Appendix II

Degradation of Poly-chlorinated Biphenyls by Micro-organisms

The fate of P.C.B.s in the environment is of considerable interest to ecologists. Among the various natural mechanisms for degrading organic materials, a significant contribution may be expected from micro-organisms. As part of a broad study designed to investigate causes for the apparent distribution of P.C.B.s in the environment we have studied the effects of selected micro-organism cultures on both single isomers and commercial mixtures of P.C.B.s.

The basic experimental procedure consists of shaking prepared substrates comprising P.C.B.s, mineral solutions, inocula, inhibitors, etc., for various periods of time and determining the loss of P.C.B. by G.L.C. analysis of extracts. In this work only primary degradation has been measured and we have not identified the products of the metabolic processes involved. Preliminary experiments showed that rates of degradation were increased by the addition of biphenyl; in many of the experiments, therefore, biphenyl is added initially and for experiments involving long exposures it is also added subsequently at intervals. Mercuric chloride was shown to be an effective inhibitor for the micro-organisms studied and is included at 100 ppm in a number of experiments for control purposes. Various types of shake-flask equipment have been used, but we have now standardised on an orbital incubator (Gallenkamp) operated at 130 cycles per minute controlled at a temperature of 25°C.

A standard procedure is adopted for the preparation of samples and final extraction for analysis. 5 mls. aliquots of di-ethyl ether solution containing 100 ppm P.C.B. plus 1000 ppm biphenyl (when included) are introduced into 250 ml. culture flasks (stoppered quick-fit conical flasks) and the ether evaporated by a slow stream of dry nitrogen, without external heating. To each flask is then added 100 mls. of culture solution. To maintain aerobic conditions the flasks are vented at weekly intervals and, when appropriate, biphenyl (0.005 gms) added periodically.

MONS 042098

Analytical Methods

Samples obtained from the biodegradation experiments are much less complex than those obtained from environmental studies since they contain only the P.C.B., biphenyl, nutrients, culture and water.

For this reason the extraction of the P.C.Bs and any degradation residues from the aqueous sample is very simple but emphasis is placed upon quantitative extraction and upon the achievement of a high concentration factor.

A brief description of the extraction and analysis procedures together with a discussion of the important features is given below:

a) Extraction and Concentration

The P.C.B. sample undergoing biodegradation is in an aqueous phase and, because it has been found that in these circumstances there is a tendency for it to adsorb on the walls of the container, the complete aqueous sample is extracted with solvent (pentane) - aliquots are not used.

The pentane extract is reduced to a volume of 2 - 3 mls (a concentration factor of 50 - 100X) on a warm water bath using a nitrogen purge. Such conditions are mild enough to selectively remove the pentane without affecting the P.C.B. components. For measurements on single isomers and simple mixtures, the pentane extracts are analysed directly using a 4 ft. column, packed with 3% Silicone Oil O.V 101 on 60 - 80 mesh gas Chrom Q, with a high temperature Ni^{63} electron capture detector.

For analysis of commercial mixtures, however, when the volume of 2 - 3 mls is reached a weighed amount of n decane (usually 0.1g) is added and the evaporation is continued, until all the pentane is removed. The n decane has a much higher boiling point than pentane and because of this the P.C.B. components dissolve in the n decane and are unaffected by the complete removal of pentane. This decane sample, in which the P.C.B. concentration is up to 500 x that originally in the pentane, is then used for capillary column analysis.

b) Measurement of the Degree of Biodegradation and composition of the Residues

To fully describe the results of the biodegradation of any P.C.B. sample three questions must be answered :

i) which of the original P.C.B. components have been reduced?

ii) by how much?

iii) what is the nature of the new components produced by biodegradation - if any?

The method used in all the recent biodegradation work has been that involving high resolution capillary column gas chromatography with electron capture detection. This is capable of giving accurate answers to questions (i) and (ii) and, combined with mass spectrometry is potentially capable of answering question (iii).

The decane solution of P.C.Bs obtained as described in section (a) is examined directly (using a suitable aliquot) on a stainless steel capillary column coated with Apiezon L (200', 30 thou bore) and the separated components are measured using a high temperature Ni^{63} electron capture detector. This produces a complex gas chromatogram similar to Figure I. Under the conditions of the analysis the capillary column is capable of resolving most of the 40 - 50 components of Aroclor 1242 and the E.C. detector can measure quantitatively a component concentration equivalent to better than 1 ppb (1 in 10^{-9}).

Two features concerning this analytical procedure need further discussion.

i) Use of Apizon L as column coating

This paraffinic stationary phase was chosen early in the investigations. It is known that the material elutes components according to their boiling point and that boiling point can be related to structure. It was believed that a set of empirical rules could be drawn up to relate the structure of any P.C.B. with its elution time on Apizon L. In many cases this has turned out to be true and Simmons and Welti (9) have published a paper showing the proposed structures of most of the components in Aroclors 1242, 1254 and 1260. Combined with our own data this has enabled us to follow the degradation of the individual components of known structure in Aroclors 1242 and 1016.

ii) Quantitative Application of the Electron Capture Detector

While the electron capture detector is ideally suited to this analysis in that it is capable of detecting exceptionally small quantities of chlorinated materials it has a potential weakness in that its response is very sensitive to molecular structure. It was expected that chlorinated biphenyls of the same chlorine content could, because of structural differences, have widely different responses. This coupled with a possible range of chlorinated biphenyls from mono to deca chloro could result in great errors of measurement of the degraded materials.

This potential weakness has not been realised in practice for the Aroclors under study (1242 and 1016) and, in fact, the capillary column chromatograms of Aroclor 1242 obtained using an electron capture detector do not differ greatly from those obtained with a flame ionisation detector (which is not seriously affected by structural differences between isomers). Reasons for this unexpected behaviour have been postulated, but the main conclusion is that for the study of the biodegradation of Aroclors 1242 and 1016 the error caused by structural differences do not affect the overall interpretation of results.

EXPERIMENTAL

Some early experiments were made using activated sludge inocula from the biological effluent treatment plant at Ruabon, N. Wales, pre-adapted to degrade biphenyl. The experimental procedures were developed during this work and some degradation of P.C.Bs was observed, but most of the results discussed here arose from experiments using specific biphenyl degrading cultures isolated by Evans and Hunt (21). Two such cultures have been investigated for the degradation of P.C.B.s. These are a nocardium of the order actinomycetes isolated from a soil sample from the Sudan (Culture C_2) and one isolated from a canal mud sample (U.K.) and reported to be of the pseudomonas family (Culture C_3). Evans and Hunt have described studies of the microbial attack by these cultures on biphenyl and have suggested the metabolic routes shown in Fig. 2.

Stocks of active organisms (C_2 and C_3), received from Professor Evans, have been prepared and maintained according to his recommendations. A prepared growth media (Table 1) was inoculated with the culture and the mixture aerated continuously in the dark at a temperature of 25°C using scrubbed and filtered air. Inocula for subsequent experimental substrates were withdrawn after stopping the air flow for a brief time to allow excess biphenyl to settle. The media were prepared in 15 litre quantities. As prepared the pH was about 5.8, but was brought to 7 by addition of NaOH solution before introducing the inoculum. When only relatively small volumes of stock culture (one or two litres) were removed for experiments, the bottle was replenished by addition of an equal volume of solution containing the inorganic constituents of the growth medium. For larger quantities the practice was adopted of starting a fresh culture.

TABLE 1. METHOD FOR CULTURE OF BIPHENYL-DEGRADING ORGANISMS

Constituent	Grm. per litre
$(NH_4)_2\ SO_4$	1.0
KH_2PO_4	1.0
$MgSO_4 . 7\ H_2O$	0.5
$Fe\ SO_4$	0.05
Biphenyl	1.0

RESULTS

A number of single isomers and simple mixtures have been studied in contact with Cultures C_2 and C_3. Packed column G.L.C., as previously described, was used to measure the loss of P.C.B. The results are summarised in Table 2.

TABLE 2

Isomer	Culture	With or without biphenyl	Time to Degrade to 50%
2,4'-dichloro-	C_2	With	2 days
	C_2	Without	5 days
4,4'-dichloro	C_2	With	>50 days
	C_2	Without	>50 days
	C_3	With	<10 days
	C_3	Without	<15 days
2,3-dichloro	C_2	With	4 days
		Without	4 days
3,4-dichloro	C_2	With	2 days
		Without	3 days
2,3,2'-trichloro-	C_2	With	3 days
		Without	7 days
2,3',4'-trichloro-	C_2	With	<2 days
		Without	3 days
3,3',4'-trichloro-	C_2	With	6 days
		Without	6 days
2,4,6-trichloro	C_2	With	>50 days
		Without	>50 days
2,2',4,4'-tetrachloro	C_2	With	>50 days
		Without	>50 days
2,2', 4,6-tetrachloro	C_2	With	>50 days
		Without	>50 days
2',3',4',5',2,3-hexachloro*	C_2	With	<15 days
		Without	>50 days

* Mixed with 2,3,2' and 2,3',4',-trichlorobiphenyls to improve solubility.

Some commercial P.C.B. mixtures have also been studied in contact with cultures C_2 and C_5. Using high resolution capillary column G.L.C., the components have been identified by the methods developed by Sissons and Welti (9) and the percentage degradation of each component calculated from comparative peak height measurements in the chromatograms. Analyses were made after 10, 20, 30 and 60 days exposure. Biphenyl was added at intervals of 10 days. For Aroclors 1242 and 1016 exposed to culture C_2 the percentage residues for a number of components are shown graphically in Figures 3 and 4.

The concentrations of individual isomers containing 5 or more chlorines in these Aroclors are so low that it has not been found possible to quantify the extent and rate of degradation. To overcome this problem we added 10% of Aroclor 1254 to increase the more highly chlorinated homologue content. This particular mixture, however, showed no significant degradation after 25 days, the presence of the more highly chlorinated Aroclor appearing to inhibit the bacterial action.

DISCUSSION

The results of the work reported here indicate quite clearly that the lower chlorinated isomers of P.C.Bs can be degraded by enzyme hydroxylation induced by micro-organisms. The results with single isomers seems to support the work of Evans and Lunt (21) and their suggested mechanisms for biphenyl degradation with the specific organisms which they isolated. It seems reasonable to explain our findings in terms of chlorine substitution interfering with the hydroxylation steps or subsequent ring opening stages. This kind of theory would indicate that the culture C_2 would not degrade 4,4'-dichlorobiphenyl, whereas culture C_3 could. It also suggests that C_2 could degrade 2',3',4',5',2,3-hexachlorobiphenyl. These indications were supported by the experimental results obtained with single isomers and simple mixtures. The conclusion to be reached from these results, coupled with the proposed mechanisms of Evans and Lunt is that the intractable low chlorine isomers (<5Cl) of P.C.Bs would be those chlorinated in the 2,6 positions on one ring, and the 2,5,2',5' and 3,5,3',5' tetra-chlorobiphenyls. The total number of intractable low chlorine isomers would, on this basis, be 20 out of a theoretical total of 82. Of these, only 7 are likely to be present in significant quantities, based on probability considerations.

When commercial mixtures of P.C.Bs are exposed to these biphenyl-degrading cultures a somewhat different pattern of degradation appears to emerge. The most noticable feature is that many of the low chlorine isomers which appeared to be intractable when present alone, degrade comparatively readily when present in the mixture. Thus 4-4'dichloro-biphenyl degrades to 50% in approximately 2 days when exposed to culture C_2 as a component of Aroclor 1016 or Aroclor 1242.

The general conclusion from the study of these commercial mixtures is that all isomers present in significant amounts in Aroclor 1016 degrade with time constants (at 20°C) of less than 20 days; in Aroclor 1242 a small percentage, consisting of more highly chlorinated homologues may degrade more slowly, probably with some time constant exceeding 50 days.

MONS 042108

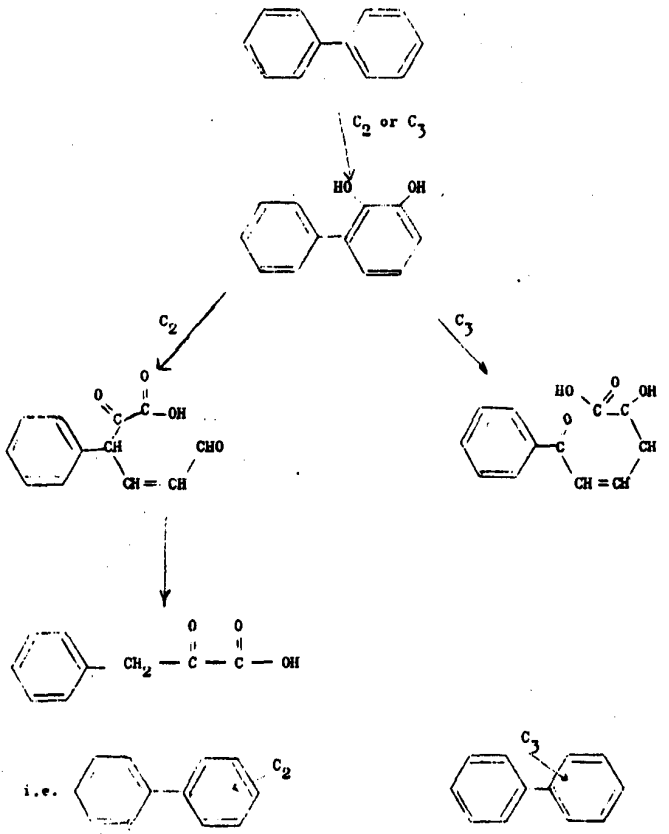
The curves showing the pattern of degradation for some isomers in Aroclors 1016 and 1242 in figures 3 and 4 tend to fall into three groups. Those isomers containing only one or two chlorines degrade rapidly, in less than 10 days. Another group consisting mainly of trichloro-biphenyls seems to degrade rapidly, after an initial induction period, but then tends to a slower rate when the level has dropped to 10% or so. The third group, primarily tetrachloro-biphenyls, degrades more slowly, but does not show such a pronounced induction period.

This pattern of degradation can possibly be explained on the basis of adaptation and reversion. For the mono- and di-chloro-biphenyls the biphenyl-degrading cultures probably need little adaptation and will readily degrade these components. For more highly chlorinated components however, some adaptation will be necessary and the ease with which this is accomplished may depend on the concentrations of precursors available. As the precursors are degraded, however, the organisms may suffer reversion and the rate of degradation thereby decrease. As the chlorine content in the molecule is increased the rate of degradation may be expected to decrease as a result of reducing the available molecular sites for oxidation. However specific tetrachloro isomers, for which suitable precursors are available, may not need a long adaptation period and could therefore follow the type of curve indicated.

fig 1

1242, 1ppm

FIG. 2 Metaholic pathways for the biodegradation of biphenyl with C_2 and C_3



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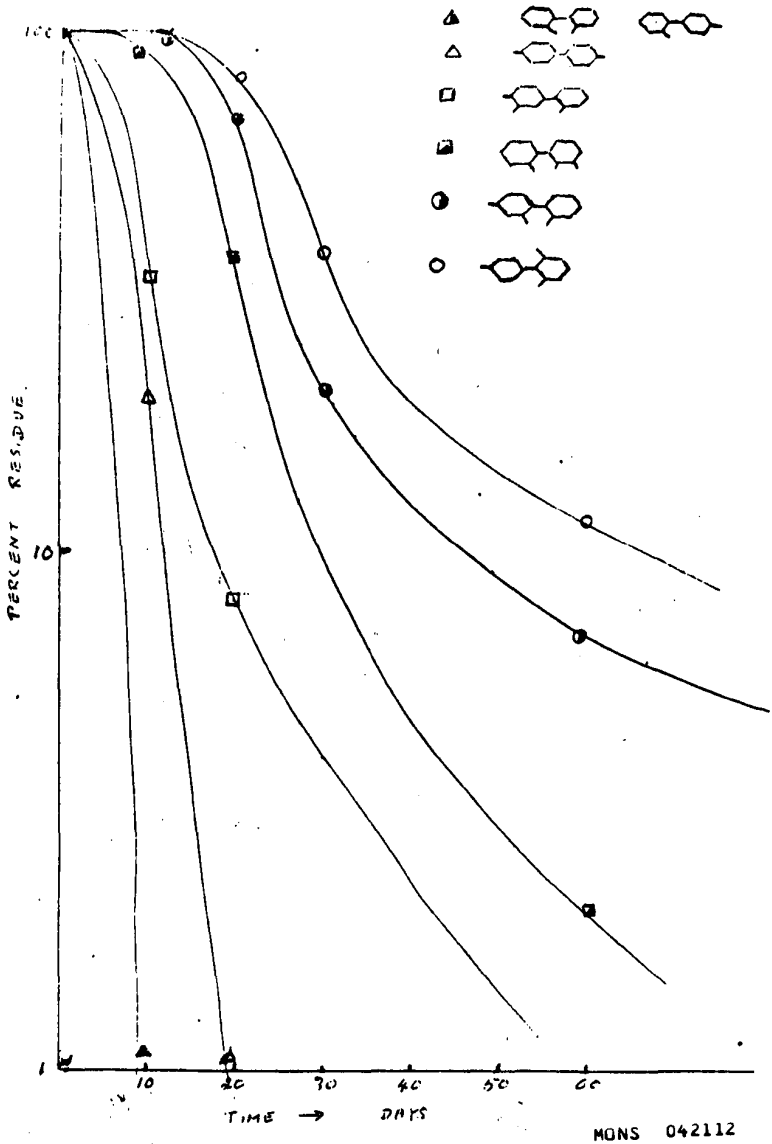
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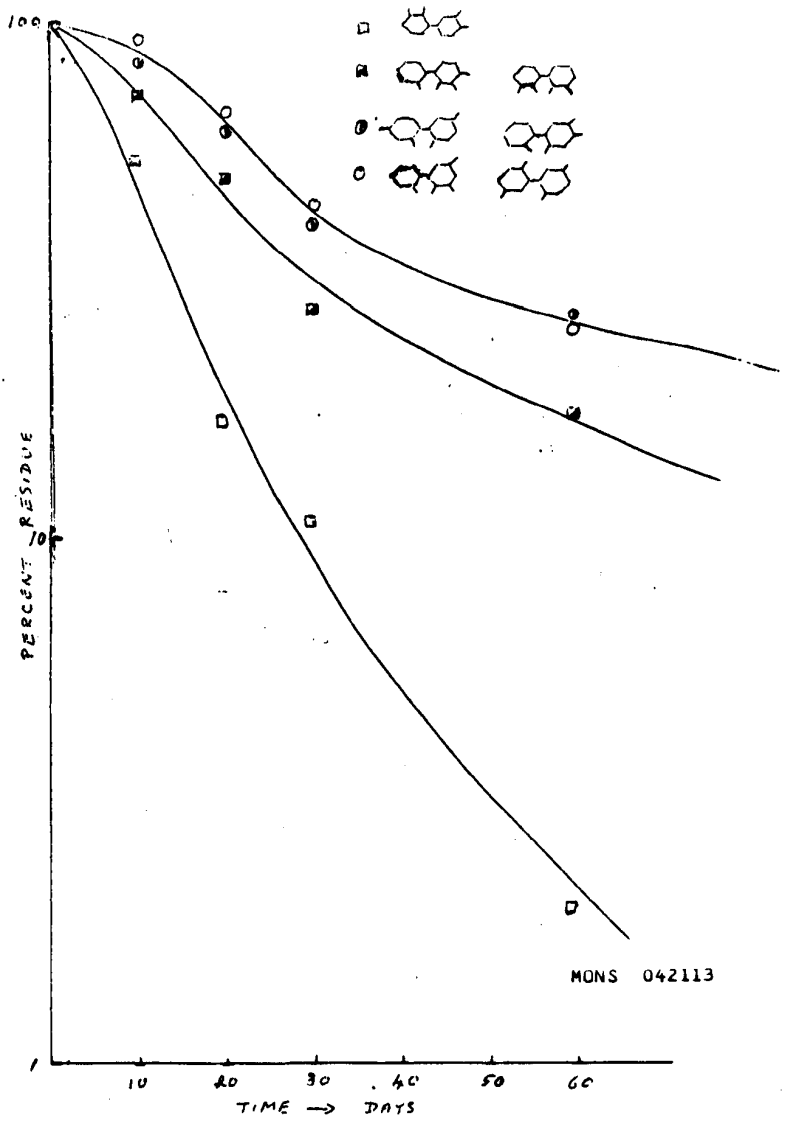
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