The Biosynthesis of Polyacetylenes

2. ORIGIN OF THE CARBON ATOMS*

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The principal acetylenic metabolites of the basidiomycete B. 841 (probably a *Poria* sp.) are known to be the hydroxy acids nemotinic acid (I) and odyssic acid (II), together with derivatives such as the corresponding lactones (Bu'Lock, Jones & Leeming, 1955, 1957). Acetylenic compounds of various kinds are now known to be relatively common constituents of certain groups of plants and fungi, and it is natural that interest should be felt in problems of their origin and their relationship to more conventional metabolites. In such a connexion the polyacetylenes from B. 841 form an apt group for further study, since they are produced fairly rapidly and in good yields (up to 1 mg./ml.) from culture media of simple constitution and, as all the compounds have strong absorption maxima at substantially the same wavelengths, their total amount is readily determined. The structures of (I) and (II) are not symmetrical so that isotopic studies are not likely to be vitiated by spurious randomization of labelling, a situation which would prevent comparable studies in many other polyacetylene-producing fungi; moreover, as is shown in this paper, the molecules of (I) and (II) are derived entirely from a single metabolic intermediate, a consideration which is bound to simplify the relationship between their synthesis and other metabolic processes in the fungus.

The gross characteristics of the formation of polyacetylenes from glucose in B. 841 have already been established (Bu'Lock & Leadbeater, 1956). It was shown that they are probably formed from an intermediate in normal glucose breakdown, and that the extent to which metabolized glucose carbon was diverted into polyacetylene synthesis was diminished during active growth of the fungus and could be increased by omitting essential growth factors from the nutrient medium. The present work is concerned with the identification of the intermediate from which the carbon atoms of (I) and (II) are derived.

METHODS

Culture of the fungus. Methods of growing the fungus B. 841 have already been described (Bu'Lock & Leadbeater, 1956). Experiments were carried out with cultures

which had been supplied with the full growth medium for 6 weeks, followed by 4% glucose for 4 weeks, after which the medium was replaced with fresh 4% glucose containing the labelled substrate. The medium was drawn off for extraction after incubation for 7-9 days.

Extraction and purification of polyacetylenes. The culture medium was repeatedly extracted with ethyl acetate, and the polyacetylene content of the combined extracts estimated spectroscopically. The extract was then resolved by countercurrent distribution as described by Bu'Lock *et al.* (1955) and the nemotinic acid fractions were purified similarly. At the same time a more hydrophilic fraction was obtained, which contains (I) in bound form ('combined nemotinic acid') and this was purified by redistribution between ethyl acetate and water. With the radioactive polyacetylenes only those fractions showing a constant specific activity (per unit extraction) were combined, and the others discarded. The radioactive compounds were diluted, when necessary, with purified material.

Incorporation of acetate and succinate and oxidation of nemotinic acid to succinic acid

The labelled substrate (20 μ C; 0.3-0.8 mg. in 20 ml. of sterile water) was added to a single culture flask; on working up, about 20 mg. of purified nemotinic acid (I) was obtained in each case. The (I) from [1- 14 C]acetate was diluted with purified material, the degree of dilution being determined from the change in specific activity; the (I) from [1:4- 14 C]₂succinate was not diluted. The (I) was counted as an 'infinitely thin' film of polymer (up to 0.6 mg. on 2.5 cm.²) deposited on planchets by the evaporation of aqueous solutions under an infrared lamp, and the original acetate and succinate solutions were counted similarly. Weighings were carried out on a microbalance and it was found that for films of up to 1 mg. on 2.5 cm.² no self-absorption correction was necessary.

Oxidation. In a typical experiment, a solution of (I) (47 mg.) in water (25 ml.) was mixed with 85 ml. of permanganate-periodate reagent (Rudloff, 1956) and sufficient K₂CO₃ to bring the solution to pH 7.7. The mixture was shaken for 6 hr., the excess of iodine removed with sodium metabisulphite, and the solution acidified to pH 3.0 with aq. 10% H₂SO₄ and continuously extracted with ether for 12 hr. The ether extract was evaporated to dryness, the residue neutralized with Na₂CO₃, reacidified with dilute HCl and evaporated to dryness. The crude succinic acid so produced was converted into the *p*-phenylazo-anil as follows (Henbest & Owen, 1955): the crude acid was heated with acetic anhydride (1 ml.) for 30 min. at 100° and the mixture evaporated to dryness under reduced pressure. The residual succinic anhydride, purified when necessary by vacuum sublimation, was dissolved in CHCl₃ (0.5 ml.) and heated under reflux with a solution of chromatographically

* Part 1: Bu'Lock & Leadbeater (1956).

pure *p*-aminoazobenzene in CHCl_3 (1 ml.) for 30 min. The anilic acid which separated on cooling was heated with acetyl chloride (2 ml.) until it dissolved; the solution was then evaporated under reduced pressure and the crystalline residue dissolved in benzene and purified by chromatography on Al_2O_3 to give succino-*p*-phenylazo-anil, m.p. 218° (yield 12–20% from nemotinic acid). A weighing pipette was used to deposit known amounts of the anil from toluene solution as 'infinitely thin' films for counting.

Incorporation of [1- ^{14}C]acetate and complete degradation of nemotinic acid

Five B. 841 cultures were incubated for 9 days with a total of 80 μC (1–2 mg.) of [1- ^{14}C]acetate. The nemotinic acid was isolated as above, counted as a thin film and then hydrogenated in ethanol with a platinum catalyst. The solution was filtered and evaporated, leaving an oil which on distillation (180°/15 mm. Hg) gave γ -undecanolactone in about 80% yield. Similarly, the 'combined nemotinic acid' was hydrogenated and the crude product treated with boiling ethanolic NaOH (5% in 95% ethanol) for 30 min. The solution was acidified and extracted with ether, and the ether extract evaporated and distilled to give a further quantity of the γ -undecanolactone.

A small sample of labelled γ -undecanolactone (90 mg.) was converted into γ -oxoundecanamide (Bu'Lock *et al.* 1955) and the purified product (m.p. 130°) counted by a thick-film technique for comparison with the BaCO_3 from subsequent degradations.

Alkali fusion of γ -undecanolactone. In a typical procedure the lactone (0.4 g.) was fused with powdered KOH (2.5 g.) in a nickel crucible contained in a Pyrex vessel, through which N_2 could be passed and which is heated in a bath of Wood's metal. The temperature was raised to about 350° over 10 min.; it was found that at approx. 250° no fission occurred, and that if the reaction was carried out in air or on a larger scale the final yields were lower. When the emerging N_2 was passed through a cold trap, no major quantity of volatile product was retained. The fusion mixture was kept at 350° until evolution of gas ceased (20–30 min.), then cooled, dissolved in water, washed twice with ether, acidified and subjected to steam-distillation. The fore-run, containing a little acetic acid, was discarded, and titration of the rest of the steam-distillate showed fission to two acids in about 56% yield. The titrated solution was acidified and extracted with ether, and the ether extract evaporated into water (40 ml.); the aqueous solution was fractionated in a six-tube countercurrent system between water and *n*-hexane. Tubes 1 and 2 contained propionic acid (identified by paper chromatography and as the *p*-phenylphenacyl ester) in 55% yield, tubes 3 and 4 were discarded and tubes 5 and 6 contained octanoic acid, similarly identified, in 54% yield. The acids were recovered by acidification and ether extraction. Vapour-phase chromatography of the methyl ester prepared from the octanoic acid fraction showed the presence of small amounts of heptanoic acid and nonanoic acid, which could be removed by reversed-phase chromatography by the method of Silk & Hahn (1954).

Degradation of propionic acid. The propionic acid was oxidized to give BaCO_3 from $\text{C}_{(1)}$ of (I) and acetic acid (Nahinsky & Ruben, 1941), and the latter degraded to two samples of BaCO_3 from $\text{C}_{(2)}$ and $\text{C}_{(3)}$ of (I) by way of acetone (Popják, French, Hunter & Martin, 1951).

Degradation of octanoic acid. A sample of the octanoic acid was freed from homologues and decarboxylated by the Schmidt reaction (Blömstrand, 1954), giving BaCO_3 from $\text{C}_{(4)}$ of (I). The remainder was converted by the method of Hunter & Popják (1951) into hexanoic acid and acetic acid, which were separated from each other and from unchanged octanoic acid by countercurrent distribution between *n*-hexane and water-ethanol (50:50, v/v) (50 tubes). The acetic acid was degraded as described above, giving BaCO_3 from $\text{C}_{(4)}$ (discarded; see below) and $\text{C}_{(6)}$ of (I). The hexanoic acid was degraded in the same way as the octanoic acid, giving butyric acid and acetic acid (separated by countercurrent distribution between CHCl_3 and water); the acetic acid afforded BaCO_3 from $\text{C}_{(6)}$ and $\text{C}_{(7)}$ of (I). Schmidt degradation of part of the butyric acid afforded BaCO_3 from $\text{C}_{(8)}$ of (I). The remainder of the butyric acid was oxidized (as for propionic acid), giving a single sample of BaCO_3 from $(\text{C}_{(8)} + \text{C}_{(9)})$ of (I), the activity of $\text{C}_{(8)}$ being obtained by difference; the other product of this reaction was acetic acid, degraded as above to give BaCO_3 from $\text{C}_{(10)}$ and $\text{C}_{(11)}$ of (I).

The BaCO_3 samples from the above degradations were counted as thick films (25 mg. on 1 cm.²) in an end-window counter, for periods sufficient to give a counting error of 4% or less; the γ -oxoundecanamide was counted similarly. The actual counting rates were comparatively low (340 counts/min. for γ -oxoundecanamide, 54 counts/min. for BaCO_3 from $\text{C}_{(11)}$) so that no correction for counter-paralysis was made; the self-absorption of the γ -oxoundecanamide was assumed to be equivalent to that of BaCO_3 . Attempts at van Slyke-Folch oxidations of the saturated fatty acid derivatives gave BaCO_3 not properly representative of the samples.

RESULTS

Incorporation of [1- ^{14}C]acetate. The total incorporation of [1- ^{14}C]acetate into polyacetylenes from 4% glucose replacement cultures was always between 15 and 20% (assuming that all the spectroscopically determined polyacetylenes have the same activity as the purified nemotinic acid). This is considerably greater than the yield of polyacetylenes from glucose metabolized under similar conditions (on a carbon-content basis, about 4%).

The figures in Table 1 show the relative activities of the individual carbon atoms of (I), derived from [1- ^{14}C]acetate, and show that this activity is virtually confined to the odd-numbered atoms. Within the limits of our experimental errors these carbon atoms are uniformly labelled. We regard the apparent slight labelling of certain even-numbered carbon atoms as spurious for the following reasons. The alkali fusion of undecanolactone gives not only octanoic acid and propionic acid, but also small amounts of other acids. In determining the activity recorded in Table 1 for $\text{C}_{(4)}$, the octanoic acid was specially purified by chromatography before being subjected to decarboxylation, but for the other degradations we used acids which had not been thus purified. The presence of these

impurities in small amounts, decreasing with successive purification steps, accounts for the low activities recorded here for $C_{(3)}$ and $C_{(6)}$.

Incorporation of $[1\text{-}^{14}\text{C}]$ succinate. Table 2 shows the relative incorporations of $[1\text{-}^{14}\text{C}]$ acetate and $[1\text{-}^{14}\text{C}]$ succinate, both into the whole molecule of (I) and also into the group, $C_{(1)}\text{--}C_{(4)}$, isolated from (I) by oxidation to succinic acid. Though succinate is nearly as effective as acetate in increasing the yield of polyacetylenes in glucose refloods (unpublished observations), it is clear that radioactivity from the succinate carboxyl group is only incorporated into (I) to a very minor extent. The resultant activity in (I) is distributed between $C_{(1)}\text{--}C_{(4)}$ and the remainder of the molecule in exactly the same (1:2) ratio as the radioactivity from the acetate carboxyl group. Since the conversion of succinate into acetate via oxaloacetate and pyruvate leads to the loss of both the carboxyl-group carbon atoms, we conclude that in B. 841 there also exists a minor pathway of succinate breakdown in which at least one carboxyl-group carbon atom is transformed into the acetate carboxyl group.

DISCUSSION

The results set out in Table 1 strongly suggest that the whole molecule of nemotinic acid (I) is derived from acetic acid and that its 11 carbon atoms are

assembled by the head-to-tail linkage of six C_2 units. Though it was impracticable to carry out a similar degradation of the homologous odyssic acid (II), it seems probable that the terminal methyl group of (II) represents the twelfth carbon atom of these six C_2 units and that in the formation of (I) this atom is somehow eliminated.

(I) Nemotinic acid

(II) Odyssic acid

(III) Mycomycin

The formation of (I) and (II) thus presents close analogies to the biosynthesis of simple fatty acids, at any rate in the assembling of the carbon skeleton. Such a conclusion has seemed probable for some other naturally occurring acetylenic compounds on quite general grounds (e.g. in the various C_{18} straight-chain fatty acids with one or more triple bonds) and is probably of general validity. It follows that the enzymic reactions leading to the formation of triple bonds are such that in nature they are associated with the enzyme systems synthesizing straight-chain fatty acids, and that the acetylenic groups are formed, directly or indirectly, from the $-\text{CO}\cdot\text{CH}_2-$ groups produced in the repeated condensation of acetyl-coenzyme A. Further, when triple-bond-forming mechanisms are active it is apparent that they do not operate to the exclusion of the normal reactions of fatty acid synthesis, by which, for example, the greater part of the molecule of tariric (6-octadecynoic) acid is clearly formed. Bearing these points in mind some tentative conclusions about the biosynthesis of (I) and related compounds can be reached.

Though hydroxyacids are intermediates in fatty acid synthesis, the hydroxyl groups of (I) and (II) are not attached to originally oxygenated (i.e. carboxyl group-derived) carbon atoms, and are therefore secondary features, such as might well arise by the hydration of a double bond. The structure of mycomycin (III) (Celmer & Solomons, 1952) therefore presents a close analogy, since in this compound there are two double bonds in place of the one postulated in a precursor of (I); we

Table 1. *Incorporation of $[1\text{-}^{14}\text{C}]$ acetate*

Activities are expressed as percentage mean activity of BaCO_3 from 'active' C atoms, counted as 'infinitely thick' samples to within $\pm 4\%$.

Substance	Activity
γ -Oxoundecanamide (corrected to mol.wt. of BaCO_3)	630
BaCO_3 from	$C_{(1)}$ 100
	$C_{(3)}$ 5
	$C_{(3)}$ 96
	$C_{(4)}$ 1
	$C_{(5)}$ 103
	$C_{(6)}$ 8
	$C_{(7)}$ 102
	$C_{(8)}$ 0
	$C_{(9)}$ 103
	$C_{(10)}$ 0
	$C_{(11)}$ 96

Table 2. *Incorporation of acetate and succinate*

Activities are expressed as counts/min./mg., counted as 'infinitely thin' films to within the indicated limits, adjusted to the mol.wt. of nemotinic acid. In each experiment, $20\text{ }\mu\text{C}$ of labelled substrate was used.

Substrate	...	...	...	...	...	$[1\text{-}^{14}\text{C}]$ Acetate	$[1\text{-}^{14}\text{C}]$ Succinate
Total incorporation into polyacetylenes (%)						15	0.3
Activity of nemotinic acid (I)						342 ± 7	32 ± 4
Activity of succinic acid from (I), as <i>p</i> -phenylazoanil						111 ± 7	10 ± 1

would suggest that the hydroxylation step is an essential part of the processes leading to the formation of the diyne-allene group. Compared with (I), mycomycin contains an extra C_2 unit, and its double bonds are so situated that they might well result from $\alpha\beta$ - $\beta\gamma$ double-bond shifts of the kind catalysed by crotonase (cf. Lynen, 1954). Such features are not, of course, peculiar to polyacetylenes; a related interplay of reactions of hydration and dehydration is almost certainly involved in the biosynthesis of compounds like ricinoleic acid.

The presence of the allene groups in (I)–(III) raises the question whether these are normal intermediates in triple-bond formation. The naturally occurring allenes are very readily transformed into non-allenic acetylenes, but it is conceivable that the allenic groups might, at an earlier stage, arise by equally ready rearrangements of acetylenic groups, since the position of allene-acetylene equilibria is largely determined by the degree of conjugation with adjacent unsaturation. Consequently in (I)–(III) the allene groups may represent either incomplete conversion into, or secondary transformations of, acetylenic groups. Whatever the process by which $-CO\cdot CH_2-$ is transformed into $-C\equiv C-$, it probably involves 'activated' intermediates, since the reverse reaction is highly exothermic.

The relationship between the C_{11} compound (I) and its C_{13} homologue (II) requires some comment. In cultures of B. 841, (I) and its derivatives are greatly in excess of (II), yet it is implicit in our results that (I) is formed from a C_{12} compound containing a terminal methyl group, either (II) itself or one of its precursors. Several other polyacetylenes with odd numbers of carbon atoms are now known as fungal metabolites (personal communication from Professor E. R. H. Jones), and these, like (I)–(III), all contain a terminal ethynyl group $-C\equiv CH$. This suggests that the loss of the terminal methyl groups occurs after the introduction of unsaturation. The mechanism of the elimination seems likely to involve oxidation to a carboxyl group followed by loss of carbon dioxide; in this connexion the apparent interconvertibility, in

Polyporus anthracophilus, of compounds with terminal $-CH_3$, $-CH_2\cdot OH$ and $-CO_2H$ groups (Bu'Lock, Jones & Turner, 1957) is perhaps significant.

SUMMARY

1. Nemotinic acid incorporating radioactivity from $[1-^{14}C]$ acetate has been completely degraded to show the labelling pattern.

2. Nemotinic acid, with 11 carbon atoms, is formed from six molecules of an acetic acid derivative, linked head to tail, with elimination of the terminal methyl group, and odyssic acid is probably formed similarly without demethylation.

3. The natural acetylenes are formed within the context of the reactions of fatty acid synthesis, the triple bonds arising directly or indirectly from $-CO\cdot CH_2-$ groups. With the addition of the triple-bond-forming mechanism and the demethylation reaction, the reactions of fatty acid synthesis will account for most features of the structure of naturally occurring acetylene derivatives.

REFERENCES

- Blömstrand, R. (1954). *Acta chem. scand.* **8**, 1487.
Bu'Lock, J. D., Jones, E. R. H. & Leeming, P. R. (1955). *J. chem. Soc.* p. 4270.
Bu'Lock, J. D., Jones, E. R. H. & Leeming, P. R. (1957). *J. chem. Soc.* p. 1097.
Bu'Lock, J. D., Jones, E. R. H. & Turner, W. B. (1957). *J. chem. Soc.* p. 1607.
Bu'Lock, J. D. & Leadbeater, E. F. (1956). *Biochem. J.* **62**, 476.
Celmer, W. D. & Solomons, I. A. (1952). *J. Amer. chem. Soc.* **74**, 1870, 2245.
Henbest, H. B. & Owen, T. C. (1955). *J. chem. Soc.* p. 2968.
Hunter, G. D. & Popják, G. (1951). *Biochem. J.* **50**, 163.
Lynen, F. (1954). *Nature, Lond.*, **174**, 962.
Nahinsky, P. & Ruben, S. (1941). *J. Amer. chem. Soc.* **63**, 2275.
Popják, G., French, T. H., Hunter, G. D. & Martin, A. J. P. (1951). *Biochem. J.* **48**, 612.
Rudloff, E. von, (1956). *Canad. J. Chem.* **34**, 1413.
Silk, M. H. & Hahn, H. H. (1954). *Biochem. J.* **56**, 406.