Sex Hormones in Mucorales. The Incorporation of C_{20} and C_{18} Precursors into Trisporic Acids

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(Received 24 July 1973)

SUMMARY

^{14}C -labelled retinol, β - C_{18} -ketone* and 4-hydroxy- β - C_{18} -ketone fed to mixed *plus* and *minus* *Blakeslea trispora* cultures were efficiently incorporated into trisporic acids. This allowed the biosynthetic route from β -carotene to these hormones to be set out, leaving some uncertainties as to the sequence of later steps in the process.

INTRODUCTION

Heterothallic Mucorales display a very simple form of sexuality in which the two mating-types, *plus* and *minus*, are primarily distinguished by the fact that both are required for the collaborative formation of a group of hormone substances, the trisporic acids (reviews: Gooday, 1973; van den Ende & Stegwee, 1971); these acids (structures in Fig. 1) elicit and control further stages of the sexual process in both mating types. Knowledge of the mechanism whereby trisporic acids are formed is fundamental for a biochemical understanding of the sexual phenomena.

Suitable *plus* and *minus* strains of *Blakeslea trispora* produce considerable amounts of trisporic acids when grown in mixed ('mated') culture (Upjohn Co., Kalamazoo, Michigan, U.S.A. 1966, Netherlands Patent no. 6512313; Bu'Lock & Winstanley, 1971) and provide the preferred system for biosynthetic studies. When separately grown *plus* and *minus* cultures are mixed, both strains contribute endogenous precursor for trisporic acid formation (van den Ende, Werkman & van den Briel, 1972). This precursor is probably β -carotene, since (i) *Phycomyces blakesleeanus* mutants blocked in β -carotene synthesis are also blocked in trisporic acid production (Bergman *et al.* 1969; R. P. Sutter, personal communication); (ii) in mated *B. trispora*, inhibition of β -carotene synthesis by diphenylamine prevents trisporic acid production (Austin, Bu'Lock & Winstanley, 1969); and (iii) both [G - ^{14}C] β -carotene and [$11, 12$ - 3H_2]retinyl acetate, added to mated *B. trispora* cultures, contribute significant labelling to trisporic acids (Austin, Bu'Lock & Drake, 1970). Structurally, the hormones are modified 13-*apo*- β -carotene derivatives, and their formation by oxidative transformations of β -carotene is chemically feasible. In this paper we present evidence for the utilization of C_{20} and C_{18} intermediates in such a pathway.

* Systematic name 3-methyl-1-(2,6,6-trimethylcyclohexenyl)octa-1,3,5-trien-7-one; the trivial name allows the use of the carotenoid numbering as for the trisporic acids (see Fig. 1).

METHODS

Culture procedures and recovery of trisporic acids. Culture methods for *plus* and *minus* *Blakeslea trispora* Thaxter, and procedures for the recovery, assay and purification of the trisporic acids, have been described in full (Bu'Lock & Winstanley, 1971; Bu'Lock, Drake & Winstanley, 1972). Mixed *plus* and *minus* *B. trispora* inocula were grown on the glucose-asparagine medium and put into 150 ml of 5 % malt extract medium in 500 ml shake flasks.

Labelled precursors. Syntheses of $[10-^{14}\text{C}]\beta\text{-C}_{18}$ ketone* (structure, Fig. 1) and its conversion into methyl $[10-^{14}\text{C}]\text{retinoate}$ are described elsewhere (Bu'Lock, Quarrie & Taylor, 1973). $[10-^{14}\text{C}]\text{Retinol}$ was obtained by LiAlH_4 reduction of the methyl ester as required. Oxidation of the enol acetate of the $\beta\text{-C}_{18}$ -ketone with $\text{Pb}(\text{OAc})_4$ and subsequent hydrolysis gave the racemic 4-acetoxy- and 4-hydroxy-derivatives respectively. Counting procedures were as previously described (Bu'Lock *et al.* 1972).

Incorporation experiments. The labelled precursors were taken up from acetone solution into water containing 0.3 % of Tween-80, and from 1 to 2 mg of the precursor were dispensed in 4.0 ml of this suspension to each culture flask. This quantity of detergent had no effect on trisporic acid synthesis. Labelled precursors were added after 32 to 40 h growth (when the cultures had attained mycelial dry weights of approx. 0.5 g/flask) and the cultures processed 2 h or more after the additions (see Tables 1 to 3). 'Total acids' were measured spectrophotometrically in the bicarbonate-soluble fraction of ether extracts from the medium and were converted with diazomethane into methyl esters for thin-layer chromatography (Bu'Lock & Winstanley, 1971). 'Total neutrals' were estimated in the residual ether phase, nominally as trisporol-C, and subjected to thin-layer chromatography directly.

RESULTS

The incorporation of the $[10-^{14}\text{C}]$ substrates retinol, $\beta\text{-C}_{18}$ -ketone, 4-acetoxy- $\beta\text{-C}_{18}$ -ketone and 4-hydroxy- $\beta\text{-C}_{18}$ -ketone into neutral metabolites and trisporic acids by mated *Blakeslea trispora* was studied in three separate experiments with the results which are set out in Tables 1, 2 and 3. In all instances the cultures produced 0.2 to 0.5 mg of trisporic acids/h during the incubation period, and for the samples from which the methyl trisporates were fully purified the yield of acids was 4 to 7 times the amount of labelled substrate. None of the substrates was particularly stable under the incubation conditions and our observation that no unchanged substrate could be recovered from any of the incubations is probably not significant; low total recoveries are ascribed partly to polymer formation (on the flask walls) and partly to degradation to $^{14}\text{CO}_2$. Similarly, some of the incorporation into 'neutral metabolites' is probably due to artefacts in the culture medium, but the recovery of highly labelled trisporol-C from this fraction (Table 2) shows that much of this incorporation into neutral substances is nevertheless significant. This was confirmed by the distribution of radioactivity on thin-layer chromatograms of the neutral products from all the incubations, which in each instance showed specific incorporation into all the normal components of this rather complex mixture of metabolites. The acid fraction was relatively simpler and in all cases over 90 % of the radioactivity was located in trisporic acids B and C.

The dilutions of specific activity observed are such that direct precursor relationships can legitimately be inferred: for methyl trisporate C, about $\times 27$ from retinol (8 h), $\times 10$ from $\beta\text{-C}_{18}$ -ketone (16 h), and $\times 7$ from the racemic 4-acetoxy- $\beta\text{-C}_{18}$ -ketone (2 h). The latter acetate clearly hydrolyses rapidly but it is not possible to say whether one or both stereoisomers is utilized.

* See footnote on previous page.

Table 1. *Incorporation of [10-¹⁴C]retinol by mixed cultures of plus and minus Blakeslea trispora*

[10-¹⁴C]Retinol (approx. 2 mg; 8.57×10^5 d.p.m.) was added to each 150 ml culture after 32 h growth. At the stated times, the mycelium was filtered off and the neutral and acid metabolites recovered from the medium. Methyl trisporates B and C (7.2 mg total) were fully purified from the 8 h incubation for determination of their specific activity.

Incubation time (h)	8	16	32	64
¹⁴ C recovered (%):				
In mycelium	14	9	8	2
In neutrals	39	32	32	26
In methyl trisporates	18	15	15	14
Specific activity (d.p.m./mmol) of:				
[10- ¹⁴ C]retinol	1.29 × 10 ⁸ (initial)			
Me trisporate B	3.76 × 10 ⁶ (at 8 h)			
Me trisporate C	4.80 × 10 ⁶ (at 8 h)			

Table 2. *Incorporation of [10-¹⁴C]β-C₁₈-ketone by mixed cultures of plus and minus Blakeslea trispora*

[10-¹⁴C]β-C₁₈-ketone (approx. 1 mg; 3.29×10^5 d.p.m.) was added to each 150 ml culture after 31 h growth. At the stated times, the mycelium was filtered off and the neutral and acid metabolites recovered from the medium. Methyl trisporates B and C (total, 6.8 mg) and trisporol-C were fully purified from the 16 h incubation for determination of their specific activity.

Incubation time (h)	16	28	48
¹⁴ C recovered (%):			
In mycelium	< 2	< 2	< 2
In neutrals	25	20	17
In trisporic acids	38	38	36
Specific activity (d.p.m./mmol) of:			
[10- ¹⁴ C]β-C ₁₈ ketone	7.90 × 10 ⁷ (initial)		
Me trisporate B	8.50 × 10 ⁶ (at 16 h)		
Me trisporate C	7.70 × 10 ⁶ (at 16 h)		
Trisporol-C	1.78 × 10 ⁷ (at 16 h)		

Table 3. *Incorporation of [10-¹⁴C]-4-acetoxy- and 4-hydroxy-β-C₁₈-ketone by mixed cultures of plus and minus Blakeslea trispora*

The labelled precursor (1.2 mg; 6.8×10^5 d.p.m.) was added to each 150 ml culture after 40 h growth. At the stated times the neutral and acid metabolites were recovered from the medium; methyl trisporate C (total with Me trisporate B, 6.5 mg) was fully purified from the 2 h incubation with the 4-acetoxy-compound for measurement of its specific activity.

From [10- ¹⁴ C]4-hydroxy-β-C ₁₈ -ketone			
Incubation time (h)	8		
¹⁴ C recovered (%):			
In neutrals	29		
In acids	33		
From [10- ¹⁴ C]4-acetoxy-β-C ₁₈ -ketone			
Incubation time (h)	2		8
¹⁴ C recovered (%):			
In neutrals	51		32
In acids	29		31
Specific activity (d.p.m./mmol) of:			
Precursor	1.78 × 10 ⁸ (initial)		
Me trisporate C	2.61 × 10 ⁷ (at 2 h)

Fig. 1. Biosynthesis of trisporic acids in mixed *plus* and *minus* *Blakeslea trispora*. The conventional 15,15'-cleavage of β -carotene would give retinal but the precise C_{20} intermediate is not identified from the tracer data for retinol.

DISCUSSION

The present results, together with the data already noted and with some important reservations, suggest a pathway for trisporic acid formation in mated *Blakeslea trispora* cultures (Fig. 1). Uncertainties attach to the sequence of steps which must intervene between the $\beta\text{-}C_{18}\text{-ketone}$ and the trisporols. Chemically it is clear that these must comprise (i) the insertion of oxygen at C-4, ultimately to give a carbonyl group but initially (as confirmed by the utilization of the 4-hydroxy- $\beta\text{-}C_{18}\text{-ketone}$) as hydroxyl; (ii) hydroxylation at C-1a to give the hydroxymethyl group of the trisporols and thence (Bu'Lock *et al.* 1972) the carboxyl group of the acids; and (iii) hydrogen transfers, either by redox processes or by prototropic rearrangements, to generate the saturated portion of the trisporic acid side-chain at C-11/C-12. There is indirect evidence for the participation of the requisite type of oxygenase reactions in this sequence (Bu'Lock & Winstanley, 1971) but no direct data as to the order in which these steps occur in the mated system. There is some evidence from the structures

of minor (biologically inactive) co-metabolites in the mated system, which are formed by reactions in the side-chain involving oxygenation at C-11 followed by cleavage to give C₁₅ products. The initial step in this side-sequence can occur with trisporic acids (Bu'Lock *et al.* 1972), but among the neutral C₁₅ metabolites formed in this way are 4-keto-compounds with a hydroxymethyl group on C-1, analogous to the C₁₈ trisporols (e.g. trisporone: Cainelli *et al.* 1967) and others with an intact methyl group in this position (unpublished observations). This suggests that in the 'mated' system introduction of the 4-keto-group precedes oxidation of the 1-methyl.

However, an attempt to define a unique sequence of reaction steps for trisporic acid biosynthesis may be misleading. The necessary transformations occur at different sites in the C₁₈ skeleton and the corresponding enzymes are unlikely to have absolute substrate-specificities throughout. In 'mated' cultures where the synthesis of these enzymes is fully derepressed, the sequence of reactions will in effect be determined by the pattern of their substrate affinities and a virtually unique sequence may exist. However, the situation is intrinsically different in single *plus* or *minus* cultures in which the enzymes of the trisporate pathway are strongly, but incompletely, repressed. Under these circumstances possible reactions are enzyme-limited and any sequence will depend upon the relative amounts of the enzymes present. From preliminary investigations of the metabolites of single strains we conclude that the pattern of repressed-level enzymes seems to differ systematically between *plus* and *minus* strains. This would be expected if the gene product from the two-allele mating-type locus is a regulator for the enzymes of hormone synthesis (Bu'Lock, 1973). Among the single-strain metabolites related to trisporic acids the substances which initiate collaborative hormone synthesis in mixed cultures are to be found (Bu'Lock & Winstanley, 1971; Werkman & van den Ende, 1973; Sutter, Capage, Harrison & Keen, 1973). This means that there are systematic differences between the minor neutral metabolites of *plus* and *minus* strains; this we can confirm, but both produce extremely complicated mixtures. Nevertheless, our preliminary results support the view that the unresolved ambiguity of sequence in the 'mated' system is functionally significant in the repressed-level metabolism of the two strains growing singly (Bu'Lock, Jones, Quarrie & Winskill, 1973).

We thank the Science Research Council for their support of this work, and Drs Gooday, Sutter and van den Ende for helpful discussions.

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