Formation of a β-Hydroxy Acid as an Intermediate in the Microbiological Conversion of Monochlorophenoxybutyric Acids to the Corresponding Substituted Acetic Acids

Wain and his colleagues[1] have clearly shown that the growth-regulating activity in plants of substituted ω-phenoxyalkylcarboxylic acids containing an uneven number of side-chain methylene groups can be explained by β-oxidation of the fatty acid side-chain within the plant to give the corresponding substituted acetic acids.

Working with the soil micro-organism *N. opaca* strain T_{16}, Webley, Duff and Farmer[2] have shown that the side-chain of ω-phenyl-substituted fatty acids also undergoes β-oxidation. It has now been demonstrated that strain T_{16} will convert 3- and 4-monochlorophenoxybutyric acids to the corresponding substituted acetic acids. Further, during these conversions an intermediate is formed which from γ-(4-chlorophenoxy)butyric acid proved to be β-hydroxy-γ-(4-chlorophenoxy)butyric acid. For this purpose the methods detailed by Webley, Duff and Farmer[2] were used. In some cases, and especially to obtain separation of the substituted butyric and acetic acids from the intermediate, it was necessary to adopt the 'multiple development' technique of paper chromatography as described by Jeanes, Wise and Dimler[3].

Experiments with strain T_{16} were set up with monochlorophenoxybutyric acids in place of the ω-phenyl substituted fatty acids used previously[2]. Other conditions remained the same. Aliquots (5 ml.) were removed aseptically, acidified, extracted with ether and the residue obtained after removal of the solvent examined by paper chromatography and infra-red analysis. Fig. 1 shows a chromatogram obtained after incubation for five days of a suspension of washed cells of strain T_{16} (4·6 mgm./ml. dry weight cells final concentration) at 25° C. in 50 ml. solution of γ-(3-chlorophenoxy)butyric acid (as sodium salt, $M/250$ concentration) under aseptic conditions. The R_F of the intermediate was 0·53 (γ-(3-chlorophenoxy)butyric acid gives 0·62 and 3-chlorophenoxyacetic acid gives 0·57). After further incubation with the addition of fresh cells as described by Webley, Duff and Farmer[4], paper chromatography of the extract showed that the spots corresponding to γ-(3-chlorophenoxy)butyric acid and the intermediate had disappeared and that only a strong spot corresponding to 3-chlorophenoxyacetic acid remained. At the end of the experiment, the product was isolated from the solution as a crystalline solid with an infra-red spectrum identical with that of an authentic sample of 3-chlorophenoxyacetic acid.

Similar results were obtained with γ-(4-chlorophenoxy)butyric acid; but in this experiment the intermediate was only slowly converted to the acetate, so that after seventeen days incubation it was possible to isolate it as a crystalline solid. The infra-red spectrum of this product (Fig. 2*a*) showed it to be principally β-hydroxy-γ-(4-chlorophenoxy)butyric acid by comparison with the spectrum of a synthetic specimen (Fig. 2*b*). An additional band in the natural product (marked by an arrow) is due to some unchanged γ-(4-chlorophenoxy)butyric acid. The intermediate was further converted to the acetate derivative by incubation with a fresh batch of cells. Finally, an experiment was set up in which the synthetic β-hydroxy compound was incubated with washed cells of T_{16}. After seven days incubation the product formed was isolated from the solution in the usual way. Infra-red examination showed it to be 4-chlorophenoxyacetic acid. The melting point (154° after three recrystallizations from benzene) was not altered on admixture with the authentic material.

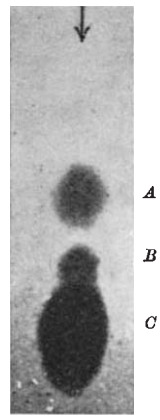

Fig. 1. *A*: γ-(3-chlorophenoxy)butyric acid; *B*: 3-chlorophenoxyacetic acid; *C*: intermediate

It seems likely that the intermediate formed from γ-(3-chlorophenoxy)butyric acid is also a β-hydroxybutyric acid. Its chromatographic behaviour resembles β-hydroxy-γ-(4-chlorophenoxy)butyric acid, which also has an R_F (0·42) lower than that of the corresponding substituted acetic acid (R_F 0·55) or substituted butyric acid (R_F 0·66).

It is established for animal tissues that one of the stages in the β-oxidation of fatty acids involves the formation of a β-hydroxy-acid derivative, although in fact such an intermediate has never been isolated[5]. The findings presented above provide direct evidence for the formation of such a compound during β-oxidation. Since no such compound was detected during the conversion of γ-(phenoxy)butyric acid to phenoxyacetic acid by the organism, it is inferred that the electronegative chlorine substituent on the ring in some way influences the β-oxidation of the fatty acid side-chain (cf. also ref. 4), presumably by weakening the attachment of the hydroxy acid to the enzyme complex. Further work is in progress on this aspect of β-oxidation and full details will be published in due course.

We are indebted to Prof. R. L. Wain, Wye College, University of London, for his encouragement during this work, and also for samples of 3- and 4-monochlorophenoxybutyric acids. We are also grateful

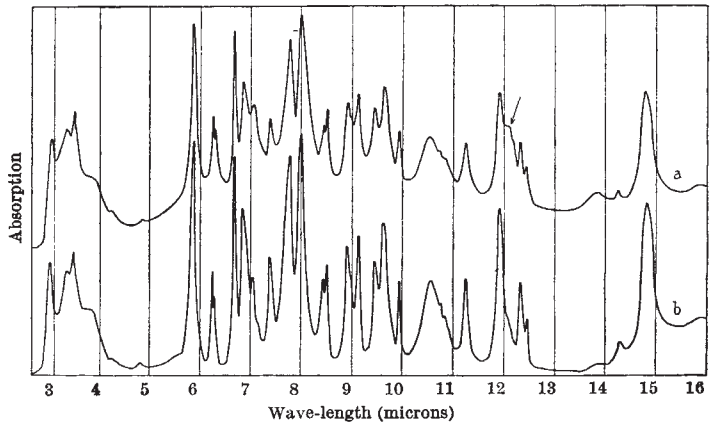

Fig. 2. Infra-red spectra of: (*a*) product from γ-(4-chlorophenoxy)butyric acid; (*b*) synthetic β-hydroxy-γ-(4-chlorophenoxy)butyric acid

to Dr. Heywood, of May and Baker, Ltd., for preparing and supplying a specimen of β-hydroxy-γ-(4-chlorophenoxy)butyric acid.

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Breakdown of Cellulose Dextrin and Gelatin in the Presence of Attapulgite

The attenuation in the breakdown of protein materials and soluble cellulose dextrin by micro-organisms with montmorillonite has been reported by Pinck *et al.*[1], Ensminger and Gieseking[2] and Lynch and Cotnoir[3]. This attenuation in the case of cellulose dextrin was attributed partially to the inactivation of the enzyme cellulase[3].

Another clay mineral, attapulgite (*p*H 8·5), intermediate in crystal structure between montmorillonite and kaolinite, has been studied using gelatin and cellulose dextrin (prepared from cellulose by degrading it with 72 per cent sulphuric acid)[4] as the substrates. Gelatin alone (100 mgm.) and gelatin plus 1 gm. of attapulgite were shaken with a phosphate buffer, *p*H 6·6, and inoculated with a soil suspension. The gelatin plus attapulgite medium evolved 4·15 m.equiv. of carbon dioxide while the gelatin alone evolved 4·03 m.equiv. of carbon dioxide. The attapulgite plus 100 mgm. of cellulose dextrin evolved 1·73 m.equiv. of carbon dioxide, while the cellulose dextrin alone evolved 2·60 m.equiv. of carbon dioxide (Table 1) after 8 days. These values have been corrected for evolution of carbon dioxide from the yeast extract control which in the presence and absence of clay gave a value of 1·82 millequivalents of carbon dioxide.

The activity of the enzyme, cellulase, was tested in an Ostwald viscometer at 25° C. using methyl cellulose (4,000 centipoise) as the substrate. 3·6 mgm. of the enzyme in 50 ml. of 0·05 *M* acetate buffer, *p*H 4·15, were shaken in the presence and absence of 100 mgm. of the clay minerals, attapulgite and montmorillonite, for 24 hr. The samples were centrifuged and 5 ml. of the supernatants were incubated for 1 hr. at 25° C. with 5 ml. of 0·5 per cent methyl cellulose. The time of efflux was then measured. It can be seen from Table 2 that, unlike the montmorillonite, the cellulase was not inactivated by the attapulgite.

Table 1. Amounts of Carbon Dioxide evolved by Soil Micro-organisms in the Presence and Absence of Attapulgite

Substrate	Amount added	Carbon dioxide (m.equiv.) corrected* for yeast extract		Protection (per cent)
		No clay	Clay	
Cellulose dextrin	(1) 100	2·66	1·80	
		2·61	1·72	
	(2) 100	2·57	1·63	
		2·51	1·79	
	(Average)	2·60	1·73	33·2
	S.D.	0·072	0·076	
Gelatin	100	4·21	4·16	
		3·85	4·14	
	(Average)	4·03	4·15	0·0

* The carbon dioxide evolved by the yeast extract control has been subtracted.

Table 2. Degradation of Methyl Cellulose (4,000 Centipoise) by Cellulase in the Presence and Absence of Clay Minerals as indicated by Efflux Time from Ostwald Viscometer

	Average time of efflux
Attapulgite	
Buffer + meth. cell.	4 min. 23 sec.
Buffer + meth. cell. + cellulase	1 min. 56 sec.
Buffer + meth. cell. + cellulase + clay	1 min. 57 sec.
Montmorillonite	
Buffer + meth. cell.	5 min. 47 sec.
Buffer + meth. cell. + cellulase	1 min. 46 sec.
Buffer + meth. cell. + cellulase + clay	2 min. 37 sec.

It is suggested, by analogy with results obtained with montmorillonite[3], that the cellulose dextrin, which exists in units of 20–30 anhydro-glucose molecules, can enter into the holes or spaces in the attapulgite lattice and is thereby protected from attack by the soil micro-organisms. The gelatin, because of its greater molecular size, is unable to do this.

Because of the prevalence of attapulgite in many soils this observation of the attenuation of breakdown of cellulose dextrin is perhaps significant in terms of soil organic matter relationships. To our knowledge, this is the first use of this soil clay mineral in this type of study.

The attapulgite, the cellulase and the methyl cellulose used in this work were commercial preparations from Ward's Natural Science Estab., Inc., the Bios and General Chemical Companies, and from Dow Chemical Co., respectively.

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Electrophoresis of Glucose Epimers

The movement of sugars in an electric field has already been used in certain analytical and preparative methods. This movement is based on the principle that borate ions form complexes with hydroxyl groups in sugars, so that these will move towards the anode in a borate buffer.

A method by which the epimerization of glucose, especially in biological material, could be estimated, would be of great interest. Therefore, electrophoresis was applied to solutions of α- and β-glucopyranose. The method used in our investigations was that of Consden and Stanier[1]. The mobilities for different sugars found by them are well reproducible.