Synthesis of Lipids from [2-¹⁴C]Acetate and [¹⁴C]Glucose by *Trichoderma viride*

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(Received 2 November 1960)

The main purpose of this work was to investigate further the synthesis of unsaturated fatty acids, particularly linoleic acid and linolenic acid, by plant tissue. The fungus *Trichoderma viride* was selected as a suitable subject and a study was made of the fate of labelled carbon supplied to it as [2-¹⁴C]acetate or [¹⁴C]glucose (generally labelled). In other organisms the synthesis of saturated fatty acids by repeated condensation of C₂ units is now well established (Bloch, 1948; Popják, French, Hunter & Martin, 1951), but, although there is evidence for the formation of oleic acid from stearic acid by dehydrogenation in animal tissues (Stetten & Schoenheimer, 1940; Schoenheimer & Rittenberg, 1936; Bernhard, Rothlin & Wagner, 1958), there is much less information about the synthesis of unsaturated fatty acids, particularly in plant tissue. Hilditch (1956) discussed two possible routes of synthesis. The first was a desaturation process starting from stearic acid and proceeding by way of oleic acid and linoleic acid to linolenic acid. The second involved a hydrogenation sequence operating in the reverse direction. Our conclusions, which favour the formation of linoleic acid and linolenic acid by the former process, have been summarized elsewhere (Crombie & Ballance, 1959).

METHODS

***Trichoderma viride* cultures.** Spores were cultivated on agar slopes of a 2% glucose-acetate medium containing (g./l.): NH₄NO₃, 5.00; KH₂PO₄, 3.50; MgSO₄·7H₂O, 2.50; FeCl₃, 0.08; ZnSO₄, 0.025; agar, 35.00; D(±)-glucose (anhydrous), 20.00; acetic acid, 5.00 (pH adjusted to 6.8 with aq. 50%, w/v, NaOH). The agar suspension was steamed (1 hr.), tubed, autoclaved (10 lb./in.² for 15 min.), cooled and each tube inoculated with concentrated spore suspension. Incubation at 20° for 10–12 days in continuous light gave rise to an even layer of spores. The spore suspension (10 ml. of sterile water) from each slope was used to inoculate five conical flasks (350 ml.), each containing 50 ml. of the above-mentioned glucose-acetate medium: these experimental mycelium cultures were agitated in the absence of light at 25° on a flask shaker (Ballance, 1961).

Feeding with radioactive compounds. The specific activities of the radioactive acetate and glucose stock solutions (0.1 mc/100 ml. of methanol or water respectively) were

determined with samples (100 μl.), which were diluted with 0.5 g. of sodium acetate or 0.25 g. of glucose and then diluted to 10 ml. with water. Samples (100 μl.) were withdrawn, evaporated on 25 mm. diameter nickel planchets and counted with a gas-flow proportional counter.

After 2 or 4 days' growth on the shaker, experimental mycelium from 20 flasks was suspended in 800 ml. of medium of similar mineral salt concentration but containing, for labelled acetate experiments, 10 g. of glucose/l. and 0.25 g. of sodium acetate/l., and for labelled glucose experiments 5 g. of glucose/l. and 0.125 g. of sodium acetate/l. After equilibration (4 hr.) in the new medium, the radioactive substrate was added. Later the mycelium was harvested, killed in boiling ethanol and the lipid was extracted. The mycelium was aerated with CO₂-free air throughout the equilibration and radioactive-feeding periods. In another series of experiments, after the radioactive-feeding period mycelium was grown for a further time in 500–800 ml. of non-radioactive 2% glucose-acetate medium before being killed and analysed.

Lipid analysis. Killed mycelium was homogenized in ethanol, the homogenate filtered and the residue washed alternately with ethanol and diethyl ether (final mixture 1:2, v/v). The solvents were evaporated and the total lipid was taken up in light petroleum (b.p. 40–60°).

With ice-cold acetone the lipid was separated into acetone-insoluble (crude phospholipid) and acetone-soluble (crude triglyceride and free fatty acid) fractions. The latter were removed with dilute Na₂CO₃ solution and regenerated with HCl. The quality of the acetone fractionation was checked by phosphorus estimations, small samples being saponified, ignited and determined colorimetrically (Hardman & Crombie, 1958). Samples (1–2 mg.) of each lipid fraction were transferred in CHCl₃ to nickel planchets and counted (no self-absorption correction was made).

The crude triglycerides and phospholipids were saponified (Crombie & Comber, 1956) and the fatty acids isolated. Reversed-phase column chromatography (Crombie, Comber & Boatman, 1955) was used for both fatty acid-mixture analysis and for the isolation of individual acids. As each acid left the column the solution was made alkaline (NaOH), the acetone was removed by evaporation and traces of liquid paraffin were extracted with diethyl ether. The solution was acidified, the fatty acids were taken up in light petroleum, washed, dried (Na₂SO₄) and evaporated, and a sample (1 mg.) was counted for radioactivity. Since oleic acid and palmitic acid were not separated by the reversed-phase column, the figures for these acids were obtained by a second analysis after degradation of the unsaturated acids (including oleic acid) to short-chain-length acids by oxidation with alkaline permanganate (Crombie *et al.* 1955). In addition a sample of each fatty acid mixture was catalyti-

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cally hydrogenated and then chromatographed: the combination of the three chromatographic procedures allows a complete fatty acid analysis to be made.

For samples too small for column chromatography, and for rapid checking of column data, analyses were carried out by the paper-chromatographic technique of Ballance & Crombie (1958): radioautographs were prepared by direct contact with Kodirex X-ray film.

RESULTS

Composition of Trichoderma viride lipid. Growth of the fungus under a variety of conditions did not markedly alter the proportion of the lipid fractions (Table 1) or their fatty acid composition. Typical fatty acid analyses are shown in Table 2.

Gross incorporation of ^{14}C into the major lipid fractions. Mycelium (4 days old) from 21 flasks of 2% glucose-acetate culture medium was added to the acetate-feeding medium (750 ml.) and equilibrated for 4 hr. Sodium [$2\text{-}^{14}\text{C}$]acetate (20 ml. of stock soln., 12.7×10^6 counts/min.) in sterile water was added, and, after 1 hr., the mycelium was filtered off and analysed. Identical runs, which were set off at the same time, were harvested after 3 and 5 hr. The incorporation of ^{14}C into the crude phospholipid, crude triglyceride and free fatty acid fractions (Fig. 1) gave no indication that one of the three fractions served as a precursor for either of the others.

Incorporation of ^{14}C into the individual fatty acids of the major lipid fractions. The individual fatty acids from the phospholipid and triglyceride fractions of the above-described experiments were isolated by column chromatography and their specific activities were determined (Table 2). In both fractions the ^{14}C rapidly accumulated in palmitic acid, oleic acid and stearic acid, particularly in palmitic acid. Accumulation in linoleic acid

is considerably slower than in oleic acid, but faster than in linolenic acid. The three corresponding samples of free fatty acids were too small for column-chromatographic analysis, but on radioautographs most of the blackening appeared at the combined oleic acid-palmitic acid spot. In further

Fig. 1. Incorporation of radioactive acetate into the major components of *Trichoderma* lipid. □, Phospholipid; Δ, triglyceride; ■, free fatty acids; ●, acetone-soluble non-saponifiable material; ○, acetone-insoluble non-saponifiable material.

Table 1. *Major lipid components of Trichoderma viride grown under different conditions*

Experimental conditions	Age (days)	Dry wt. (g./flask or tube)	Lipid (g./100 g. dry wt.)	Acetone-insoluble phospholipid* (g./100 g. of lipid)	Acetone-soluble triglyceride† (g./100 g. of lipid)	Free fatty acids (g./100 g. of lipid)	Non-saponifiable (g./100 g. of lipid)
Spores (20% glucose-agar slopes)	12	0.017	9.6	21 (82)	64.3 (4.0)	14.5	6.2
Mycelium (aerated 20% glucose soln.)	7	0.43	4.8	61.6 (79)	37.4 (6.0)	1.0	14.0
Mycelium (non-aerated 20% glucose soln.)	14	0.22	5.4	54.9 (99)	35.5 (1.0)	9.6	12.0
Mycelium (7 days in aerated 20% glucose soln., then 7 days in aerated water)	14	0.48	5.3	40.7 (88)	54.3 (5)	5.0	6.3
Mycelium (aerated 2% glucose-acetate soln.)	4	0.44	7.6	45.7 (79)	52.7 (8)	1.6	0.6

* Percentage purity (given in parentheses) was obtained from 3.8 μg. of P≡100 μg. of phospholipid.

† Percentage phospholipid contaminant was obtained from phosphorus determinations as described above.

Table 2. *Distribution of ^{14}C among fatty acids synthesized from $[2-^{14}\text{C}]\text{acetate}$*

Fatty acid* (mg./100 mg.)	Specific activities† (counts/min./mg.)		
	1 hr.	3 hr.	5 hr.
Phospholipid fraction			
Linolenic 2.8	25	83	116
Linoleic 53.9	36	153	254
Oleic 20.4	219	971	1519
Palmitic 19.8	485	1409	2463
Stearic 1.8	74	743	1978
Behenic 1.4	89	100	422
Triglyceride fraction			
Linolenic 4.6	27	67	21
Linoleic 38.6	28	99	197
Oleic 32.7	—	272	631
Palmitic 13.9	114	912	561
Stearic 8.7	119	252	292
Behenic 1.5	102	20	29

* Composition of the fatty acid mixtures remained steady throughout the 5 hr. period.

† Fatty acids obtained from lipid fractions shown in Fig. 1.

Table 3. *Effect of varying substrate and age of culture on the distribution of ^{14}C among the total synthesized fatty acids*

Fatty acids	$[^{14}\text{C}]\text{Glucose}$ specific activity (counts/ min./mg.)	2-day Culture: fatty acid composition (mg./100 mg.)	$[2-^{14}\text{C}]\text{Acetate}$: specific activity (counts/min./mg.)	2-day Culture: fatty acid composition (mg./100 mg.)	$[2-^{14}\text{C}]\text{Acetate}$: specific activity (counts/ min./mg.)	4-day Culture: fatty acid composition (mg./100 mg.)
Linolenic	65	2.2	434	2.2	48	5.0
Linoleic	53	42.3	1522	42.3	149	44.0
Oleic	329	29.0	5806	29.0	701	24.0
Palmitic	508	20.9	9628	20.9	1129	21.3
Stearic	367	5.4	2570	5.4	940	5.6
Total activity supplied (counts/min.)	54.3×10^6		17.4×10^6		14.5×10^6	

work the separation into phospholipid, triglyceride and free fatty acids was not carried out, but the component fatty acids were examined as a whole. Table 3 compares the use of $[^{14}\text{C}]\text{glucose}$ and $[2-^{14}\text{C}]\text{-acetate}$ as substrates for a 2-day-old culture and the effect of $[2-^{14}\text{C}]\text{acetate}$ feeding to a 4-day-old culture. The pattern of incorporation is similar to that in Table 2. Tables 2 and 3 emphasize that the extent of labelling does not correspond with quantity of the acid that occurs. For instance, linoleic acid, which represents 40–50 % of each fatty acid mixture, accumulates only 8–20 % of the radioactivity incorporated into the whole mixture.

A small acid peak that emerged from the reversed-phase columns ahead of linolenic acid (the first to emerge) had low specific activity. It is thought to represent the oxidation product of one or more of the unsaturated acids [cf. Schlenk, Gellerman, Tillotson & Mangold (1957), who noted radioautographic peaks which accumulated at the solvent front during study of the auto-oxidative breakdown of radioactive methyl esters of oleic acid, linoleic acid and linolenic acid].

Table 4. *Specific activity (counts/min./mg.) of fatty acids after growth of mycelium (for 3 hr.) in radioactive acetate medium and continued growth (to 20 hr.) in non-radioactive medium (4-day-old mycelium)*

Fatty acids	Times of sampling (hr.)			
	3*	7	15	20*
Total fatty acids	630	689	555	518
Linolenic	48	33	76	95
Linoleic	149	146	277	213
Oleic + palmitic	902	704	716	585

* Percentage composition (g./100 g. of fatty acid) at 3 hr. (and at 20 hr., in parentheses): oleic acid + palmitic acid, 47.1 (43.2); linoleic acid, 40.5 (42.5); linolenic acid, 4.5 (4.4).

Consequences of transferring mycelium from ^{14}C -labelled to unlabelled substrate. Mycelium (4 days old; 270 g. fresh wt.) was equilibrated (4 hr.) with acetate-feeding medium (2.4 l.); sodium $[2-^{14}\text{C}]\text{-acetate}$ (43.4×10^6 counts/min.) was added and metabolism allowed to continue for 3 hr. The mycelium was filtered, thoroughly washed and transferred to non-radioactive 2 % glucose-acetate medium. Mycelium was harvested in 45 g. portions

3, 7, 15 and 20 hr. from the introduction of the labelled acetate. The total fatty acids were isolated from each portion and, as is shown in Table 4, there was first a rise and then a slight decline in activity consequent on transfer to the non-radioactive medium. Examination of the specific activities of the individual fatty acids in Table 4 reveals again this rise and slight fall in activity. Thus first oleic acid and palmitic acid show a decline in activity, linoleic acid shows a rise and decline and linolenic acid still shows a rise in activity after 20 hr. It may be inferred at this stage that oleic acid and palmitic acid (formed before the others) might be undergoing conversion first into linoleic acid then into linolenic acid. It is difficult to explain the fact that at 7 hr. the specific activity of the total fatty acids had risen, whereas all the values of the individual acids (except stearic acid and behenic acid, whose specific activities were not determined in this experiment) had fallen, compared with their 3 hr. values; however, these changes in the labelling of the acids (Table 4) are not great and are therefore more subject to experimental error than are the changes (in the labelling pattern) which were much more forcibly demonstrated with the more actively metabolizing 2-day-old mycelium.

Twenty-four 2-day-old mycelial mats were equilibrated (4 hr.) in aerated acetate-feeding medium (600 ml.). Sodium [^{14}C]acetate (17.36×10^6 counts/min.) was added and growth allowed to proceed for 3 hr. The mycelium was harvested; the total fatty acids were extracted and analysed. A second sample of 24 mycelial mats was treated in exactly the same manner, but, instead of being harvested, was thoroughly washed and growth was continued in non-radioactive 2% glucose-acetate medium for 2 days more, before harvesting and analysis occurred. The results from both these experiments have been compared in Table 5.

Total radioactivity in the fatty acids at the end of the [^{14}C]acetate feeding (Table 5) was 43×10^4 counts/min., but, after continued growth in non-

radioactive medium, increased to 71×10^4 counts/min. When 4-day-old mycelium was transferred from active to non-active culture conditions, a slight rise in activity and then a decline took place; here the increase in activity is marked and no decline has occurred. The more marked increase in activity is thought to result from the high speed of metabolism of the 2-day-old mycelium, coupled with the presence of a large fatty acid-precursor pool within the organism. Simmons & Quackenbush (1954) encountered a similar effect in work on soya bean, and offered the same explanation.

The increase in activity, however, does not obscure the substantial movement of the ^{14}C label into linoleic acid and the decline in label present in palmitic acid. During this change in the labelling pattern the percentage of linoleic acid by weight has remained stationary, but palmitic acid has declined and oleic acid has risen. These results were confirmed by paper chromatography and the preparation of radioautographs. During the period between 2 and 4 days of growth, considerable fatty acid synthesis took place; the average weight of the total fatty acids from 24 mycelial mats was 253 mg. at 2 days, and had increased to 418 mg. at the end of 4 days.

The position with respect to feeding with generally labelled [^{14}C]glucose is similar to that found for [^{14}C]acetate. Labelled glucose (54×10^6 counts/min.) was introduced to 2-day-old mycelium (from 24 flasks) contained in glucose-feeding medium (400 ml.) and growth allowed to continue for 3 hr. Three-quarters of the mycelium was killed and analysed. The remainder was returned after washing to non-radioactive 2% glucose-acetate-feeding medium for a further 2 days and then harvested and analysed. After growth for 3 hr. on radioactive glucose 10 times as much of the incorporated radioactivity had appeared in the oleic acid and palmitic acid as had entered linoleic acid. On further growth for 2 days, the incorporation of ^{14}C into linoleic acid is greatly increased, whereas that of the oleic acid and palmitic acid fraction altered only a little

Table 5. *Distribution of ^{14}C in fatty acids from 2-day-old mycelium grown (3 hr.) in a radioactive acetate medium and then allowed to grow for 2 days in non-radioactive medium*

Fatty acid	Before continued growth			After continued growth		
	Composition (mg./100 mg.)	$10^3 \times$ Specific activity (counts/min./mg.)	$10^3 \times$ Total activity (counts/min.)	Composition (mg./100 mg.)	$10^3 \times$ Specific activity (counts/min./mg.)	$10^3 \times$ Total activity (counts/min.)
Linolenic	2.2	4.3	9.5	0.7	23.1	17.1
Linoleic	40.4	15.2	614.7	39.9	59.4	2370.9
Oleic	27.7	58.1	1608.3	38.2	84.4	3224.5
Palmitic	20.0	96.3	1921.8	13.9	96.1	1336.1
Stearic	5.2	25.7	133.4	5.5	29.2	160.0
Arachidic*	1.0	8.9	9.3	0.7	9.7	6.4
Behenic	3.5	4.0	14.1	1.2	4.9	5.9

* Formation of arachidic acid seems to be associated with high acetate concentration and conditions of good aeration.

Table 6. *Distribution of ^{14}C in fatty acids from 2-day-old mycelium grown (3 hr.) in the presence of [^{14}C]glucose and transferred for a further 2 days to a non-radioactive medium*

Fatty acids	Before transfer		After transfer	
	Composition (mg./100 mg.)	$10^3 \times$ Total activity (counts/min.)	Composition (mg./100 mg.)	$10^3 \times$ Total activity (counts/min.)
Linolenic	2.1	1.4	0.7	1.0
Linoleic	40.4	21.4	40.4	160.4
Oleic + palmitic	47.3	192.3	52.0	174.7

(Table 6). The composition (mg./100 mg.) of the fatty acid mixture did not change significantly during the experiment.

DISCUSSION

In considering the synthesis of the major fractions of *Trichoderma* lipid, it seems from the specific activities of the fractions (Fig. 1) that deacylation of the fatty acyl-coenzyme A to form free fatty acids is not the major pathway to phospholipids and triglycerides; the latter fractions probably arise directly from activated fatty acids.

The finding that *Trichoderma* incorporated radioactive carbon chiefly into palmitic acid is consistent with results obtained by Wakil (1958), Wakil & Ganguly (1959), Long & Porter (1959), Martin, Horning & Vagelos (1960) and Kusunose, Kusunose, Kowa & Yamamura (1960). The enzyme systems used by these groups of investigators all gave rise initially to highly labelled palmitic acid. It would appear (Table 7) that in *Trichoderma* the remaining saturated fatty acids arise by addition of one or more activated coenzyme A acetate or C_2 units to the first-formed palmitic acid.

Throughout the experiments with *Trichoderma*, palmitic acid always had an initially higher specific activity than oleic acid. On transferring mycelium from labelled to unlabelled substrate, the specific activity of oleic acid rose relative to the level of activity in palmitic acid, a result which could suggest a conversion of palmitic acid into oleic acid. This conversion would be expected to be by way of stearic acid, the desaturation of which has been demonstrated in animal tissue by Schoenheimer & Rittenberg (1936), Stetten & Schoenheimer (1940) and by Bernhard *et al.* (1958). More recently Bu'Lock & Ridyard (1960) described the conversion of [9:10- $^{14}\text{C}_2$]stearic acid into [9:10- $^{14}\text{C}_2$]oleic acid in rabbit mammary gland tissue, and, although this conversion did not occur in germinating peanut cotyledons, Bloomfield & Bloch (1960) showed the desaturation of stearic acid to oleic acid in cell-free yeast extracts. In the present work, however, the low specific activity of stearic acid implies that this acid, as such, is not an intermediate in the rapid conversion of palmitic acid into oleic acid. A similar

Table 7. *Specific activities (counts/min./mg.) of the saturated fatty acids from 2-day-old mycelium grown for 3 hr. in the presence of labelled substrate*

Fatty acid	No. of carbon atoms	Radioactive substrate	
		Glucose	Acetate
Palmitic	16	508	9628
Stearic	18	367	2570
Arachidic	20	121	890
Behenic	22	81	460

finding was made by Dittmer & Hanahan (1959), but this does not exclude the possibility of activated or modified stearic acid being an intermediate between activated palmitic acid and oleic acid. Later work by the same authors (Hanahan & Dittmer, 1960) revealed that when labelled palmitic acid was fed to rats, a very rapid conversion into stearic acid and thence into oleic acid took place; unfortunately no experimental details were given.

The use of triose units derived from glucose to synthesize linolenic acid, and the subsequent successive hydrogenation of linolenic acid to linoleic acid to oleic acid has been discussed by Hilditch (1956). But when *Trichoderma* was grown on labelled glucose there was only a slight incorporation of radioactivity; what little did enter the fatty acids was found initially mainly in palmitic acid and oleic acid, suggesting that the glucose had given rise to acetate units which resulted in the typical palmitic acid/oleic acid-labelling pattern normally produced by feeding with labelled acetate.

The arguments in favour of the introduction of one or two double bonds into the oleic acid molecule are based on the change in the labelling pattern that took place when *Trichoderma* mycelium containing strongly labelled oleic acid and palmitic acid was transferred (for 2 days) to unlabelled substrate. Only when such a transfer was effected did the specific activity of linoleic acid reach a value similar to that of palmitic acid or oleic acid. During the 2 days of continued growth on unlabelled substrate 40% of the fatty acids synthesized was linoleic acid, and it is therefore reasonable to postulate a conversion of palmitic acid or oleic (most probably oleic) acid into linoleic acid. The increased specific activity of linolenic acid could be interpreted as a further

dehydrogenation of linoleic acid. The alternate labelling of carbon atoms of both saturated and unsaturated long-chain fatty acids discovered by Gibble & Kurtz (1956) when flax was fed with [^{14}C]acetate can be construed as evidence favouring dehydrogenation of saturated fatty acids. The only apparent alternative to the dehydrogenation pathway would be a route direct from acetate to linoleic acid, the reactions for which were slow to restart after the transfer of mycelium from the original medium to the feeding medium. This route appears highly unlikely on several counts. In our view the continued synthesis of linoleic acid from unlabelled oleic (or less likely palmitic) acid is the best explanation of the initial low specific activity of linoleic acid.

Since completion of the above-described work and publication of our preliminary communication (Crombie & Ballance, 1959) these deductions have received support from Yuan & Bloch (1960), who have described the production of labelled linoleic acid from labelled oleic acid in *Torulopsis utilis*.

SUMMARY

1. Phospholipids closely followed by triglycerides had higher specific activities than the free fatty acids in the lipid obtained from 4-day-old *Trichoderma* mycelium that had been grown for 1, 3 or 5 hr. in the presence of [$2\text{-}^{14}\text{C}$]acetate.

2. After only 1 hr. in the presence of labelled acetate, radioactive carbon was found in all the long-chain fatty acids, especially oleic acid and palmitic acid, in all three major fractions of the lipid.

3. When the 4-day-old mycelium containing high-specific-activity palmitic acid and oleic acid was transferred to unlabelled substrate there was slight indication of conversion of these acids into linoleic acid and linolenic acid.

4. When 2-day-old mycelium, containing high-specific-activity palmitic acid and oleic acid (this activity was obtained from either [^{14}C]acetate or [^{14}C]glucose), was transferred to an unlabelled medium for 2 days the specific activity of linoleic acid (representing 40–50% of the fatty acids present), which was always initially very low, rose to a value equal to the activities of palmitic acid and oleic acid.

We are grateful to Professor W. T. Williams for his encouragement and to Dr D. W. Turner for access to a pro-

portional counter and instruction in isotopic carbon technique. Advice on the culturing of *Trichoderma viride* was given by Dr J. G. Manners. One of us (P. E. B.) acknowledges the receipt of a D.S.I.R. award.

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