5 (9.5-11.5)</td>
<td>24.3 (19.5-33.0)</td>
<td>32.2 (27.5-35.0)</td>
<td>26.3 (20.5-33.0)</td>
</tr>
<tr>
<td>Seawater-control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>58.8 (55.5-65.5)</td>
<td>57.3 (46.0-69.0)</td>
<td>31.8 (25.5-37.0)</td>
<td>19.5 (12.5-29.0)</td>
</tr>
<tr>
<td>40</td>
<td>15.0 (7.5-22.5)</td>
<td>45.5 (40.5-48.0)</td>
<td>33.5 (25.5-39.0)</td>
<td>25.0 (19.5-30.0)</td>
</tr>
<tr>
<td>80</td>
<td>4.0 (3.0-4.5)</td>
<td>39.5 (27.5-53.5)</td>
<td>33.2 (28.5-36.0)</td>
<td>25.7 (20.5-31.0)</td>
</tr>
<tr>
<td>140</td>
<td>5.8 (3.0-9.5)</td>
<td>41.5 (28.5-49.0)</td>
<td>37.7 (35.0-41.0)</td>
<td>25.3 (18.5-34.0)</td>
</tr>
</tbody>
</table>
Fig. 4 - Ammonia levels in haemolymph of Carcinus maenas after injection of 8.8% ammonium chloride solution.

- DDT-treated
- Ethanol control
- Seawater control

559 x 10^{-4} \text{g NH}_3 \text{ injected at zero time}

\( \mu \text{g NH}_3 \text{ cm}^{-3} \)

Time after injection (mins)
Fig. 5 - Ammonia levels in haemolymph of *Carcinus maenas* after injection of ammonium chloride solution.

a) **0.18% NH₄Cl solution**

![Graph showing ammonia levels in haemolymph after injection of 0.18% NH₄Cl solution.](image)

- Dashed line: DDT-treated
- Solid line: Seawater Control

b) **0.88% NH₄Cl solution**

![Graph showing ammonia levels in haemolymph after injection of 0.88% NH₄Cl solution.](image)
Fig. 5 (Cont.)

(c) 30% NH₄Cl Solution

194 x 10⁻⁴ g NH₃ injected at zero time

μg NH₃ cm⁻³

0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150

time after injection (mins.)

(d) 8.8% NH₄Cl solution

- DDT-treated
- Seawater Control

μg NH₃ cm⁻³

0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150

time after injection (mins.)
The significant differences in ammonia levels between DDT-treated individuals and the controls when an 8.8% solution was injected (Fig. 4), led to a repeat of the experiment at this concentration. However the second set of results, and those when lower concentration solutions were used, failed to show a similar pattern (Fig. 5). There was either very little difference between experimental and control individuals, or a reverse situation from the initial results, was found.

c) EXPERIMENT 3

The effect of 200x10^{-9} g.dm^{-3} DDT on the rate of loss of ammonia from the haemolymph of Carcinus maenas after feeding.

Ammonia levels are elevated in the haemolymph of Carcinus maenas as a result of digestion of food. This was chosen as another means to monitor the rate of ammonia loss from the haemolymph under experimental and control situations.

Crabs were exposed to approximately 200x10^{-9} g.dm^{-3} DDT for 10 days, and the relevant control animals maintained for 10 days prior to feeding. A large quantity of mussels (without valves) were fed to the crabs in both tanks. After one hour, the water was changed and the first haemolymph sample (0.2cm^{3}) taken. Three individuals in each tank were used.

There was no difference in the rate of loss of ammonia between the crabs in experimental and control conditions when measured over 11 hours (Fig. 6). The initial ammonia concentration was not sufficiently high to detect the ammonia loss over any length of time. Also, it was difficult to be certain that all the crabs ate similar quantities of food. This variability can be seen in the
Fig. 6 - Ammonia levels of haemolymph of *Carcinus maenas* after being fed mussels.
large ranges of values after 1 hour.

d) EXPERIMENT 4

The effect of $200 \times 10^{-9}$ g.dm$^{-3}$ DDT on the levels of ammonia, and sodium and potassium ions in the haemolymph of *Carcinus maenas* under reduced salinity.

On transferring from a high to a low salinity, the ammonia level in the haemolymph of Crustacea is increased as a result of deamination of the amino acid pool - mainly from muscle tissue. By transferring *Carcinus maenas* from 100% to 5% seawater, it was planned to monitor ammonia levels of the haemolymph to determine whether exposure to DDT had any effect on the rate or time of ammonia increase. From this it might be concluded whether DDT affected the animal's ability to cope under reduced salinity.

Under these conditions, haemolymph samples were taken for Na$^+$ and K$^+$ ion analysis. Previous experiments had been carried out (Chapter II) monitoring ion levels over a period of several days, but this present experiment gave an opportunity to monitor any changes occurring over the first few hours after the seawater had been changed.

Haemolymph samples ($0.2 \text{cm}^3$ for ammonia estimation and $0.3 \text{cm}^3$ for ion analysis) were taken from three individuals one hour after the seawater was diluted and then over the following 11 hours. Samples for Na$^+$ and K$^+$ analysis were diluted four times with distilled water before estimation by flame photometer.

The whole experiment was repeated under identical conditions. Ammonia levels are shown in Fig.7, and sodium and potassium ion concentrations in Tables 4 and 5.
Fig. 7a - Ammonia levels in haemolymph of Carcinus maenas after transferring from 100% to 5% seawater.
Ammonia levels in haemolymph of *Carcinus maenas* after transferring from 100% to 5% seawater. (Repeat experiment)
TABLE 4 - Sodium ion concentrations in haemolymph of *Carcinus maenas* after transferring from 100% to 5% seawater, in duplicate experiments.

<table>
<thead>
<tr>
<th>Time after change (hours)</th>
<th>Mean (Range)</th>
<th>t-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DDT-treated</td>
<td>Control</td>
</tr>
<tr>
<td>Sodium ions (mEq.dm(^{-3}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>442 (421-468)</td>
<td>428 (422-433)</td>
</tr>
<tr>
<td>2</td>
<td>407 (393-417)</td>
<td>404 (402-405)</td>
</tr>
<tr>
<td>3</td>
<td>372 (331-406)</td>
<td>404 (398-409)</td>
</tr>
<tr>
<td>5</td>
<td>328 (301-365)</td>
<td>375 (372-377)</td>
</tr>
<tr>
<td>7</td>
<td>285 (258-325)</td>
<td>349 (343-354)</td>
</tr>
<tr>
<td>9</td>
<td>265 (240-288)</td>
<td>318 (314-322)</td>
</tr>
<tr>
<td>11</td>
<td>246 (245-248)</td>
<td>301 (286-316)</td>
</tr>
<tr>
<td>Sodium ions (mEq.dm(^{-3}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% seawater = 48mEq.dm(^{-3})</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* denotes values different from control at p=0.05.

** denotes values different from control at p=0.01.
TABLE 5 - Potassium ion concentrations in haemolymph of *Carcinus maenas* after transferring from 100% to 5% seawater, in duplicate experiments.

<table>
<thead>
<tr>
<th>Time after change (hours)</th>
<th>Mean(Range)</th>
<th>t-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DDT-treated</td>
<td>Control</td>
</tr>
<tr>
<td>Potassium ions (mEq.dm(^{-3}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9.0(7.9-10.2)</td>
<td>9.0(6.6-10.3)</td>
</tr>
<tr>
<td>2</td>
<td>8.0(7.4-8.7)</td>
<td>8.1(5.4-9.6)</td>
</tr>
<tr>
<td>3</td>
<td>7.6(6.6-8.6)</td>
<td>6.9(5.1-7.8)</td>
</tr>
<tr>
<td>5</td>
<td>6.1(5.8-6.4)</td>
<td>6.6(3.9-8.1)</td>
</tr>
<tr>
<td>7</td>
<td>6.6(5.8-7.4)</td>
<td>5.9(3.6-7.6)</td>
</tr>
<tr>
<td>9</td>
<td>6.7(6.4-7.0)</td>
<td>6.8(5.8-7.8)</td>
</tr>
<tr>
<td>11</td>
<td>5.2(4.6-5.6)</td>
<td>6.2(5.2-6.8)</td>
</tr>
</tbody>
</table>

Potassium ions (mEq.dm\(^{-3}\))

| 1 | 8.0(7.6-8.4) | 10.7(9.6-12.4) | 2.983* |
| 2 | 7.1(5.2-8.4) | 8.0(5.2-12.0)  | 0.396 |
| 3 | 6.3(6.0-6.4) | 7.3(6.0-8.8)  | 1.216 |
| 5 | 6.4(4.8-8.0) | 6.7(6.0-7.6)  | 0.144 |
| 7 | 10.7(7.6-16.4)| 6.7(5.2-8.0)  | 1.341 |
| 9 | 20.4(18.0-24.4)| 6.8(6.4-7.2)  | 6.712** |

5% seawater = 1.1mEq.dm\(^{-3}\).

* denotes values different from control at p=0.05.
** denotes values different from control at p=0.01.

Examination of Fig. 7 shows no consistent difference in the pattern of ammonia increase in the haemolymph, between DDT-exposed and control crabs. This suggests that the break-down of amino acids in the body, or the mechanism of transport to the site of deamination, has not been affected by exposure to DDT at 200x10\(^{-9}\)g.dm\(^{-3}\). Also, the rate of ammonia loss from the haemolymph does not show any consistent patterns when comparing control and experimental animals.

Tables 4 and 5 show that the sodium and potassium ion...
concentrations in the haemolymph have been affected by exposure to DDT at this concentration. The marked difference in levels between experimental and control animals apparent in the latter parts of the experiments, indicates that the ability of Carcinus maenas to maintain the sodium ion concentration at a consistent level above that of the external medium, has broken down.

The sudden rise in potassium ion concentrations after 9 hours in the second experiment is difficult to explain. There has been some disruption of osmoregulatory ability, but not in the manner expected. Weisbart and Feiner (1974) demonstrated a significant but inconsistent increase in potassium concentration, and a significant but inconsistent decrease in osmotic and sodium concentrations when goldfish (hyperosmotic to environment) were exposed to DDT. They did not attempt to explain this difference.
BIBLIOGRAPHY


CHAPTER V

This chapter is concerned with two small sections - histological effects of DDT; and some levels of DDT in the tissues found as a result of dosing Carcinus maenas in the previous experiments.

1. HISTOLOGICAL STUDIES

a) INTRODUCTION

Pollutant-carrying water enters the crab body mainly through the gills, where the pollutant can exert its first effects. It was decided to carry out some light-microscopic investigations on the structure of Carcinus gills, noting changes which may occur as a result of exposure to water containing ethanol and water containing ethanol plus DDT.

Histological changes in tissues noted by other authors after exposure to DDT have been discussed in I.7.i. However, these are concerned mainly with electron microscopical studies, examining more detailed changes in structure. It has been found that DDT exposure often results in an accumulation of smooth endoplasmic reticulum in the cell, increased vacuolation of the cytoplasm, localised necrosis, parenchymatous degeneration of cells and hypertrophy of hepatic cells.

In this work, I have examined only the structure as seen under the light microscope, when a much larger proportion of the gill surface can be sampled.

Walsh & Ribelin (1975) reported no significant changes in gills of lake trout and coho salmon exposed to DDT. Their article gives a review of types of lesions in fish caused by pesticides. Of the organochlorines, only mirex is reported to cause noticeable gill changes. Fused lamellae were noticed in cutthroat trout, and edema and aneurysms of the lamellae
in goldfish (Van Valin et al, 1968).

Hughes & Perry (1976) devised a method for evaluating the action of pollutants on the structure of trout gills, as seen under the light microscope. The diffusing capacity of gases could be estimated by measuring the diffusion distances between water and blood, together with determination of gill area.

In this work, the ATPase activity has been examined histochemically. Also, the quantity of mucopolysaccharides around the gill lamellae, and the D.N.A./R.N.A. ratio in the tissue, as an indication of changes in protein synthesis.

b) PREPARATION OF TISSUE FOR STAINING

The gills were removed immediately after the animals had been killed. This work was combined with the in vivo ATPase assays on the gills after exposure to \(200 \times 10^{-9} \text{ g.dm}^{-3} \text{ }^{14} \text{C-DDT}\). Tissues from the ethanol and seawater controls were also prepared.

For the mucopolysaccharide and D.N.A./R.N.A. staining, the tissues were fixed in formal saline (10%), dehydrated and then embedded in paraffin wax. Sections (10\(\mu\)) were cut.

This method would be of no use for the histochemical staining of ATPase, as the process of dehydration and embedding would deactivate the enzyme. Fresh-frozen sections were therefore prepared.

A small beaker containing hexane was placed into liquid nitrogen, until frozen. It was then removed and a metal cryostat chuck taken and pressed onto the hexane in order to melt the surface. The gills were placed on the now cold chuck, to which they adhered. This was inverted onto the melted hexane and left for 40 - 60 seconds to freeze the gill tissue. The
whole chuck was covered with Parafilm and kept in solid carbon dioxide until sections could be taken. Sections (10 μ) were cut using a cryostat at -26°C, the knife having been previously cooled with solid CO₂. This work was carried out in the laboratories of the Institute for Marine Environmental Research at Plymouth by Dr. M. Moore.

c) STAINING FOR MUCOPOLYSACCHARIDES

The use of Alcian blue or Alcian green for the specific staining of mucins was introduced by Steedman (1950). The dye is a copper phthalocyanin, being highly coloured and water-insoluble (Pearse, 1968). In fixed tissues and paraffin sections, Alcian green in acid solution stains connective tissue mucins and most epithelial mucins, with usually negligible staining of the background proteins. Spicer (1960) and Lev & Spicer (1964) showed that at pH 2 the dye reacted mainly with uronic acid groups of mucosubstances, whereas at pH 1 it could be made specific for sulphated mucopolysaccharides alone.

By the use of a strong counterstain, the staining of connective tissues by Alcian green can be overlaid, leaving only the connective tissue mucins stained green.

Staining Solutions:

**Alcian Green pH 2.5**

Alcian green (lg)

3% acetic acid (100cm³)

**Alcian Green pH 0.2**

Alcian green (lg)

10% sulphuric acid (100cm³)
Method (after Steedman, 1950)

1. Bring to water.
2. Stain in Alcian green solution for 5 minutes.
3. Wash briefly in distilled water.
4. Counterstain in Mayer's carmalum for 2 minutes.
5. Wash in tap water.
6. Dehydrate, clear and mount.

The use of the solution at pH 2.5 would result in most acid mucosubstances being stained green. At pH 0.2, the result would be strongly sulphated acid mucosubstances stained blue. In this work, the former solution was used.

The results of this staining are shown in Plate 1,a,b,c, being DDT-exposed, ethanol control and seawater control tissues, respectively. Although these photographs show only a small area of the gill tissue, this section is representative of most of the surface.

The photographs show that after exposure to DDT, the thickness of the mucopolysaccharide layer around the gills has been reduced (Plate 1a).

Under exposure to heavy metals such as zinc, copper and a zinc/copper combination, no change in mucus levels around the gills of rainbow trout has been reported (Lloyd, 1960; Sellers et al, 1975). However, increased amounts of mucus on the body surface were observed when treated with zinc or a zinc/copper combination.

Other authors (Schweiger, 1957; Gardner & Yevick, 1970) have reported a higher incidence of mucous cells on exposure to heavy metals. Strik et al (1975) noted a higher incidence of swollen mucous cells in the gills of rainbow trout exposed to
10mg.dm$^{-3}$ of chromium. It was thought that mucus acts as a protective mechanism against irritation caused by heavy metals and other chemicals (Sawyer, 1959).
Fig. 1  L.S. Lamellar gill of *Carcinus maenas*

---

Fig. 2  Details of gill lamellae

---

Fig. 3  T.S. Crab gill (diagramatic)
Alcian green staining of gills of *Carcinus maenas*

Magnification: 40 x 2 x 3 using green filter

a) After exposure to p,p'-DDT in ethanol

b) After exposure to ethanol

c) In seawater control
d) STAINING FOR RIBONUCLEIC ACID

The staining of tissue sections to show ribonucleic acid (R.N.A.) and deoxyribonucleic acid (D.N.A.) was first demonstrated by Brachet (1940, 1942, 1944) using methyl green and pyronin G. This was an indirect method, depending upon the specific depolymerization of R.N.A. by the enzyme ribonuclease. The methyl green stained D.N.A. in chromatin, while pyronin G stained R.N.A. in both nucleus and cytoplasm. Using two sections, one is exposed to ribonuclease and then both stained with methyl green - pyronin. Material stained red with pyronin, and removable by treatment with ribonuclease is considered to be R.N.A.; that which is not removable, is not R.N.A.

Kurnick (1955) devised a modification in which chloroform-washed pyronin-Y is used. This method is more selective for R.N.A. than previous methods.

Methyl green - Pyronin Y method (after Kurnick, 1955)

Methyl green solution

Methyl green (2g) was dissolved in distilled water by stirring well. This was poured into a separating funnel and 6 to 8 aliquots of chloroform (100cm³) added and shaken. Each time the chloroform layer was discarded. The process was continued until no more violet colour was extracted.

Pyronin Y solution

Pyronin Y (2g) was dissolved in distilled water (100cm³).

Staining solution

Methyl green (7.5cm³)
Pyronin Y (12.5cm³)
0.1M acetate buffer pH 4.8 (30cm³)
Method

1. Bring sections to water.
2. Stain in methyl green-pyronin solution for 4 - 10 minutes.
3. Blot dry.
4. Rinse rapidly in absolute acetone.
5. Rinse rapidly in 50% acetone in xylene.
6. Rinse rapidly in 10% acetone in xylene.
7. Rinse in xylene.
8. Place sections in fresh xylene and mount.

The results are shown in Plate 2,a,b,c. These are DDT-exposed, ethanol control and seawater control tissues respectively.

Although showing only a small area of gill tissue, these photographs are representative of most of the surface shown on the slide. The red areas represent R.N.A. and the green areas, D.N.A.

Exposure to DDT has resulted in a decrease in R.N.A. content of the gill tissue when compared with the controls. The effect of DDT and drugs on protein synthesis and microsomal enzymes has previously been discussed (1.7.f). Exposure generally leads to an increase in protein and enzyme synthesis caused by, among other factors, the increase of R.N.A. in the cell.

This is the reverse of the work reported here on Carcinus gill tissue.
Methyl green-Pyronin Y staining of gill tissue of *Carcinus maenas*

Magnification : 2.5 x 1.6 x 3

a) After exposure to p,p'-DDT in ethanol
b) After exposure to ethanol
c) In seawater control
e) **STAINING FOR ATPASE ACTIVITY**

This was according to the method of Padykula & Herman (1955, a, b) described by Pearse (1968).

**Incubation Medium**

This must be freshly prepared.

- 0.1M sodium barbiturate (2.062g. \(100\text{cm}^{-3}\)) \(20\text{cm}^3\)
- 0.18M calcium chloride (1.998g. \(100\text{cm}^{-3}\)) \(10\text{cm}^3\)
- Distilled water \(30\text{cm}^3\)
- Adenosine triphosphate (disodium salt) \(152\text{mg}\).

As soon as the ATP was dissolved, the pH was adjusted to 9.4 with 0.1M NaOH and made up to \(100\text{cm}^3\) with distilled water. If turbid, it was filtered.

**Method**

1. Incubate sections for 5 minutes to 3 hours at 37°C.
2. Wash in three changes of CaCl\(_2\) (1%).
3. Transfer to CoCl\(_2\) (2%) for 3 minutes.
4. Wash in distilled water for 1 minute.
5. Develop in dilute yellow ammonium sulphide.
6. Wash and counterstain if desired.

The sections were then mounted with glycerol-gelatine. No dehydration was necessary with cryostat sections.

Incubation for 2 hours was found to give the most suitable results. ATPase activity was shown by black deposits. A control slide was also carried through the incubation and staining procedure. This had been heated for one minute over a beaker of boiling water. This deactivated the enzymes, and there were therefore no dark deposits seen in the tissue.

An alternative ATPase staining method is the lead method of Wachstein and Meisel (1957) using Pb\(^{2+}\) ions to capture the
initial reaction product of ATP hydrolysis. However, Moses et al. (1966) found that decreasing the concentration of Pb\textsuperscript{2+} or increasing the concentration of ATP completely altered the localisation of the reaction product. At 0.9mM nuclear staining was predominant, and at 0.45mM a cytoplasmic pattern was obtained which suggested mitochondrial localisation. It was suggested that plasma membrane phospholipids, by acting as phosphate activators in a lead catalysed transphosphorylation of ATP, could result in local deposition of metal phosphate precipitates.

Lowenstein (1958) and Tetas & Lowenstein (1963) showed that other bivalent cations, including Ca\textsuperscript{2+}, Cu\textsuperscript{2+}, Mn\textsuperscript{2+} and Mg\textsuperscript{2+}, could act in the same way as Pb\textsuperscript{2+}. Therefore, it was suggested that Ca\textsuperscript{2+} ions in the histochemical calcium-cobalt method for ATPase might produce a similar false localisation. The presence of a visible product may not in fact indicate ATPase activity, and that the localisation of the product may not be that of the enzyme.

Tormey (1966) obtained results which indicated that the histochemical localisation of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase was impossible.

The results of this staining method demonstrated a lower activity of the enzyme in tissue from DDT-treated animals, than in the two controls. This was shown only by the degree of darkness created by the stain; the darker the stain, the greater the activity.

This staining method will indicate Ca\textsuperscript{2+}-ATPase activity, bearing in mind the comments of Lowenstein (1958) and Tetas and Lowenstein (1963) reported previously.
Ca\(^{2+}\)-ATPase is usually associated with muscle tissue, of which there is none in the part of the gill tissues sectioned. As *Carcinus* is an organism which often moults, needing to have a reserve of calcium for this process, it is probable that a Ca\(^{2+}\)-ATPase may exist in the gill tissues. Here it may be involved in the transport of Ca\(^{2+}\) ions between water and tissues. Burdick et al (1976) have reported a Ca\(^{2+}\)-ATPase in the gills of the killifish. In calcium-enriched freshwater, the enzyme is more active than in calcium-deficient water.

This histochemical enzyme assay correlates well with the biochemical work which demonstrates effects on other ATPase systems - Mg\(^{2+}\)-ATPase and Na\(^+\),K\(^+\)-ATPase.

f) PHOTOGRAPHY

Photographs of slides were taken using a Zeis Photomicroscope 2.

Black and white photographs were taken using an Ilford Pan F film, and colour using Agfachrome 80S film.

2. UPTAKE OF \(^{14}\)C-DDT BY VARIOUS ORGANS OF *Carcinus maenas*

Since various physiological and biochemical parameters have been measured which are connected with the gills, it was an interesting corollary to estimate the levels of \(^{14}\)C-DDT taken up by these tissues.

a) TISSUE-EXTRACTION OF DDT FOR ELECTRON-CAPTURE GAS-LIQUID CHROMATOGRAPHY

The gills of one individual crab were extracted before any exposure to DDT. It was taken from the Tamar Estuary and
almost immediately killed for extraction. If the animal had been left in fresh water for some time prior to extraction, there would have been a chance for some pollutants to have been lost from the body.

The animal was killed by destruction of the thoracic ganglion and the gills removed. These were ground up with solvent-cleaned sand and anhydrous sodium sulphate. Nanograde-hexane (5cm$^3$) (Mallinckrodt Chemical Works) and nanograde-propan-2-ol (20cm$^3$) were added. The cells were broken up by ultrasonication for 10 minutes using an M.S.E. Ultrasonic Disintegrator. The mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant was removed and collected.

Solvents were again added to the sediment and the whole procedure repeated twice more.

The supernatants were filtered under vacuum using a sintered glass funnel, then rotary evaporated to reduce the volume. The final extract was cleaned using a Florisil column to remove lipids. The Florisil had previously been activated by heating at 300°C overnight. The column was eluted with nanograde-hexane (30cm$^3$) followed by nanograde-toluene (20cm$^3$).

The liquid was evaporated to 1cm$^3$ and diluted for electron-capture gas-liquid chromatography (G.L.C.).

Conditions of G.L.C.
Pye Unicam 104 gas-liquid chromatogram fitted with $^{63}$Ni electron-capture detector.
Column : 10% DC-200.
Column temp : 195°C.
Detector temp. : 250°C.
Suitably diluted gill extract (0.6mm$^3$) was injected into the G.L.C., resulting in a trace which showed mainly polychlorinated biphenyls (P.C.B.) and a little DDE present. There was no DDT present in detectable quantities. The compounds were not quantified.

b) **TISSUE-EXTRACTION OF $^{14}$C-DDT AND DERIVATIVES FOR RADIOACTIVE-COUNTING**

Individual crabs which died during Experiment 2(II.2.c.ii) and Experiment 4 (II.2.c.iv) were analysed for $^{14}$C-radioactivity at the point of death. That from Experiment 2 had been exposed to an average of $2 - 4 \times 10^{-9} \text{g.dm}^{-3} \text{C-DDT}$ in a static 100% seawater system. That from Experiment 4 had been exposed to an average of $50 \times 10^{-9} \text{g.dm}^{-3} \text{C-DDT}$ in a static 50% seawater system.

The organs dissected out were gills, digestive gland, gonad and stomach. Gills were rinsed with acetone to remove any adsorbed $^{14}$C-DDT prior to extraction. The extraction procedure was similar to that for electron-capture GLC, except that there was no necessity to use nanograde solvents. After the final extract had been evaporated to near-dryness, the scintillant, butyl-PBD (10cm$^3$) was added, and the solution transferred to a scintillation vial for radioactive counting.

This method would estimate all DDT residues (DDTR) present, since the $^{14}$C-label will be present in all the metabolites of DDT.
c) AMOUNTS OF $^{14}$C-DDT AND DERIVATIVES PRESENT IN SOME ORGANS OF CARCINUS MAENAS

Table 1  
(Experiment 2)  
(died after 9 day's exposure to DDT)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>dpm (mg. tissue)$^{-1}$</th>
<th>$10^{-12}$ g.g$^{-1}$ DDTR</th>
<th>No. of times conc. from water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gills</td>
<td>137.60</td>
<td>6.82</td>
<td>2</td>
</tr>
<tr>
<td>Digestive gland</td>
<td>73.11</td>
<td>3.62</td>
<td>1</td>
</tr>
<tr>
<td>Stomach</td>
<td>29.40</td>
<td>1.46</td>
<td>-</td>
</tr>
<tr>
<td>Gonad (male)</td>
<td>29.10</td>
<td>1.44</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2  
(Experiment 4)  
(Died after 20 day's exposure to DDT)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>dpm (mg. tissue)$^{-1}$</th>
<th>$10^{-12}$ g.g$^{-1}$ DDTR</th>
<th>No. of times conc. from water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gills</td>
<td>16,103</td>
<td>87,107</td>
<td>1,742</td>
</tr>
<tr>
<td>Digestive gland</td>
<td>2,765</td>
<td>14,956</td>
<td>299</td>
</tr>
<tr>
<td>Stomach</td>
<td>1,799</td>
<td>9,896</td>
<td>198</td>
</tr>
<tr>
<td>Gonad (male)</td>
<td>1,829</td>
<td>9,731</td>
<td>195</td>
</tr>
</tbody>
</table>

DDTR represents DDT and its metabolites.

The difference in levels of $^{14}$C-DDTR between Tables 1 and 2 can be attributed to the difference in $^{14}$C-DDT in the water in which the crabs were kept, and the difference in exposure time before death. The levels follow the same order of concentration in both situations i.e. gills > digestive gland > stomach > gonad.

DDT is taken up from the water through the gills (Holden, 1962); consequently it was expected that high levels would
collect here. The digestive gland is comparable with the vertebrate's liver, the organ in which many compounds are stored in the body, and detoxified if poisonous. The gonads are tissues with a high lipid content. It would, therefore, be expected that higher concentrations may have been found here, than indicated in Tables 1 and 2.

Grzenda et al (1970) fed $^{14}$C-DDT to goldfish and estimated the residues in various parts of the body. These results are tabulated in I.6.c. The testes are very high in the order of concentrations relative to muscle (having the lowest concentration), while gills were found to have relatively low DDTR levels. Tissues connected with the digestive system, and the faeces contained higher levels than the gills.

The differences in order of concentration of DDTR in tissues of these goldfish and tissues of Carcinus, can be explained in this instance by the fact that the former was fed $^{14}$C-DDT, while the latter took up the pesticide from the water.
BIBLIOGRAPHY


DISCUSSION

This work follows a sequence, from using low concentrations of DDT, to using higher concentrations; and from measuring one parameter without effect, to measuring associated mechanisms, with effect.

The investigations have centred on the gills, and the haemolymph which is intimately associated with them. These were chosen, as the pollutant-carrying water makes initial contact with the gills. It has been shown (V.2) that the gills show the highest $^{14}$C-DDTR levels of several tissues examined, after exposure to $^{14}$C-DDT in seawater.

After 20 days' exposure to an average of $50 \times 10^{-9}\, g.dm^{-3}$ $^{14}$C-DDT in the water, the concentration of DDT and its metabolites was found to have been concentrated over 1700 times in the gill tissue.

The only other major organ which could have been examined easily was the hepatopancreas or digestive gland, in which the second highest concentrations of DDTR were found. In higher animals, the liver, which is comparable to the hepatopancreas, is the principal storage organ for chemicals such as DDT, and this is where many of the breakdown processes occur. Although the tissue would have been suitable for histological studies, the ATPase activity would probably be at a lower level than in the gills. This is especially so for Na$^+$, K$^+$-ATPase, since the gills are known to be involved in the maintenance of ionic balance (Webb, 1940).

Initially an attempt was made to use DDT concentrations which could be related to those found in the natural environment at the present day. Cox (1971) estimated levels in the sea around the U.S.A., and found $2.3 \times 10^{-9}\, g.dm^{-3}$ DDT in water off Oregon and Washington, to $5.6 \times 10^{-9}\, g.dm^{-3}$ in water off Southern California. The levels found in estuaries are
much more variable than these estimates. A report by the Agricultural Research Council (1970) estimated levels of organochlorine pesticides being transported in Britain's rivers to average $180 \times 10^{-6} \text{g.dm}^{-3}$. However, this is the level of total organochlorine pesticides, a very small part of which, today, is DDT. Although not banned in Britain, as in North America and Scandinavia, its use is under strict control. DDT levels would, therefore, be very much lower than $180 \times 10^{-6} \text{g.dm}^{-3}$.

By increasing the DDT levels used in the experiments to 100 and then $200 \times 10^{-9} \text{g.dm}^{-3}$, a relationship could be established with levels found in estuaries, or in localised regions such as rock pools or around outflows from certain industries.

Other authors often use very much higher levels, in the range of $x 10^{-3}$ to $x 10^{-6} \text{g.dm}^{-3}$ while determining the effects of DDT on organisms, but these bear little resemblance to present-day concentrations.

It was realised, from reading papers from many authors, that DDT affected the action of the membrane-bound ATPase enzymes. Usually inhibition was noted, but also occasionally stimulation. The main result of this is a change in ion levels of the body fluids and cellular fluids. In many cases these are maintained constant, by the process of active transport with which ATPases are usually involved. Assuming that DDT would affect the ATPases of Carcinus, the ion levels of the haemolymph were monitored.

When no changes were detected, the central ATPase enzyme system was studied. Under the same conditions which caused no effect on ion levels, a change was noted in activities. As a result of this disruption in the active transport process,
an effect must be exerted somewhere. This led to the work on ammonia excretion. One of the more obvious results of lack of energy available, would be with respect to movement or oxygen consumption. General inactivity in DDT-exposed crabs was noticed when compared with controls, but at higher DDT levels, hyper-activity has been noted in many organisms (Yeager & Munson, 1945). Measurement of oxygen consumption by animals is a relatively difficult process to carry out and obtain meaningful results.

a) **ATP-ASE ACTIVITY**

The *in vitro* experiment (III.3.a.) showed that Mg\(^{2+}\)-ATPase was inhibited at concentrations of DDT from 10\(^{-10}\)M to 10\(^{-5}\)M. Na\(^+\), K\(^+\)-ATPase showed an increase in activity, reaching a maximum of 217.1% stimulation at 10\(^{-9}\)M. As the concentration of DDT was increased, the activity decreased. At the highest concentration, 10\(^{-5}\)M DDT (354.5 x 10\(^{-5}\)g.dm\(^{-3}\)), activity was 141.8% of the control.

Inhibition of ATPases by DDT has been reported by many authors, and in many tissues. The occurrence of stimulation of activity has been reported less frequently.

Desaiah *et al* (1975) divided Mg\(^{2+}\)-ATPase into oligomycin-sensitive (mitochondrial) and oligomycin-insensitive. They demonstrated inhibition of the former, but stimulation of the latter using 0.5 and 2 x 10\(^{-6}\)g.dm\(^{-3}\) DDT in water, and 50 x 10\(^{-3}\)g.kg\(^{-1}\) DDT in food. There was very little change in total Mg\(^{2+}\)-ATPase activity. Na\(^+\), K\(^+\)-ATPase activity was stimulated in brain tissue using the same DDT concentration. However, all activities were inhibited when using gill tissue.
The in vitro experiment on Carcinus gill tissue did not distinguish between the two Mg\(^{2+}\)-ATPases. The work of Desaiah *et al* (1975) demonstrated the variability of effect between different tissues.

Phillips & Wells (1974) have demonstrated ATPase inhibition and stimulation in various tissues of turtles. Mg\(^{2+}\)-ATPase was stimulated in almost all the tissues in the presence of 5.3 x 10\(^{-7}\)M DDT, the lowest concentration used. The incidence of stimulation was reduced to about 50% of cases at 5.3 x 10\(^{-6}\)M DDT, and then at 5.3 x 10\(^{-5}\)M, inhibition was almost always reported. The incidence of stimulation of Na\(^+\), K\(^+\)-ATPase was very much lower than for Mg\(^{2+}\)-ATPase. If the results of their work are examined in detail, it is evident that there is considerable variation in activities between different tissues in the same turtle species, and also between species.

Care must be taken when comparing *in vitro* and *in vivo* work. That of Desaiah *et al* (1975) is an *in vivo* study, while that of Phillips & Wells (1974), an *in vitro* study. The *in vitro* work on Carcinus gill tissue also shows very different results from the *in vivo* work. Mg\(^{2+}\)-ATPase was inhibited in Experiments III.3.c., III.3.d. and III.3.e. after exposure to an average of 200 x 10\(^{-9}\)g.dm\(^{-3}\) DDT. The same occurred in the *in vitro* study. However, whereas Na\(^+\), K\(^+\)-ATPase was previously stimulated, its activity was inhibited in these three *in vivo* studies. 200 x 10\(^{-9}\)g.dm\(^{-3}\) would have been a high enough concentration to cause these effects, for during the *in vitro* experiment, at a similar concentration (354.5 x 10\(^{-9}\)g.dm\(^{-3}\) or 10\(^{-9}\)M DDT) Mg\(^{2+}\)-ATPase activity was inhibited 25%, and Na\(^+\), K\(^+\)-ATPase activity stimulated 217% from the controls.
This discrepancy in results is difficult to explain. Jackson & Gardner (1973) stressed the difference between *in vivo* and *in vitro* experiments on ATPases using organochlorines. When administered *in vivo*, the DDT is carried to the target organs by the blood lipids, being very lipophilic. However, when administered *in vitro*, because of the hydrophobic nature of DDT, precipitation tends to occur, even when a water-miscible solvent is used as the carrier. These authors found, using salmon and trout brain preparations, that the organochlorines, including DDT, brought about reductions in activity only at concentrations which exceeded the limits of solubility. These effects were reduced or abolished in the presence of Corexit 7664, a surfactant used to prevent precipitation.

This theory, though, does not explain the results found with *Carcinus*. The *in vivo* work, in which precipitation would not occur, showed comparable percentage Mg$^{2+}$-ATPase inhibition levels at similar concentrations (comparing Experiments III.3.a. and III.3.d.).

During the *in vivo* studies, the DDT-carrying water is continually bathing the gill tissues, thus allowing the DDT to exert an effect on Na$^+$, K$^+$-ATPase over a long period of time. Although the gill tissue has been well-broken up for the *in vitro* work, a very much shorter time was allowed for the insecticide to reach the enzymes. Before sacrificing the crabs for enzyme assays, very high concentrations of DDT had accumulated in the gills. This time difference and the fact that *in vivo* studies allow time for accumulation in tissues, may explain the differing results. Reference to Fig.2 (III.3.a.) shows that after stimulation at low DDT concentrations ($10^{-10}$ M and $10^{-9}$ M), inhibition from these activities was starting to occur. If a range of higher
DDT concentrations had been used, inhibition of enzyme activity may have been evident. Higher concentrations in vitro would have been comparable with DDT accumulation in tissues.

Another explanation is that the whole body may exert some effect on the response during in vivo studies. Those in vitro are concerned with a very small area of tissue totally isolated from other tissues.

The inhibition of Ca\(^{2+}\)-ATPase was demonstrated by the histochemical work (V.I.e.). This enzyme is associated with the transport of Ca\(^{2+}\) ions, and therefore impairment of this function may lead to muscular and skeletal disorders.

Valentine & Soulé (1973) discussed fluctuating asymmetry in the body of an animal, as a measure of environmental stress. They demonstrated that deviation from perfect symmetry of several anatomical characters occurred under the influence of DDT. These characters were mainly associated with the skeletal structure, and it was suggested that calcium metabolism may have been affected.

Further evidence for this is the inhibition of growth of shells of bivalve molluscs (Butler et al, 1962). Yearling oysters showed a decrease in growth rate after 24 hours' exposure to 0.1 mg dm\(^{-3}\) DDT.

The phenomenon of eggshell-thinning in birds has been discussed (I.7.g.iv). Miller et al (1976) found that eggshell-thinning in ducks was accompanied by a decrease in the activities of two shell gland enzymes - Ca\(^{2+}\)-ATPase and carbonic anhydrase.

The demonstration of Ca\(^{2+}\)-ATPase inhibition in Carcinus gills may, therefore have some adverse effect on formation of
the exoskeleton. As the crab regularly moults, it is possible that after a prolonged time in water polluted with organochlorines, the exoskeleton may be reduced in thickness over successive moults. This may lead to death by crushing.

Inhibition of Mg$^{2+}$-ATPase activity may lead to impairment of oxidative phosphorylation and thus respiration. Wilber (1971) suggested that uncoupling of oxidative phosphorylation would account for an increase in oxygen requirements, as reported by several authors (I.7.k).

Na$^+$, K$^+$-ATPase is involved with the active transport of Na$^+$ and K$^+$ against a concentration gradient. In an aquatic organism, such as Carcinus maenas, which must maintain a distinct relationship between the concentration of the external medium and the body fluids, impairment of Na$^+$, K$^+$-ATPase activity in gills will lead to an imbalance of ions and possibly death. This ATPase is involved in active transport of Na$^+$ and K$^+$ in other body organs (e.g. intestine, cloacal bladder and kidney - Phillips & Wells, 1974). Inhibition would create an inability to effectively utilise metabolites and cause serious electrolyte imbalances.

b) **ION LEVELS IN HAEMOLYMPH OF CARCINUS MAENAS**

The consequence of ATPase inhibition by DDT may be observed in a number of ways. One of these is a change in ion levels of the body fluids, and another, a change in the rate of ammonia excretion.

In dilute media, Carcinus maenas maintains its body fluids at a higher concentration than the concentration of the water (Fig.3,II.1.b). In order to maintain this difference, active transport of ions occurs between the body fluids and water, mainly through the gills. Na$^+$, K$^+$-ATPase is
involved in this. Inhibition of the enzyme should result in this concentration difference being reduced, i.e. the body fluids being diluted. This was examined with respect to Na\(^+\), K\(^+\) and Cl\(^-\) ions and osmolality (total fluid concentration) while exposed to up to 200 x 10\(^{-9}\) g.dm\(^{-3}\) DDT in salinities down to 15% seawater. Under these conditions, there was no evidence to suggest that the haemolymph ion levels had altered, even though it was known that the enzyme involved was inhibited.

Experiments III.3.d. and III.3.e. were carried out in order to put the crabs under greater physiological stress. This was a final attempt to obtain some change in ion levels. It was thought that although the animals may be able to withstand exposure to DDT in 50% seawater, the extra stress exerted by DDT and 30% or 15% seawater may not be withstood. Even under these conditions no marked changes were evident.

It was noted that under exposure to DDT in low salinities, the ion levels between individuals varied more than in the control (Experiment III.3.e.). This suggests that DDT is having some effect on the osmoregulatory mechanism, and the effect is varying in degree between individuals.

During a later experiment (IV.3.d.), haemolymph samples were taken for ion analysis over the first few hours after the salinity of the water was reduced. Previous experiments had involved a minimum of a day's acclimation before sampling. It seemed that this period allowed the animal's osmoregulatory mechanism to adjust to the new environment. Prior to this it was apparent that the mechanism had been affected. The inhibition of the ATPases, already demonstrated, was therefore affecting the crab's ability to maintain an osmotic balance.
(with respect to Na\(^+\) and K\(^+\) ions) between its internal medium and the environment.

As *Carcinus* inhabits estuaries, throughout its life it will be exposed to many pollutants and unfavourable conditions. A mechanism seems to have developed whereby the organism is able to resist or compensate for changes occurring as a result of ATPase inhibition. Much greater changes in body fluid ion levels have been observed in more sensitive organisms, such as freshwater or open-ocean fish.

c) **AMMONIA EXCRETION IN *CARCINUS MAENAS***

As Crustacea adapt to a lower salinity, the body fluid concentration decreases slightly. This can partly be attributed to a lowering of the amino acid content of the body fluids. The amino acids are catabolised forming ammonia, which results in an increase in pH value of the haemolymph. The ammonia then has to be lost from the body.

In dilute salinities, although the ion levels of the haemolymph are decreased the difference between ion levels of this and the seawater, increases. To maintain this ratio, certain ions are pumped into the body. One of these is Na\(^+\). Mangum (1976) has suggested that a Na\(^+\) - NH\(_4^+\) exchange pump exists in the gills of crabs (Ch.IV), which is catalysed by Na\(^+\), K\(^+\)-ATPase. This theory is supported by the evidence that Na\(^+\), K\(^+\)-ATPase activity is similar when NH\(_4^+\) is substituted for K\(^+\) in the incubation medium (III.2.c.).

Although most of the experiments on *Carcinus* were not carried out in dilute seawater, where ammonia excretion would be enhanced, the same result was achieved by injection of ammonium chloride solution (IV.3.b.) and feeding (IV.3.c.). The latter proved inadequate at supplying sufficient ammonia
in the haemolymph for the rate of excretion to be monitored successfully.

Initial results (Fig. 4, IV.3.b.) suggested that when the animals had been exposed to $200 \times 10^{-9}$ g dm$^{-3}$ DDT for 10 days, loss of ammonia occurred at a slower rate than when not exposed to DDT. This supported the work of Mangum and co-workers, that there existed a $\text{Na}^+ - \text{NH}_4^+$ exchange pump catalysed by $\text{Na}^+, \text{K}^+$-ATPase. Repeat experiments, however, failed to confirm this.

In summary, the experimental results suggested a number of subtle effects of ATPase inhibition on the crabs' physiology, including a disturbance in ionic balance over a short time period, and some evidence (albeit unconfirmed) of disturbance in ammonia excretion. However, in none of these experiments were the physiological effects very apparent. This was surprising in view of the clear effects of DDT on enzyme activity in vitro. There are many possible explanations, including the existence of compensatory biochemical processes that are able to overcome the effects of ATPase inhibition. However, the experimental results give sufficient indication of the physiological consequences of enzyme inhibition to suggest the possibility of damage to crabs exposed to these levels of DDT in the environment.

Progress in this study would now depend upon a much better understanding of the role of ATPases and other enzyme systems in crab gills, and particularly in the processes of ionic exchange and ammonia excretion.
d) MUCOPOLYSACCHARIDE AND R.N.A. CONTENT OF GILLS OF

*CARCINUS MAENAS*

Freeman (1966) has proposed a mechanism relating the endoplasmic reticulum (ER) and Golgi apparatus to the synthesis of mucus. He postulated that the ER of goblet cells synthesises a protein moiety which is transported to the Golgi apparatus where it is combined with acid mucopolysaccharides and glycoproteins. Shearman & Muir (1960) noted in studies on goblet cells that smooth membranes formed the lamellae of the ER nearest the goblet, and these merged with membranes containing denser material and with a roughened surface (rough-ER). It was shown that the mucous globules are in direct continuity with the clear material in the spaces between the lamellae.

An increase in the amount of mucus produced may, therefore, be accounted for by a proliferation of smooth-ER, or may be a result of a change in some other process concerned with mucus production. For a decrease in mucus production to occur, the reverse may happen.

If this theory is correct, the results using *Carcinus* gills suggest a decrease in smooth-ER as a result of DDT-exposure. This is, however, inconsistent with previous reports on histological changes induced by DDT (I.7.i). Weis (1974) showed that the relatively DDT-resistant guppy developed an increase in smooth-ER in liver cells after treatment with DDT, while there was no change in the smooth-ER content in the relatively DDT-susceptible zebrafish. This change was believed to be associated with an increase in microsomal drug-metabolising enzymes.
From results obtained with *Carcinus*, it appears that this animal is not especially susceptible to DDT. One would therefore expect to observe a proliferation of smooth-ER and microsomal enzymes.

Plate 2,a,b,c, in which ribonucleic acid (R.N.A.) is indicated by pink staining, shows a smaller amount of RNA present in the tissue treated with DDT, than the control. This work is inconsistent with observations noted previously (I.7.f), that protein synthesis is stimulated after exposure to DDT, with an increase in drug-metabolosing enzymes. The microsomal enzymes are believed to be associated with smooth-ER.

If the theory connecting decreased mucus production with a decrease in smooth-ER is correct, the observations on *Carcinus* gill mucopolysaccharides correlate well with evidence for decreased RNA, indicating also a possible decrease in smooth-ER.

These statements suggest that *Carcinus maenas* is a relatively susceptible organism to DDT. Usually, animals are able to increase the synthesis of microsomal enzymes in order to effectively metabolise the pollutant, and consequently show reduced effects.

e) CONCLUSIONS

This work has been successful in revealing some of the effects caused by sublethal concentrations of DDT on the shore crab, *Carcinus maenas*. Although some negative effects have been demonstrated, these are as equally important as positive effects, with respect to our knowledge of pollutants and their actions.

*Carcinus* is a common coastal and estuarine organism, being easily kept under laboratory conditions, but it has
its disadvantages. These are connected mainly with the great variation in values when measuring a particular parameter. This is seen between individuals, and in one individual over a period of time.

Inhibition of Na$^+$, K$^+$, Mg$^{2+}$- and Ca$^{2+}$-ATPases has been demonstrated. It has been possible to provide evidence for the existence of a connection between Na$^+$, K$^+$-ATPase and ammonia excretion in *Carcinus maenas*. Histological damage has been shown in light microscope studies on gill tissue.

In many of the experiments, it has been possible to demonstrate that *Carcinus maenas* can adapt to drastic changes in its environment, having, therefore, a type of physiology which would lead to a successful existence for the animal in estuarine and some polluted habitats.
BIBLIOGRAPHY


