THE DEGRADATION OF POLYCHLORINATED BIPHENYLS BY MICRO-ORGANISMS.

Polychlorinated Biphenyls (PCBs) have been indicted as environmental pollutants. Jensen (aron) 1966) showed that PCBs were probably the substances present in wildlife specimens, which were interfering with gas chromatographic analyses for organochlorine insecticides.

Since then considerable evidence has accumulated to support the view that some PCBs are widespread in nature and may reach levels which are significant in many aquatic animals and ~~surprisingly high in~~ some fish-eating birds. This evidence has been comprehensively reviewed by Edwards (1971).

For functional and manufacturing reasons, commercial PCB products consist of complex mixtures of homologues and their isomers. They are sold under various trade names, often designated by the chlorine content. ~~Thus Aroclor* 1242 contains approximately 42% by weight chlorine, Aroclor 1254 contains 54% and so on.~~ The possible fate of a PCB product in a natural environment is of interest to ecologists and conservationists; the pattern of accumulation may depend not only on production and use, but also on its degradation. Of the various natural mechanisms for degrading organic materials, a significant contribution might be expected from micro-organisms; we have therefore studied microbial degradation of some PCB compounds and commercial mixtures.

Some early experiments were made using activated sludge inocula from the biological effluent treatment plant of Monsanto Chemicals Ltd., at Ruabon, North Wales, pre-adapted to degrade biphenyl. During this phase of the work the experimental techniques were developed and some degradation

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of PCBs was observed. Meanwhile, work by Lunt and Evans (1970) on microbial degradation of biphenyl was brought to our attention; we obtained from Professor Evans some of the culture, classified as a Nocardia spp. (NCIB 10603), and studied its effects on PCBs. Later we obtained another organism, Pseudonomas spp. (NCIB 10643), also described by Lunt and Evans (1972) and studied the effects of this culture on selected PCBs.

Experimental

The basic experimental procedure consisted of shaking prepared substrates comprising PCBs, inocula, mineral solutions, inhibitors, etc., for various periods of time and determining the loss of PCB by gas chromatographic analysis of extracts. In this work only primary degradation was measured and the products of metabolism have not been identified. Preliminary experiments showed that the rates of degradation were increased by the addition of biphenyl; in many of the experiments, therefore, biphenyl was added initially and subsequently, at intervals, during extended periods of exposure.

Stocks of the active organisms received from Professor Evans were prepared and maintained according to his recommendation as follows:-

A prepared growth medium (Table 1) was inoculated with the culture and the mixture was continuously areated in the dark at 25°C using filtered and scrubbed air. Inocula for subsequent experimental substrates were withdrawn after stopping the air flow for a short time to allow the biphenyl to settle.

The media were prepared in 15 litre quantities and, as prepared, had a pH of approximately 5.8; this was adjusted to pH 7.0 with sodium hydroxide

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solution before introducing the inoculum. When small volumes (1 - 2 litres) of stock culture solution were needed the bottle was replenished with an equal volume of growth medium. For larger quantities a fresh culture was started.

TABLE I

Media for Culture of Biphenyl-Degrading Organisms

Constituent	Concentration g./litre
Ammonium Sulphate	1.0
Potassium Dihydrogen Phosphate	1.0
Magnesium Sulphate ($7H_2O$)	0.5
Ferrous Sulphate	0.5
Biphenyl	1.0

The following procedure was used to prepare samples for biodegradation:-

A solution (5 ml) of diethyl ether containing 100 ppm of PCB was pipetted into a 250 ml glass stoppered conical flask. To this was added (for those samples subjected to biodegradation in the presence of biphenyl) a second diethyl ether solution (5 ml) containing 1000 ppm biphenyl. The ether was completely removed by evaporation using dry nitrogen at ambient temperature and this was followed by addition of the culture solution (100 mls).

Two agitation systems were used in this study. A Monsanto built shaker (120 cycles/minute, ambient temperature) was used for all the single compounds and simple mixtures while a Gallenkamp orbital incubator (130 cycles/min., 25°C) was used for commercial PCB mixtures.

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Flasks were vented at 7 day intervals to ensure that an adequate supply of air was available in the system and, for those flasks containing excess biphenyl, this was maintained by adding solid biphenyl during the venting procedure.

For each experiment with a single compound in which biodegradation was attempted(either in the presence of biphenyl or alone) a control sample duplicating the test sample but containing 100 ppm of mercuric chloride was run simultaneously. The mercuric chloride prevented bacterial action and enabled a check to be made on any PCB losses arising from the experimental method, e.g. from evaporation, adsorption etc. In practically all cases it was found that more than 90% of the PCB was recovered in the procedure described here; later experiments dispensed with the mercuric chloride controls, giving greater shaker capacity for test samples.

On removal from the shaker or incubator the total contents of each shake-flask were transferred to a separating funnel (250 ml), the flask was washed with pentane (2 x 10 ml) and the washings added to the contents of the separating funnel. The aqueous phase was then extracted with pentane (4 x 25 mls); the pentane extracts were bulked ready for concentration and gas chromatographic analysis.

In all experiments the total contents of each shake-flask were used for analysis, since it was suspected that adsorption effects might invalidate the results if aliquots were taken. This suspicion was verified subsequently by direct experiments, which indicated that up to 90% of the PCB

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could remain on the flask wall, although virtually 100% recovery was achieved using the solvent extraction method described. Thus the study of each compound or mixture required several flasks as follows:-

PCB plus culture

PCB plus culture plus biphenyl

PCB plus culture plus biphenyl plus $HgCl_2$

When studying single compounds and simple mixtures three sets of flasks were used. Concentrations of PCB, initially and after several days shake-flask exposure were determined and significant biological degradation assessed by comparison with the inhibited controls. If substantial degradation was observed the experiment was terminated, but, where necessary, exposure was continued for a further period to establish whether the compound should be classified as intractable. When commercial PCB mixtures were studied, however, five sets of flasks were used so that residual concentrations of PCB components could be determined initially and after 10, 20, 30 and 60 days exposure.

Concentration and Gas Chromatography

Two methods for concentration and gas chromatography were used for the samples as follows:-

Single Compounds and Simple Mixtures - For these samples simple concentration and gas chromatographic methods were used because the PCBs were present in relatively large amounts and were easily measured using a packed column and electron capture detector:-

The pentane was reduced to a volume of approximately 2 - 3 mls using a stream of dry nitrogen and a warm water bath. The concentrate was then

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analysed for PCB using the following gas chromatographic conditions:

Instrument	- Hewlett Packard 402
Column	- 4'4' Silicone Oil OV101 on Gas Chrom Q 60/80 mesh.
Temperature	- Between 150°C and 250°C depending upon compound.
Sample size	- 5 µl
Internal Standard	- Usually a second PCB compound
Detector	- Ni^{63} Electron capture operated at 280°C.
Carrier Gas	- Argon/Methane 95/5

Complex Commercial Samples - For these samples special concentration and gas chromatographic methods were needed.

Because of the complexity of the samples (up to 70 components) a method capable of separating as many components as possible prior to measurement was required and a capillary column system was therefore used. Since each component could be as little as 1% in the original PCB (i.e. 0.05 ppm) and upon degradation this could drop to 1% of that concentration (i.e. 0.0005 ppm) a method with a high concentration factor was essential.

The pentane solution was therefore initially reduced to approximately 5 mls when a known weight (0.1g) of decane was added. The solution was further concentrated using dry nitrogen and a water bath until the pentane had been completely removed (checked by reweighing). For an initial solution of 100 mls containing 5 ppm of PCB this was equivalent to concentrating to 5000 ppm and for a residual PCB of 0.1 ppm a final level of 100 ppm could be obtained.

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The PCB concentrate in decane was then analysed by gas chromatography using the following conditions:-

Instrument	-	F & M Model 810
Column	-	300' x 0.030" I.D. Stainless Steel capillary coated with Apezon L according to the method of E.J. Malec (1971).
Temperature	-	220°C
Sample Size	-	2 µl Split 30:1
Detector	-	Ni^{63} Electron Capture operated @ 280°C
Carrier Gas	-	Argon/Methane 95/5

As the biodegradation study was concerned with relative losses of PCB isomers the known variability in response of the electron capture detector to components if different chlorine level and structure was not a problem. Reduction in any component was determined by comparison of the component peak area in the chromatogram of the degraded sample with the peak area in the original sample, correction being made if necessary for the use of different sample sizes or concentrations.

PCB Samples

The following PCB samples have been used in the work described here:

Compound	Source
2,4'-dichloro-biphenyl	Monsanto U.S.A. Lab, St. Louis
4,4'-dichloro-biphenyl	Monsanto U.S.A. Lab, St. Louis
2,3-dichloro-biphenyl) + 3,4-dichloro-biphenyl)	Monsanto U.K. Lab, Ruabon (prepared as mixture)
2,3,2'-trichloro-biphenyl) + 3,4,2'-trichloro-biphenyl)	Monsanto U.K. Lab, Ruabon (prepared as mixture)
3,4,3'-trichloro-biphenyl	Monsanto U.S.A. Lab, St. Louis
2,4,6-trichloro-biphenyl	Monsanto U.K. Lab, Ruabon
2,4,2',4'-tetra-chloro-biphenyl	Monsanto U.S.A. Lab, St.Louis
2,4,6,2'-tetra-chloro-biphenyl	Monsanto U.S.A.Lab, St.Louis
2,3,4,5,2',3'-hexachloro-biphenyl	Monsanto U.K. Lab, Ruabon
Aroclor 1242	Monsanto U.K. Plant, Newport

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Results

Six single compounds, one three component and two two component mixtures have been exposed to the micro-organism cultures described by Lunt and Evans. The loss of PCB was measured in each case using packed column g.l.c. after exposures varying between 7 and 33 days. The approximate times for 50% loss were estimated from these measurements and are recorded in Table II. Where no significant loss was measured during the course of the experiment the PCB compound was recorded as intractable, but this is intended as a relative term and does not necessarily mean that no degradation occurs.

TABLE II

Compound	Culture (NCIB No.)	Biphenyl	Time to degrade 50% (days)
2,4'-dichloro-	10603	yes	2
	10603	No	5
4,4'-dichloro-	10603	Yes	Intractable
	10603	No	Intractable
	10643	Yes	10
	10643	No	15
2,3-dichloro-	10603	Yes	4
	10603	No	4
3,4-dichloro-	10603	Yes	2
		No	3
2,3,2'-trichloro-	10603	Yes	3
		No	7
3,4,2'-trichloro-	10603	Yes	<2
		No	3
3,4,3'-trichloro-	10603	Yes	6
		No	6
2,4,6-trichloro-	10603	Yes	Intractable
		No	Intractable
2,4,2',4'-tetrachloro-	10603	Yes	Intractable
		No	Intractable
2,4,6,2'-tetrachloro-	10603	Yes	Intractable
		No	Intractable
2,3,4,5,2',3',-hexachloro*	10603	Yes	<15
		No	Intractable

* mixed with 232' and 23'4'-trichlorobiphenyls to improve solubility.

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Some commercial PCB mixtures have also been exposed to these cultures. The components, separated by capillary column gas chromatography, have been identified by the methods described by Sissons and Welti (1971) and the percentage degradation for each component calculated from comparative peak height measurements. Analyses were made after 0, 10, 20, 30 and 60 days exposure and biphenyl was added at ' days intervals. Typical chromatograms for Aroclor* 1242 and the same material after degradation are shown in Fig.1; percentage residues for a number of significant components, following exposure to NICB 10603, are shown graphically in Figs.3 and 4.

The concentrations of individual components, containing five or more chlorine atoms, in Aroclor 1242 are low and it has not been possible to quantify the extent and rate of degradation of these highly chlorinated homologues, although many of them have been observed to degrade significantly.

Discussion

The work reported here shows quite clearly that many PCBs can be degraded by enzyme hydroxylation induced by micro-organisms. The results with single compounds are consistent with the work of Lunt and Evans (1972) and their suggested mechanisms for the metabolism of biphenyl by the two specific organisms which they have isolated (Fig.2). It seems reasonable to suppose that chlorine substitution in specific positions on the biphenyl molecule will interfere with either the initial hydroxylation step or subsequent ring-opening stages. Thus chlorine substitution in the 4,4'-positions might be expected to interfere with metabolism by NICB 10603, but not necessarily with that by the organism NICB 10643. The results

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in Table II for 4,4'-dichloro-biphenyl support this reasoning. Although it has not been possible to explore this structure/degradation relationship fully, due to shortage of specific compounds, the results given in Table II are consistent with a plausible theory of this type, including the indication that 2,3,4,5,2',3'-hexachlrobiphenyl can be degraded by the organism NICB 10603. Such a theory suggests that the intractable compounds containing less than five chlorine atoms would be those chlorinated in the 2,6 position on one ring and the 2,5,2',5'- and 3,5,3',5'-tetrachloro-biphenyls.

When commercial mixtures of PCBs are exposed to these biphenyl-degrading cultures a rather different pattern emerges. Some of the low chlorine compounds which appear intractable when alone, degrade quite readily when present in the mixture. For example, 4,4'-dichloro-biphenyl degrades to 50% in approximately two days when exposed to NICB 10603, as a component in Aroclor 1242. Most of the components present in significant amounts in Aroclor 1242 degrade with time constants (at 25°C) less than 20 days; a small proportion, consisting of highly chlorinated homologues, may degrade more slowly.

The curves showing the pattern of degradation for some of the significant components in Aroclor 1242 (Figs.3 and 4) tend to fall into three groups. Those compounds containing only one or two chlorine atoms 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 about 10%. The third group, primarily of tetrachloro-biphenyls, degrades more slowly.

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This pattern of degradation might be explained in terms of adaptation and reversion. Mono- and dichloro-biphenyls can probably be degraded with little or no adaptation of the biphenyl-degrading organisms. For more highly chlorinated components however, adaptation may be necessary and the ease with which this may be accomplished will depend on the concentrations of precursors available. As these precursors degrade, however, reversion may occur and the rate of degradation therefore decrease. Also, as the chlorine content in the PCB molecule is increased, the rate of degradation might be expected to decrease as the number of sites available for oxidation is reduced. Thus, tetrachloro-biphenyls will, in general, degrade less rapidly than trichloro-biphenyls, except for some specific structures.

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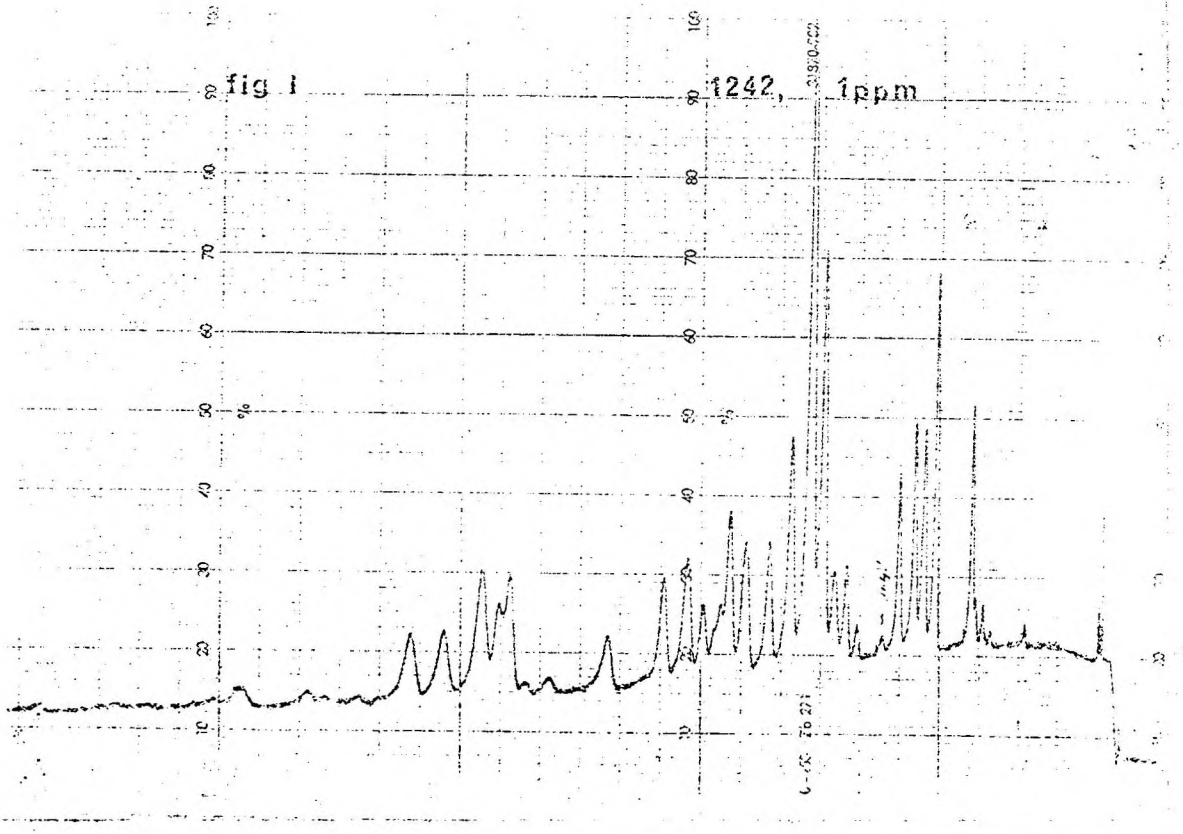
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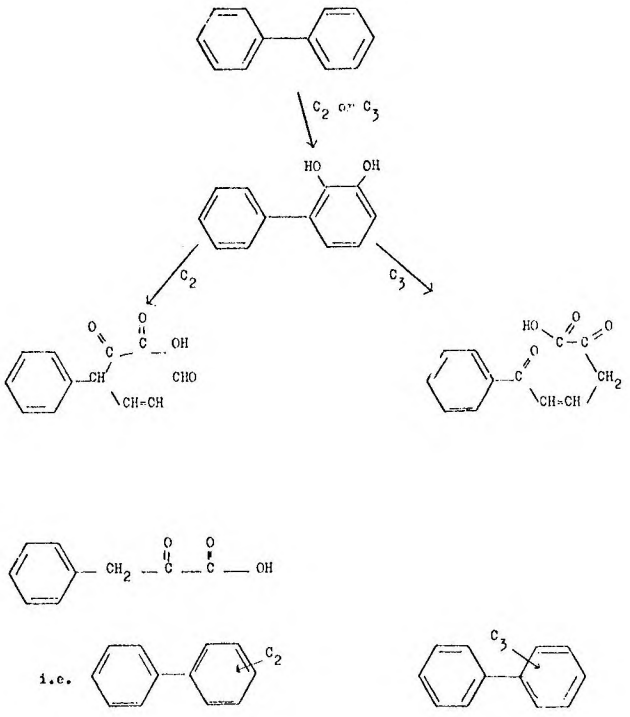
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FIG. 2 Metabolic pathways for the biodegradation of biphenyl with C_2 and C_3



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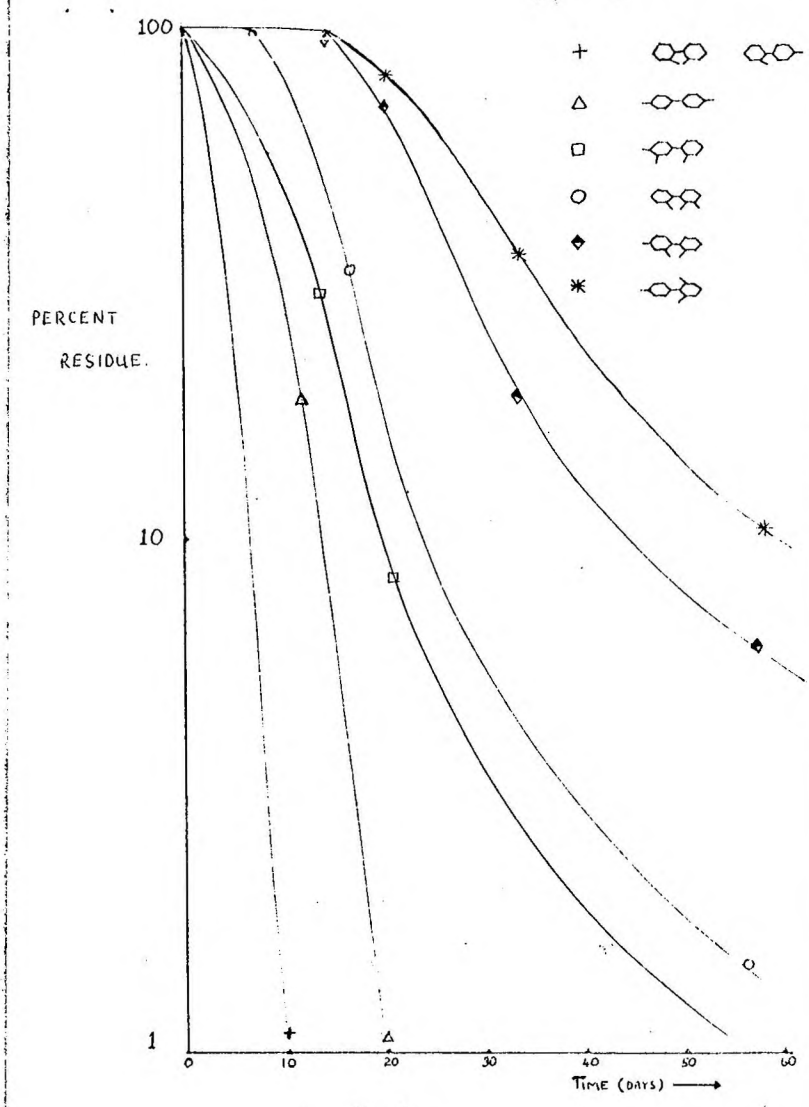


Fig. 3.

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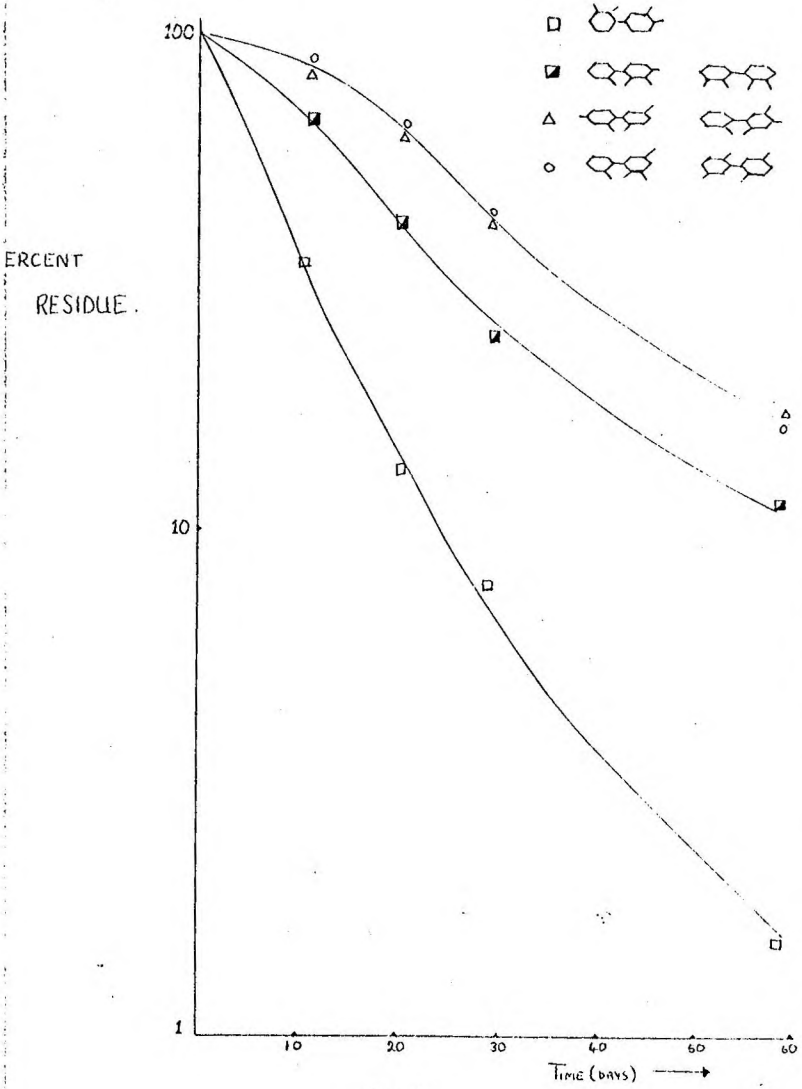


FIG 4

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