J. Lipid Research, January, 1962
Volume 3, Number 1

Disproportionate labeling of oleic and linoleic acids during the biosynthesis of uniformly labeled fatty acids

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[Received for publication August 17, 1961]

SUMMARY

Fatty acids uniformly labeled with C^{14} were obtained by growing *Penicillium javanicum*, in the presence of uniformly labeled acetate, on a nitrogen-deficient medium. When uniformly labeled acetate was the C^{14} source, the specific activity of the linoleic acid was greater than the specific activity of the oleic acid. The reverse was true when uniformly labeled sucrose was the C^{14} source. It is suggested that the disproportionate labeling observed between oleic acid and linoleic acid is the result of changing relative rates of synthesis of these acids coupled with an acetate pool of changing specific activity.

In the course of preparing uniformly labeled C^{14} fatty acids, it was observed that when the mold *Penicillium javanicum* was grown in the presence of U-C^{14}-acetate,[1] the specific activity of the isolated linoleic acid was significantly higher than the specific activity of the oleic acid.

Disproportionate labeling has been observed also by Bernhard *et al.* (1) among the fatty acids of *Phycomyces blakesleeanus*, and by Simmons and Quackenbush (2) among the fatty acids isolated from developing soybean seeds. Preferential synthesis of the fatty acids with high specific activity was the explanation offered by both groups; however, experimental evidence in support of this conclusion is lacking. A hypothesis of differing rates of fatty acid synthesis coupled with an acetate pool of changing specific activity is proposed to explain the disproportionate labeling observed in the fatty acids produced by *P. javanicum* and evidence is presented that supports this hypothesis.

MATERIALS AND METHODS

P. javanicum (ATCC-9099) was obtained from the American Type Culture Collection.

The U-C^{14}-acetate was prepared by mixing equal amounts, on a radioactivity basis, of acetate-1-C^{14} and acetate-2-C^{14}. The acetate-1-C^{14} was synthesized[2] according to the procedure of Ruben *et al.* (3). The acetate-2-C^{14} was purchased from Nuclear Research Chemicals, Orlando, Florida. The U-C^{14}-sucrose was purchased from Tracerlab, Waltham, Massachusetts. These materials, when used, were substituted for an equal weight of the unlabeled compounds in the nutrient medium.

Unless otherwise indicated, *P. javanicum* was grown in stationary culture at 27° for 7 days in Kolle flasks. The composition of the nutrient medium, in grams per liter, was: Na acetate, 0.3; $NaNO_3$, 0.08; $MgSO_4 \cdot 7H_2O$, 0.05; KCl, 0.05; $FeSO_4 \cdot 7H_2O$, 0.001; KH_2PO_4, 0.58; sucrose, 3.0; and Difco yeast extract, 0.1. The pH was adjusted to 5.5. This is the nitrogen-free medium of MacGee[3] that was modified by adjusting the nitrate concentration to give the desired lipid yield and composition. This modified medium will be referred to as the nitrogen-deficient medium.

At the end of the incubation period, the mold was washed free of nutrient medium, lyophilized, and saponified with alcoholic KOH. After acidification, the fatty acids and nonsaponifiable matter were extracted into petroleum ether. The fatty acids were isolated by ion-exchange chromatography using a procedure similar to the one described by Benedict (4). The fatty acids were separated into crude saturated and unsaturated

* With the technical assistance of W. R. King.

[1] The abbreviations used are: U-C^{14}-acetate, uniformly labeled acetate; U-C^{14}-sucrose, uniformly labeled sucrose.

[2] Acetate-1-C^{14} was synthesized by H. W. Lampe.

[3] J. MacGee, unpublished data.

fractions by low temperature crystallization (−30°) from acetone. Further purification of each fatty acid fraction was achieved by refluxing the fatty acid methyl esters with mercuric acetate in methanol to form the methanol-mercuric acetate addition complex of the unsaturated esters. The mixture of saturated fatty acid esters and mercury derivatives from each fatty acid fraction was then chromatographed on Merck acid-washed alumina[4] containing 10% water. The saturated esters were eluted with hexane; the mercury derivatives were then decomposed and the freed unsaturated esters concomitantly eluted by passing HCl-methanol, 1:10 (v/v) through the column. This procedure is a modification[5] of that reported by Jantzen and Andreas (5). Oleic acid and linoleic acid were obtained separately from the unsaturated fatty acid fraction by liquid-liquid countercurrent distribution. Two runs of 400 transfers each were required for complete separation. The two-phase solvent system resulting from a mixture of heptane, dimethyl formamide, and glacial acetic acid, 3:2:1 (v/v) was used for the distribution.

The degradative procedures used in determining the label distribution in the oleic and linoleic acids were as follows. The stearic acid formed by the catalytic reduction of the oleic or linoleic acid was decarboxylated according to the method of Anker (6). Oleic and linoleic acids were oxidatively cleaved at the double bonds by a procedure similar to that of von Rudloff (7). Prior to degradation the labeled acids were diluted with unlabeled carrier.

Fatty acid composition of the mixtures and purity of the isolated fatty acids were determined by gas-liquid chromatography using an "Aerograph" gas chromatograph. The conditions were: column length, 200 cm (0.5 cm i.d.); packing, 12% (w/w) ethylene glycol adipate polyester on acid-washed 60 to 80 mesh Chromosorb W; temperature, 200°; and helium flow rate, 50 ml per minute, standard temperature and pressure. Peak identification was based upon the chromatographic behavior of reference fatty acids. The Beckman "Megachrom" gas-liquid chromatograph was used for preparative work under the following conditions: column length, 366 cm (1.6 cm i.d.); packing, 25% (w/w) ethylene glycol adipate polyester on acid-washed 60–80 mesh Chromosorb W; temperature 158° and 167°; helium inlet pressure 25 psi; and outlet pressure 1 psi.

Radioactivity measurements were made with a "Tri-Carb" liquid scintillation counter (8). The CO_2 resulting from the decarboxylation of stearic acid was converted to barium carbonate, which was suspended in "Thixcin" scintillation gel for counting (9).

Acetate was determined by a method similar to that of Olmsted *et al.* (10) and sucrose was determined, as total reducing sugar following acid hydrolysis, by a method similar to that of Munson and Walker (11).

[4] Obtained from Merck and Company, Inc., Rahway, New Jersey.

[5] D. F. Kuemmel, unpublished data.

TABLE 1. FATTY ACID COMPOSITION OF THE LIPID EXTRACTED FROM *P. javanicum*

Fatty Acid	Per Cent of Total Fatty Acids
<16*	0.8
16:0	20.3
16:1	0.6
18:0	17.4
18:1	25.1
18:2	31.8
18:3	0.5
>18	3.5

* The abbreviation system used is that of Dole *et al.* (12).

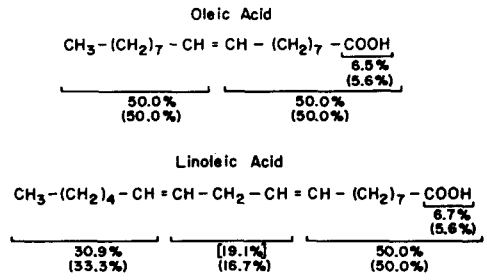

FIG. 1. Distribution of radioactivity in biosynthesized oleic and linoleic acids. *P. javanicum*, from which these acids were isolated, was grown under the conditions described in Table 2. The values in parentheses are theoretical values calculated for a uniformly labeled 18-carbon fatty acid. The value in brackets was calculated, by difference, from the experimental values.

RESULTS AND DISCUSSION

When grown under the conditions described, 18% of the dry weight of *P. javanicum* was found to be attributable to fatty acids plus nonsaponifiable lipids. These lipids represented a radiochemical yield of 35% when the mold was grown in the presence of U-C^{14}-acetate. The fatty acids represented a weight yield of 16% and a radiochemical yield of 28%. The composition of these fatty acids is shown in Table 1.

The C^{14} distribution in the oleic and linoleic acids isolated from *P. javanicum* grown in the presence of U-C^{14}-acetate is given in Figure 1. The C_3 fragment produced by the oxidative cleavage of linoleic acid was not recovered. In parentheses are the theoretical values for a uniformly labeled molecule. It is obvious

TABLE 2. SPECIFIC ACTIVITY OF LIPIDS ISOLATED FROM *P. javanicum* GROWN IN THE PRESENCE OF C^{14}-ACETATE*

Fraction	Specific Activity
	$\mu c/g$
Linoleic acid	187
Oleic acid	119
Saturated fatty acids	112
Nonsaponifiable lipid	71

* One millicurie of uniformly labeled acetate was substituted for an equal weight of unlabeled acetate in the nutrient medium. The sucrose in the medium was unlabeled.

that these fatty acids were uniformly labeled with C^{14}.

The specific activities of the different lipid fractions are shown in Table 2. The nonsaponifiable and saturated fatty acid fractions have not yet been investigated further.

As can be seen from Table 2, the specific activity of the linoleic acid produced by *P. javanicum*, grown in the presence of U-C^{14}-acetate, was significantly greater than that of the oleic acid. This observation has been verified in a large-scale synthesis of uniformly labeled oleic and linoleic acids.

This phenomenon has been investigated further. Figure 2 shows the relative concentrations of oleic and linoleic acids in the mold at intervals throughout the incubation period. The relative concentration of linoleic acid was considerably greater than that of the oleic acid during the early part of the incubation period. After the fourth day, however, the relative concentration of the oleic acid in the lipid increased while that of the linoleic acid decreased. Therefore, there was a difference in the way in which the rates of synthesis of these acids varied during the incubation. The rate of linoleic acid synthesis was high during the first half of the incubation period and later fell. The rate of oleic acid synthesis rose during the last half of the incubation. Total fatty acid synthesis was essentially linear throughout the incubation period (Fig. 2).

The changes in acetate and sugar concentrations in the nutrient medium during incubation are shown in Figure 3. The acetate disappeared quite rapidly during the first few days of the incubation while sugar utilization was much slower during this period. The initial rise in acetate concentration at day 1 is a reproducible but unexplained phenomenon.

From these data, it can be presumed that the acetate in the medium, exogenous acetate, was the major source of the acetate pool used for fatty acid synthesis during the first part of the incubation period and that the acetate formed by sucrose metabolism, endogenous acetate, was the major source of acetate for fatty acid

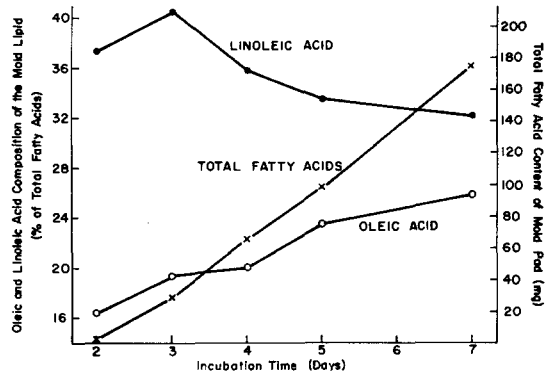

FIG. 2. Oleic acid, linoleic acid, and total fatty acid content of *P. javanicum*.

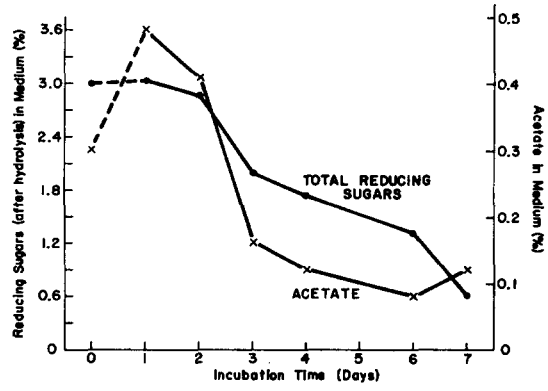

FIG. 3. Disappearance of sucrose and acetate from the nutrient medium during the growth of *P. javanicum*.

synthesis during the latter part of the incubation period. Therefore, when exogenous acetate is the C^{14} source, the specific activity of the acetate pool would be maximal at the start of the incubation period. As shown in Figure 2, linoleic acid synthesis was greatest during this same period. During the final half of the incubation period, when oleic acid synthesis was greatest, the specific activity of the acetate pool would decrease since endogenous acetate from the metabolism of unlabeled sucrose was now the major contributor to the acetate pool. Therefore, linoleic acid synthesis was maximal at the time when the acetate pool had its maximum specific activity and oleic acid synthesis was maximal at the time when the acetate pool had its minimum specific activity. This would account for the observation that the specific activity of the linoleic acid was higher than that of the oleic acid when U-C^{14}-acetate was the C^{14} source.

Assuming that the explanation given above is correct, a reversal of the observed specific activity pattern should be seen if U-C^{14}-sucrose were used as the exo-

TABLE 3. SPECIFIC ACTIVITY OF OLEIC AND LINOLEIC ACID FROM *P. javanicum* GROWN IN THE PRESENCE OF C^{14}-SUCROSE*

Fraction	Specific Activity
	$\mu c/g$
Oleic acid	4.23
Linoleic acid	3.79

* Uniformly labeled sucrose, 0.05 mc, was substituted for an equal weight of unlabeled sucrose in the nutrient medium. The acetate in the medium was unlabeled.

genous C^{14} source and the acetate were unlabeled, other conditions being the same. The data in Table 3 show that, when U-C^{14}-sucrose was used, this was the case. The specific activity of the oleic acid, at the end of the 7-day incubation, was greater than that of the linoleic acid.

The proposed explanation of the differing rates of fatty acid synthesis coupled with an acetate pool of decreasing specific activity can account for the different fatty acid specific activities observed when U-C^{14}-acetate was the exogenous C^{14} source. This sequence could also explain the specific activity differences observed by Bernhard *et al.* (1) since the medium used by them also contained two carbon sources, acetate-1-C^{14} and unlabeled glucose. It is possible that the disproportionate labeling reported by Simmons and Quackenbush (2) was the result of a different mechanism since the nutrient medium used by these workers contained only one carbon source, U-C^{14}-sucrose. However, it is not necessary that the mechanism be different from that proposed above because unlabeled acetate precursors were undoubtedly already present in the soybean cuttings used by these workers even though not present in the nutrient medium.

The author wishes to express his gratitude to the following people for their contribution to the success of this work: Dr. J. MacGee for his advice and comments and for the valuable background information on *P. javanicum* arising from his initial work with this organism, Dr. D. F. Kuemmel for his help on the fatty acid degradations, Drs. C H. Orr and K. T. Knapp for the gas-liquid chromatographic analyses and preparative gas-liquid chromatography, Dr. E. S. Lutton and Mr. C. B. Stewart for the liquid-liquid countercurrent separations, and Dr. J. C. Alexander for his comments and suggestions.

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