From the desk of
W B PAPAGEORGE

3/20/75

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PCB
Feeding Studies

MONS 029035

Monsanto

MAR 20 1975

FROM (NAME & LOCATION): H A Vodden - LLN

DATE: 14th March, 1975

cc: R A Baxter - LLN

SUBJECT:

REFERENCE: HAV/SB/622

TO: W R Richard - St Louis

I am sending a copy of the presentation I made in St Louis in September on the status of our studies of diodegradation.

We have some more results on the Ruabon Pond experiment, but Ron Lidgett is currently analysing the last batch of samples and I will send you those results together with the updated comments next week.

You may also like the enclosed copy of the paper on microbial degradation, which is shortly being published in the Science of the Total Environment. We have seen the proof copies of the paper and will send you reprints when they arrive.

all

H A Vodden

MONS 029036

THE DEGRADATION OF POLYCHLORINATED BIPHENYLS BY MICRO-ORGANISMS

(Biodegradation of Polychlorinated biphenyls)

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ABSTRACT

An investigation of the effects of two micro-organism cultures on single compounds and multi component mixtures of polychlorinated biphenyls (PCBs) is described.

Several low chlorine PCB-compounds can be degraded easily with these cultures and, in favourable circumstances, some compounds containing upto six chlorine atoms per molecule can be degraded.

In certain multi component commercial mixtures some PCB compounds are degraded more readily than if present singly. The significance of this is discussed, particularly the fact that work on single compounds does not enable predictions to be made about the behaviour of commercial mixtures in the environment. Some tentative explanations are suggested for the behaviour of the mixtures exposed to these cultures.

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INTRODUCTION

Multi-component mixtures of polychlorinated biphenyls (PCBs) have been used in a variety of industrial applications and some of these components have been found in the environment. This subject has been reviewed extensively -for example, Edwards (1971). The persistence of some PCBs has been a cause for concern and it is therefore important to establish possible fates of PCBs in the environment. The metabolism of PCBs in pigeons and quail has been studied by Bailey and Bunyan (1972), who found that compounds with a relatively low degree of chlorination were fairly rapidly metabolised and are not as persistent as the more highly chlorinated compounds. Similar results had been obtained by Grant et al (1971) in metabolic studies in the rat, where it was found that tissue residues of the lower chlorinated components were significantly less than the residues of the more highly chlorinated ones. Of the various natural mechanisms for degrading organic materials, micro-organisms would be expected to make a significant contribution. We have therefore studied microbial degradation of some PCB compounds and commercial mixtures in the laboratory.

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 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, Pseudomonas spp. (NCIB 10643), from Professor Evans and studied the effects of this culture on selected PCBs. Since this work was completed, Ahmed and Focht (1973) have reported on the biodegradation of PCBs by a species of Achromobacter, and Gibson et al (1973) have confirmed the metabolic pathway for biphenyl oxidation which was postulated by Lunt and Evans.

EXPERIMENTAL

Essentially two series of experiments have been carried out, the first (Series A) dealing with single substances or simple two or three compound mixtures and the second (Series B) investigating the behaviour of complex multicompound mixtures obtained commercially by chlorination of biphenyl.

The basic experimental procedure consisted of shaking prepared substrate 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 recommendations as follows :

A prepared growth medium (Table 1) was inoculated with the culture and the mixture was continuously aerated 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 solution before introduction 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.

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TABLE 1

Media for Culture of Biphenyl - Degrading Organisms	
Consituent	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 (5ml) 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 (5ml) 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 (100mls).

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

Flasks were vented at 7 days 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 control giving greater shaker capacity for test samples.

On removal from the shaker or inoculator 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 could remain on the flask wall, although virtually 100% recovery was achieved using the solvent extraction method described. Thus this 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 any degradation could be observed. 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 Chormatography

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

Series A

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 concentration was then analysed for PCB using the following gas chromatographic conditions .

Instrument	-	Hewlett Packard 402
Column	-	4.76 mm id X 1.2 m - 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

Series B

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 methc 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 ppr a method with a high concentration factor was essential.

The pentane solution was therefore initially reduced to approximately 5 mls when a known weight (0.1 g) of decane was added. The solution was further concentration 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.

The PCB concentrate in decane was then analysed by gas chromatography (g.c.) using the following conditions :

Instrument	-	F & M Model 810
Column	-	0.76 mm id X 90 m. Stainless steel capillary coated with Apiezon L according to the method of E.J.Maice (1971).
Temperature	-	220°C
Sample size	-	2 µl Split 30:1
Detector	-	Ni^{63} Electron Capture operated at 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 of different chlorine level and structure was not a problem. Reduction in any component was determined by comparison of the component peak height in the chromatogram of the degraded sample with the peak height in the original sample, correction being made if necessary for the use of different sample sizes or concentrations.

PCB samples were either obtained from laboratory suppliers or were synthesised by the method of Bain et al (1962) which is particularly well suited for laboratory preparation of specific chlorinated biphenyls. The purity of all samples was checked by gas chromatography (g.c.) and shown to be $> 99\%$ in all cases.

RESULTS

Series A

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 by packed column g.c. with the results shown in Table II which gives the percentage loss after a given period of time. Results are also shown in which biphenyl was added as additional substrate.

Series B

Two multicomponent PCB mixtures have also been exposed to these cultures. The first of these, Aroclor 1242 (Monsanto Chemical Ltd., London), is a chlorination product of biphenyl containing 42% chlorine. The second, Aroclor 1016 (Monsanto Co, St Louis), is a similar mixture from which the high boiling components have been removed. The percentage degradation was determined after a period of 52 and 100 days using capillary column g.c. with the results shown in Table III. In all experiments with these commercial mixtures an excess of biphenyl was maintained in the culture flasks.

The components separated by capillary g.c. have been tentatively identified by the methods described by Sissons and Welti (1971) and the percentage degradation for each component calculated from comparative peak heights. Typical chromatograms of Aroclor 1242 and the same material after degradation are shown in figures 1 and 2. Analyses were made after 0, 10, 20, 30 and 60 days exposure; the percentage residues for a number of significant components of Aroclor 1242 exposed to NCIB 10603 are shown as functions of time in figures 3 and 4. More than 90% of the original Aroclor 1242 consists of components which appear to degrade similarly. Exposure to NCIB 10643 gave similar results, but these are not given in detail .

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TABLE II

Compound	% Degradation/Days	
	NCIB 10603	NCIB 10613
2,4'-dichlorobiphenyl	70/7	60/73
" + biphenyl	100/7	100/73
4,4'-dichlorobiphenyl	nil/121	50/15
" + biphenyl	nil/121	50/10
2,3-dichlorobiphenyl	67/8	-
" + biphenyl	64/8	-
3,4-dichlorobiphenyl	80/8	-
" + biphenyl	100/8	-
2,3,2'-trichlorobiphenyl	50/7	-
" + biphenyl	95/7	-
2,3',4'-trichlorobiphenyl	94/7	-
" + biphenyl	100/7	-
2,5,4'-trichlorobiphenyl	nil/73	15/73
" + biphenyl	60/73	60/73
3,4,3'-trichlorobiphenyl	76/12	-
" + biphenyl	70/12	-
2,4,6-trichlorobiphenyl	nil/12	nil/84
" + biphenyl	nil/12	nil/84
2,4,2',4-tetrachlorobiphenyl	nil/9	-
" + biphenyl	nil/9	-
2,4,6,2'-tetrachlorobiphenyl	nil/9	-
" + biphenyl	nil/9	-
2,3,4,5,2',3'-hexachlorobiphenyl + 2,3,2' and 2,3',4'-trichlorobiphenyl	nil	-
" " " + biphenyl	50/11	-

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TABLE III

PERCENTAGE DEGRADATION				
Time (days)	Aroclor 1242		Aroclor 1016	
	NCIB 10603	NCIB 10643	NCIB 10603	NCIB 10643
52	88	76	96	91
100	95	85	>98	>96

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DISCUSSION

The work reported here shows quite clearly and in agreement with Ahmed and Focht (1973), that many PCBs can be degraded by micro-organisms.

The results from series A on single compounds and simple mixtures show that under the experimental conditions many of the lower chlorinated biphenyls ($\ll 3$ chlorine atoms/molecule) are readily degraded and that even certain compounds containing as many as 6 Cl atoms could be degraded in favourable circumstances. There is some evidence that the pattern of chlorination will influence the ease of biodegradation but insufficient specific compounds were available to explore this fully. However, it is clear that under the experimental conditions some compounds are much more readily degraded than others. When the results from Series B on the complex mixtures were examined, a different pattern emerged which was not expected from the single compound work. Some of the lower chlorinated compounds which appeared to be only slowly degraded when alone, degraded quite readily when present in the mixture. For example, 4,4'-dichlorobiphenyl degraded to 50% in approximately 2 days when exposed to NCIB 10603 as a component of Aroclor 1242 (fig.3) whereas it was virtually unaffected after 12 days when exposed alone (Table II). Mutual solubilisation may play some part in the effect and this conclusion is supported by the observation that 2,3,4,5,2',3'-hexachlorobiphenyl could be degraded in a three component mixture, but not when exposed alone (Table III). However, 4',4-dichlorobiphenyl could not be similarly induced to degrade when mixed with 3,4,2' and 2,3,2'-trichlorobiphenyls and a more complex mechanism seems likely.

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 degraded rapidly, in less than 10 days. Another group, consisting mainly of trichlorobiphenyls, seemed to degrade rapidly after an initial induction period, but then tended to

a slower rate when the concentration had dropped to about 10%. The third group, primarily of tetrachlorobiphenyls, degraded more slowly.

This pattern of degradation can be explained in terms of adaptation of the organisms. Mono- and dichlorobiphenyls can probably be degraded with little or no further adaptation of the bipehnyl 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. The induction times observed for many of the components probably indicate these adaptation periods. There are a number of possible explanations for the apparent decrease in rate of degradation later on. The viable cell population may have dropped considerably, the g.c. analysis may not give adequate isomer resolution, or, simply, the accuracy available at such low concentrations of individual components may not reflect the true shapes of the degradation curves in this region.

The observation of enhanced biodegradation of certain PCBs by addition of biphenyl to the substrate may indicate a co-metabolic mechanism (Horvath 1972). Similarly, organisms which have become adapted to the simple PCBs may then become capable of metabolising the more complex structures. More detailed study of the nature of the metabolic products would be necessary to elucidate these mechanisms but it seems clear from the present study that examination of the behaviour of single PCBs does not enable predictions to be made on the biodegradability of complex mixtures.

In the natural environment it can be expected that there will be a number of organisms capable of degrading the PCB and a mixed culture acting on a multicomponent mixture of PCBs might be expected to give more complete degradation than do single organisms. Futher work in a natural environment is intended to investigate this possibility.

ACKNOWLEDGEMENTS

It is a pleasure to record our thanks to Professor W.C.Evans, Mr.D.Lunt and Mr.C.Harrison of University College, Bangor, for supplies of micro-organisms and useful advice. We are also indebted to Dr.J.P.Brown of Monsanto Chemicals Ltd. for stimulating discussion.

LEGENDS FOR FIGURES

Figure 1 Capillary Column/ EC Chromatogram Aroclor 1242 (5 ppm) Peak numbers follow Sissons & Welti (1971)

Figure 2 Capillary Column/EC Chromatogram Aroclor 1242 (1 ppm) after exposure to NCIB 10603 for 52 days.

Figure 3 Degradation of typical di- and tri-chlorobiphenyl components of Aroclor 1242 following exposure to NCIB 10603

○ Peak 13 (2 Cl) △ Peaks 15 & 18 (3 Cl) □ Peak 10 (3 Cl)

● Peaks 11 & 17 (3 Cl) ▲ Peaks 9 & 12 (3 Cl)

Figure 4 Degradation of typical tetra-chlorobiphenyl components of Aroclor 1242 following exposure to NCIB 10603

○ Peak 34 △ Peak 21 □ Peak 28

● Peak 35 ▲ Peak 22

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REFERENCES

Ahmed, M and Focht, D.D.(1973). Degradation of polychlorinated biphenyls by two species of Achromobacter. Can. J. Microbiol. 19 47 - 52.

Bailey, S. and Bunyan, P.J.(1972). Interpretation of persistence and effects of polychlorinated biphenyls in birds. Nature (Lond.) 236 34 - 36.

Bain, P.J., Blackman, E.J., Cummings, W., Hughes, S.A., Lynch, E.R., McCall, E.B. and Roberts, R.J., (1962). Homolytic Aromatic Substitution by Radicals Derived from Sulphonyl Halides. Proc.Chem.Soc., 186.

Edwards, R. (1971). The polychlorobiphenyls, their occurrence and significance : a review. Chem. & Ind. 1340-1348.

Gibson, D.T., Roberts, R.L., Wells, M.C. and Kobal, V.A. (1973). Oxidation of biphenyl by a Beijerinckia species. Biochemical and Biophysical Research Comm. 50, 211 - 19.

Grant, D.L., Phillips, W.E.J. and Villeneuve, D.C. (1971). Metabolism of a polychlorinated biphenyl (Aroclor 1254) mixture in the rat. Bull. Environ.Contam. & Toxicol., 6, 102 - 12.

Horvath, R.S.(1972). Bacteriological Reviews, 36, 146 - 155.

Lunt, D. and Evans, W.C. (1970). The microbial metabolism of biphenyl. Biochem.J., 118 54 p.

Malec, E.J. (1971). Improved open tubular gas chromatographic columns for use at 250°C and above. J.Chromatogr. Sci. 9. 318.

Sissons, D. and Welti, D. (1971). Structural Identification of Polychlorinated Biphenyls in Commercial Mixtures by Gas-liquid Chromatography, Nuclear Magnetic Resonance and Mass Spectrometry. J.Chromat. 60 15 - 32.

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Fig 1

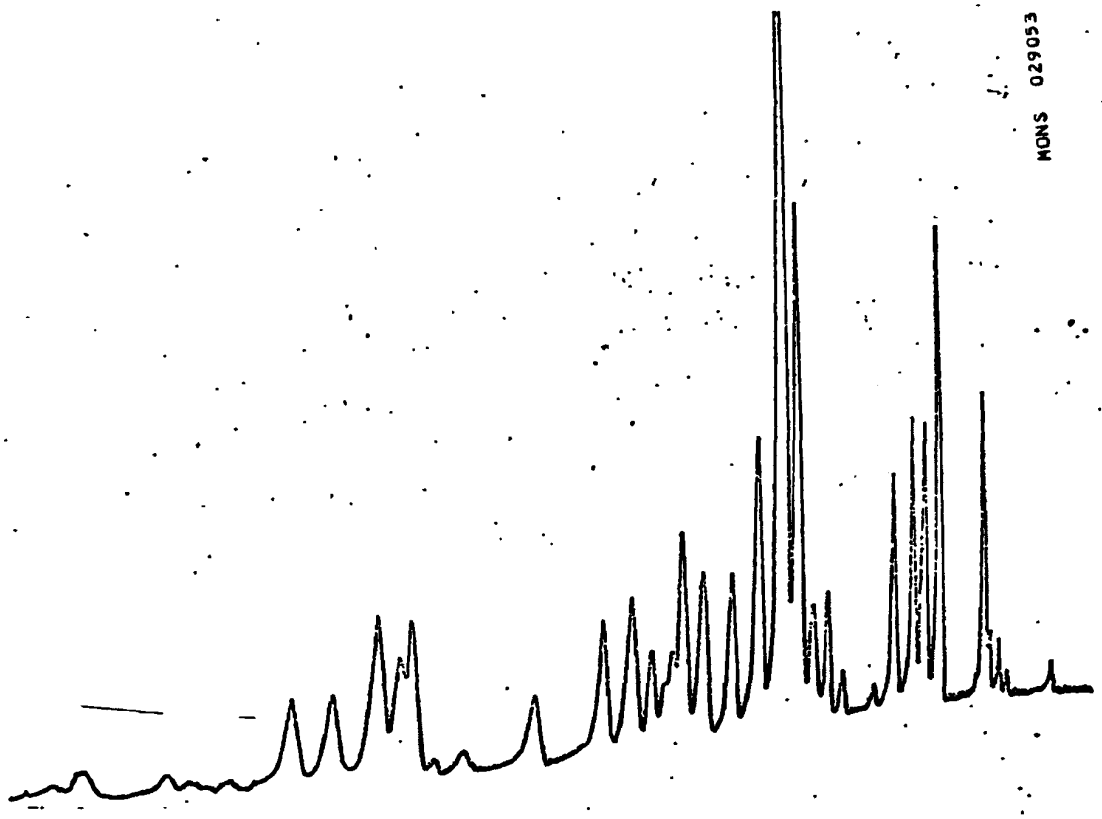

Fig 8.

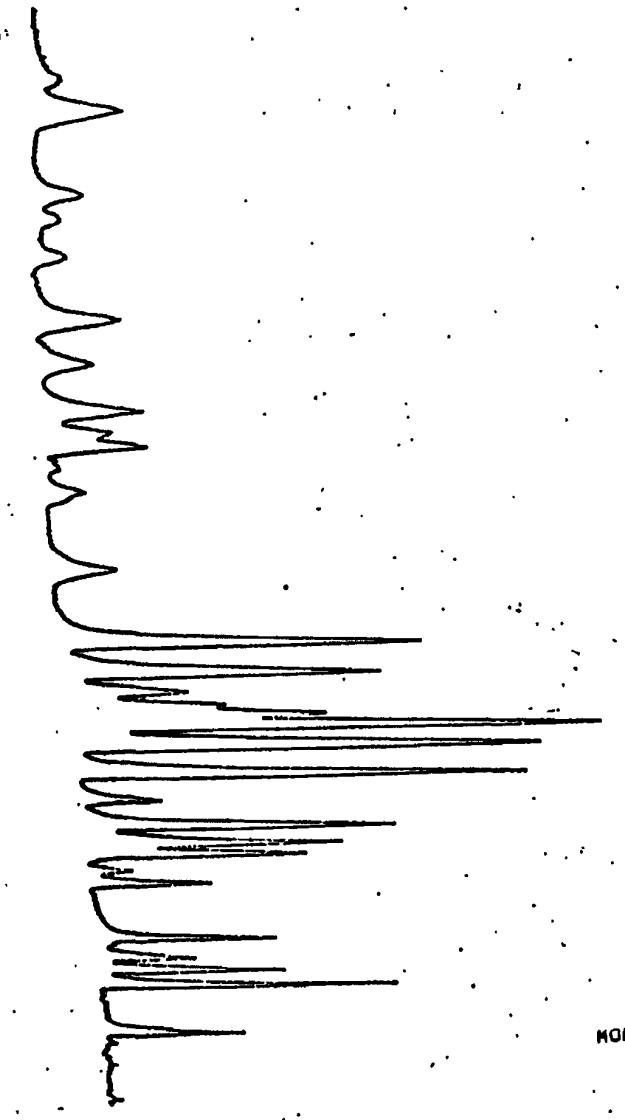

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Fig 3.

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Fig 4.

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