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Incorporation of Branched-Chain C_6-Fatty Acid Isomers into the Related Long-Chain Fatty Acids by Growing Cells of *Bacillus subtilis**

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ABSTRACT: The incorporation of eight C_6-alkanoic fatty acids into long-chain fatty acids by the growing cells of *Bacillus subtilis* (ATCC-7059) has been studied. Among them, five branched C_6-fatty acids are found to be incorporated into long-chain fatty acids and in each case a set of three new fatty acids are produced. The new fatty acids produced (3–24% of the total fatty acids) are 10-methyltridecanoic, 12-methylpentadecanoic, and 14-methylheptadecanoic from 2-methylpentanoic; 11-methyltridecanoic, 13-methylpentadecanoic, and 15-methylheptadecanoic from 3-methylpentanoic; 10,10-dimethyldodecanoic, 12,12-dimethyltetradecanoic, and 14,14-dimethylhexadecanoic from 2,2-dimethylbutyric; 11,11-dimethyldodecanoic, 13,13-dimethyltetradecanoic, and 15,15-dimethylhexadecanoic from 3,3-dimethylbutyric; 10-ethyldodecanoic, 12-ethyltetradecanoic, and 14-ethylhexadecanoic from 2-ethylbutyric. These are identified by gas–liquid chromatography and mass spectrometry using the structually related chemical C_{15}-fatty acids as standard. The major long-chain fatty acid is, in all cases, the C_{16}-fatty acid, and the two others, C_{14}- and C_{18}-fatty acids, are produced in much smaller proportions. This fatty acid distribution is very similar to that obtained when C_2- and C_4-fatty acids are incorporated into long-chain fatty acids. Thus it is concluded that the chain length of fatty acids achieved in the synthesis is not affected by the chain length or nature of chain initiator used: the C_{16}-fatty acids being always the major fatty acids regardless of whether C_2-, C_4-, or C_6-fatty acid is used. The relative activity of the five active C_6-fatty acids as the chain initiator is 2-ethylbutyric > 2-methylpentanoic > 3-methylpentanoic > 2,2- or 3,3-dimethylbutyric. Apparently C_6-fatty acids having single branching (either methyl or ethyl) at α position are excellent chain initiators. The other C_6-fatty acids tested but found to give no increase in expected or related long-chain fatty acids are caproic, isocaproic, and 2,3-dimethylbutyric.

Synthesis (*de novo*) of long-chain alkanoic acids has been shown to include the repeated condensation of the chain extender, C_2 precursor, with the chain initiator to extend its chain length to 12–18 carbon atoms. The chain extender is, in all known systems, malonyl-CoA and no exception has been reported (Brady, 1958; Wakil, 1958; Vagelos, 1964).

The chain initiator, however, could be one of several acyl-CoA esters. In the majority of organisms, acetyl-CoA serves as chain initiator and the main product is palmitic acid. In some organisms, on the other hand, branched-chain acyl-CoA esters, namely, isobutyryl, α-methylbutyryl, and iso-

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TABLE I: Kolbe Synthesis of Branched-Chain C_{15}-Fatty Acids and Their Equivalent Carbon Number.[a]

Monocarboxylic Acid	Semiester of Dicarboxylic Acid	Product	Equiv C No. (ECN)	ΔC (15.00 − ECN)
3-Methylhexanoic[b]	di-C_{10}	11-Methyltetradecanoic	14.65	0.35
3-Methylpentanoic	di-C_{11}	12-Methyltetradecanoic (anteiso)[c]	14.75	0.25
4-Methylpentanoic	di-C_{11}	13-Methyltetradecanoic (iso)[c]	14.62	0.38
3,3-Dimethylpentanoic[b]	di-C_{10}	11,11-Dimethyltridecanoic	14.40	0.60
3,3-Dimethylbutyric	di-C_{11}	12,12-Dimethyltridecanoic (neo)	14.10	0.90
3-Ethylpentanoic[b]	di-C_{10}	11-Ethyltridecanoic	14.68	0.32

[a] Measured as methyl ester on EGA SCOT column (50 ft) using methyl esters of *n*-C_{13}-, *n*-C_{14}-, *n*-C_{15}-, *n*-C_{16}-, and *n*-C_{17}-fatty acids as standard (Hawke *et al.*, 1959). [b] Prepared from the related fatty acid with one less carbon atom by Arndt–Eistert synthesis (see text). [c] Prepared previously (Kaneda, 1963a).

valeryl, related to valine, isoleucine, and leucine, respectively, also serve as chain initiators in the synthesis of fatty acids. In particular a number of grampositive and aerobic or facultative anerobic microorganisms produce branched-chain fatty acids in the latter way. We have studied this latter system extensively, mainly in *Bacillus subtilis*. The activities of branched or normal amino acids and fatty acids with 2–5 carbon atoms as chain initiators in the fatty acid synthesis have been previously reported (Kaneda, 1963b, 1966b). The work is now extended to various C_6-fatty acid isomers and the results obtained are herein reported.

The purpose of the present investigation is (a) to examine which of the eight possible isomers of alkanoic C_6-fatty acids are used as the chain initiator in the fatty acids synthesis, (b) to correlate their activity with chemical structure, and (c) to examine the factors determining the total chain length achieved in the snythesis.

Experimental Section

Microbiological Procedure. B. subtilis (ATCC 7059) was used throughout the present work. The organism was maintained on nutrient agar (Difco) and transferred to a slant of the standard medium containing 2% agar. The inoculum was incubated overnight at 37° and the fresh culture thus obtained was used to inoculate the appropriate liquid media. The standard culture conditions were as follows.

The standard culture medium (100 ml) containing glucose (1%), yeast extracts (0.1%, Difco), and inorganic salts (Kaneda, 1963a) in a 500-ml erlenmeyer flask was inoculated with the fresh culture from the slant and incubated at 37° for 16 hr on a rotary shaker. The cells which had attained the stationary phase were centrifuged and washed once with 0.85% NaCl solution. The washed cells were transferred to a test tube with 50% aqueous methanol and a known amount of either *n*-C_{13} or *n*-C_{15} methyl ester standard was added. After addition of methanolic KOH, the test tube was sealed and saponified overnight at 80° as before (Kaneda, 1963a).

Growth was measured by a Klett–Summerson colorimeter with a no. 66 filter. A culture of 100 ml with 100 Klett units was equivalent to 59 mg of the dry cell weight and this value was used to convert Klett units into the dry cell weight of *B. subtilis*.

Isolation, Methylation, and Estimation of Bacterial Fatty Acids. Fatty acids in the saponified cells were extracted with purified *n*-hexane and methylated with diazomethane as before (Kaneda, 1963a). The amount of bacterial fatty acids was determined by gas–liquid chromatography with peak areas measured by triangulation and the internal standard, *n*-C_{13} or *n*-C_{15}, was used to calculate the actual amount present in the original cells.

Chemical Fatty Acids. Methyl laurate, tridecanoate, myristate, pentadecanoate, palmitate, margarate (C_{17}), and stearate were purchased from Applied Science Laboratories; caproic and 2-ethylbutyric acids were from Eastman Kodak Co.; 2-methylpentanoic, 3-methylpentanoic (anteiso-C_6), 4-methylpentanoic (iso-C_6), 2,2-dimethylbutyric, and 2,3-dimethylbutyric acids were from K & K Laboratories, and 3,3-dimethylbutyric (neo-C_6) acid was from Aldrich Chemical Co. Chemical identity and purity of the commercial products were confirmed first by gas–liquid chromatography and then by nuclear magnetic resonance spectroscopy. Some products, however, were purified by gas–liquid chromatography to obtain purities of 98% or over. Branched-chain C_{15}-fatty acids were prepared in this laboratory from the related C_6-fatty acids through a sequence of Arndt–Eistert and Kolbe syntheses as shown in Table I. The procedures are described below for 11-methyltetradecanoic acid.

A typical example of the synthesis was as follows. 3-METHYLHEXANOIC ACID. In a 100-ml round-bottom flask fitted with a drying tube, in an ice bath, were placed 5 ml of dry benzene, 3 drops of pyridine, 3.6 ml of thionyl chloride, and 2.2 ml of 2-methylpentanoic acid. After standing at room temperature for 30 min, the benzene was removed in a rotary evaporator. The oily product was dissolved in 5 ml of benzene and the benzene was evaporated again as above, in order to remove an excess of thionyl chloride. This procedure was repeated once more. The acid chloride thus obtained was dissolved in 20 ml of benzene. To this solution was added dropwise, in cold (5°) an ethyl ether solution of diazomethane prepared from 10 g of Diazalid (Aldrich Chemical) by the procedure of Schlenk and Gellerman (1960). After standing in an ice bath for 20 min, excess diazomethane was removed by a nitrogen stream and then the ether was removed in a rotary evaporator. The diazoketone produced was dissolved in 35 ml of methanol. To this solution was added 5.9 g of Ag_2O in 5 ml of methanol and the reaction mixture was warmed to 50°. In addition 5.8 g of Ag_2O in 5 ml of methanol was added every 5 min to the reaction mixture in six portions. Then the mixture was refluxed for 15 min, treated with charcoal, and

TABLE II: Effect of C_6-Fatty Acid Isomers on Growth and Total Fatty Acids of *B. subtilis*.

Fatty Acid Substrate Added (3 mM)	Growth[a] (A) (mg of Dry Cells in 100-ml Culture)	Total Fatty Acids[b] (B) (mg)	Fatty Acid Contents (B/A × 100)
None	165	3.54	2.14
2-Methylpentanoic	172	2.97	1.72
3-Methylpentanoic	148	2.26	1.53
4-Methylpentanoic	135	2.25	1.67
2,2-Dimethylbutyric	169	3.39	2.00
2,3-Dimethylbutyric	167	2.90	1.74
3,3-Dimethylbutyric	156	3.52	1.62
2-Ethylbutyric	159	2.84	1.78
Caproic	171	3.94	2.30

[a] *B. subtilis* was grown on 100 ml of glucose (1%)–yeast extract (0.1%) medium with or without a C_6-fatty acid substrate (3 mM) at pH 7.0, 37° for 16 hr. The values were calculated from colorimetric measurement by a Klett–Summerson colorimeter (see text). [b] Measured by gas–liquid chromatography using an internal standard of methylpentadecanoate.

concentrated to a small volume. The product was checked by gas–liquid chromatography. The yield was generally 50% of the theoretical value based on the gas–liquid chromatographic measurement.

METHYL 11-METHYLTETRADECANOATE. The product was dissolved in 50 ml of methanol. To this solution, in a 100-ml beaker, was added 5 g of methyl hydrogen sebacate and 0.2 ml of 60% KOH. The electrolysis was carried out with a current of one ampere until the reaction mixture became alkaline, usually for 5 hr. The product, methyl 11-methyltetradecanoate, was confirmed by gas–liquid chromatography and purified by preparative gas–liquid chromatography on a SE-30 column.

Gas–Liquid Chromatography. Two columns were used to analyse fatty acids as methyl esters. A column of 2.5% SE-30 on Chromosorb W packed in a standard $\frac{1}{8}$ in. × 6 ft stainless steel tube was operated at 162° for 2 min and then the column temperature was programmed at a rate of 4°/min up to a final temperature of 265°. Under these conditions fatty acid methyl esters with up to 24 carbon atoms could be detected. Another column used was an ethylene glycol adipate (EGA)[1] support-coated open tubular (SCOT) column (Perkin-Elmer Corp.) with dimensions of 0.02 in. × 50 ft. It was operated at 160° for 2 min and then the temperature was programmed at 1°/min up to a final temperature of 190°. The carrier gas was helium with a flow rate of 15 ~ 20 ml/min for the SE-30 packed column and 1.2 ~ 1.5 ml/min for the EGA SCOT column. A GC-2000R model (Micro-Tek Corp.) was used throughout the present work.

Mass Spectrometry. Mass spectrograms were obtained by two ways: by Morgan–Schaffer Corp., Montreal, using a Hitachi RMU-6D mass spectrometer and in this institution, using a Perkin-Elmer Model 270 GC mass spectrometer. In the case of the former, each peak component was first separated by preparative gas–liquid chromatography, whereas in the case of the latter, peak components were analyzed by the mass spectrometer as they emerged from the gas–liquid chromatographic system, using the EGA SCOT column. Ionizing voltage was, in both cases, 70 eV.

[1] Abbreviations used are: EGA, ethylene glycol adipate; SCOT, support-coated open tubular.

Results

Effect of Added C_6-Fatty Acid Substrates on Growth and Total Fatty Acids. The medium containing glucose (1%) and yeast extract (0.1%) was used as the standard and one of the C_6-fatty acid isomers was added to the standard medium in a concentration of 3 mM. The results summarized in Table II suggest that among the singly methyl-branched isomers, growth of *B. subtilis* was suppressed more by an isomer having methyl side chain located further from the carboxyl carbon. Hence 4-methylpentanoic acid (iso-C_6) suppressed growth to the greatest extent, 3-methylpentanoic acid the next, and 2-methylpentanoic acid gave no suppression.

The fatty acid content of *B. subtilis* was decreased to about 80% of the control value in most cases except that of the addition of 2,2-dimethylbutyric or caproic acid.

Effect of Added C_6-Fatty Acid Isomers on Fatty Acid Composition. Fatty acid composition was, however, affected by the addition of some C_6-fatty acid isomers. It has been shown that the major fatty acids present in the lipids of *B. subtilis* grown on the standard culture medium are iso-C_{14}, n-C_{14}, iso-C_{15}, anteiso-C_{15}, iso-C_{16} n-C_{16}, iso-C_{17}, and anteiso-C_{17}, in order of retention time on either SE-30 or ethylene glycol adipate columns (Kaneda, 1963a). This is seen in Figure 1a. The initial chromatography of the total fatty acid samples with the short packed SE-30 column indicated that the amount of iso-C_{14} and iso-C_{16} peak components increased twofold when one of 2-ethylbutyric, 2-methylpentanoic, or 3-methylpentanoic acids was added to the standard culture medium, and that, in all cases, no fatty acids with more than 18 carbon atoms were produced. Further chromatography with the more efficient EGA SCOT column showed that three extra fatty acids were produced in each case. Three additional acids were also produced by the addition of either 2,2-dimethylbutyric or 3,3-dimethylbutyric acid to the culture medium. The extra peaks were different in all cases; *i.e.*, a total of 15 additional fatty acids were produced by the five precursors. The chromatograms are shown in Figure 1 in which the numbers on the peaks refer to the normally found fatty acid components and the letters to the new fatty acids. In the cases of 2-ethylbutyrate and 2-methylpentanoic acids, the three respective peaks were not well resolved from iso-C_{14}, iso-C_{16}-, and iso-C_{18}-fatty acid peaks, and appeared as their shoulders.

n-Hexanoic (caproic), 4-methylpentanoic (isocaproic), and 2,3-dimethylbutyric acids caused no change in fatty acid composition.

Identification of Regular Bacterial Fatty Acids. *B. subtilis* normally produces eight fatty acids shown in Figure 1 as the numbered peaks; 1, iso-C_{14}; 2, *n*-C_{14}; 3, iso-C_{15}; 4, anteiso-C_{15}; 5, iso-C_{16}; 6, *n*-C_{16}; 8, iso-C_{17}; and 9, anteiso-C_{18}. These fatty acids have been previously identified by gas–liquid chromatography, infrared spectroscopy, X-ray data, melting point, and other physical data (Kaneda, 1963a). The identification was further confirmed in the present study by use of a Perkin-Elmer GC mass spectrometer. In addition, three minor fatty acids (peak 7, *n*-hexadecenoic; 10, iso-C_{18}; 11, stearic) were identified.

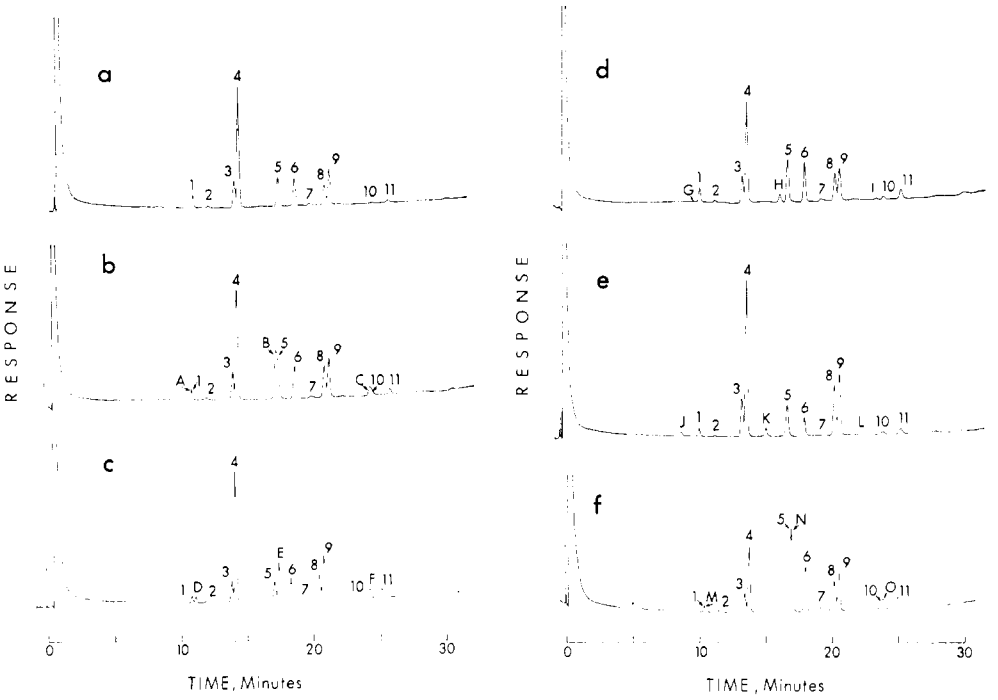

FIGURE 1: Gas–liquid chromatograms of total fatty acids isolated from the cells of *B. subtilis* grown on standard culture medium with or without a C_6-fatty acid. (a) No C_6-fatty acid added, (b) 2-methylpentanoic, (c) 3-methylpentanoic, (d) 2,2-dimethylbutyric, (e) 3,3-dimethylbutyric, and (f) 2-ethylbutyric. The numbered peaks are 1, iso-C_{14}; 2, *n*-C_{14}; 3, iso-C_{15}; 4, anteiso-C_{15}; 5, iso-C_{16}; 6, *n*-C_{16}; 7, *n*-$C_{16}{}^{-1}$; 8, iso-C_{17}; 9, anteiso-C_{17}; 10, iso-C_{18}; 11, *n*-C_{18}. The lettered peaks are not normally found in the lipids of *B. subtilis* and, as discussed in the text, are identified as (A) 10-methyltridecanoic, (B) 12-methylpentadecanoic, (C) 14-methylheptadecanoic, (D) 11-methyltridecanoic, (E) 13-methylpentadecanoic, (F) 15-methylheptadecanoic, (G) 10,10-dimethyldodecanoic, (H) 12,12-dimethyltetradecanoic, (I) 14,14-dimethylhexadecanoic, (J) 11,11-dimethyldodecanoic, (K) 13,13-dimethyltetradecanoic, (L) 15,15-dimethylhexadecanoic, (M) 10-ethyldodecanoic, (N) 12-ethyltetradecanoic, and (O) 14-ethylhexadecanoic.

TABLE III: New Bacterial Fatty Acids Produced by Addition of C_6-Fatty Acid Substrates and Their Equivalent Carbon Number.

Fatty Acid Substrate Added	Chromatographic Peak Component								
		ECN[b]			ECN[b]			ECN[b]	
	Peak[a]	Obsd	Calcd[c]	Peak[a]	Obsd	Calcd[c]	Peak[a]	Obsd	Calcd[c]
2-Methylpentanoic	A	13.63	13.65	B	15.62	15.65	C	17.61	17.65
3-Methylpentanoic	D	13.75	13.75	E	15.75	15.75	F	17.75	17.75
2,2-Dimethylbutyric	G	13.42	13.40	H	15.36	15.40	I	17.42	17.40
3,3-Dimethylbutyric	J	13.17	13.10	K	15.13	15.10	L	17.15	17.10
2-Ethylbutyric	M	13.65	13.68	N	15.69	15.68	O	17.72	17.68

[a] See Figure 1. [b] Represents equivalent carbon number and measured peaks 2, 6, and 11 shown in Figure 1 (*n*-C_{14}, *n*-C_{16}, and *n*-C_{18}) as standard. [c] Calculated from ΔC values given in Table I, assuming same terminal-chain-branching structure.

Identification of New Bacterial Fatty Acids. Any one of the five C_6-fatty acids listed earlier, when added to the standard culture medium, caused the production of a set of three new fatty acids. Thus 15 new fatty acids were produced. These fatty acids were initially identified by gas–liquid chromatography and their final identification was carried out by mass spectrometry.

The gas–liquid chromatographic identification was carried out by comparing equivalent carbon numbers of the standard branched-chain fatty acid methyl esters to those of the bacterial fatty acid methyl esters on the EGA SCOT column. The principle used is that differences, ΔC, between equivalent carbon numbers of each member of a homologous series of terminally branched-chain fatty acid methyl esters and those of each of the corresponding normal fatty acid methyl esters are the same; measurements with several sets of acids give ΔC for iso and normal fatty acids on the EGA column as 0.40, whereas for anteiso and normal fatty acids it is 0.25. In the present work, the standard branched-chain C_{15}-fatty acids were used as representative to calculate values of ΔC for each of the fatty acid homologs. The values of ΔC are shown in the last column of Table I, whereas observed equivalent carbon numbers of the new bacterial fatty acids are shown in Table III.

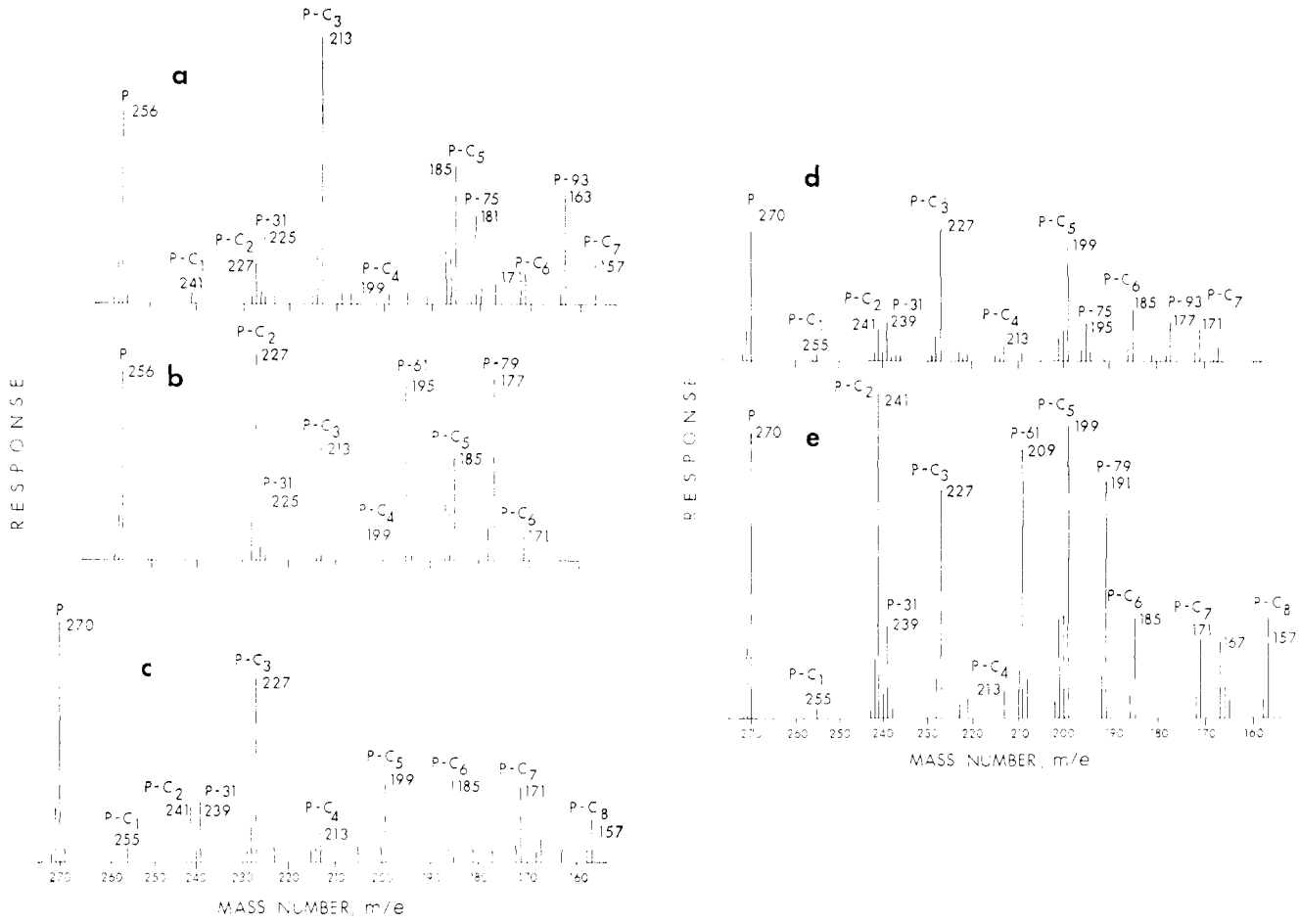

FIGURE 2: Mass spectra of bacterial and chemical fatty acid methyl ester samples produced by a Hitachi RMU-6D mass spectrometer with ionization voltage of 70 eV. (a) Methyl 11-methyltetradecanoate, (b) methyl 11-ethyltridecanoate, (c) methyl 14- methylpentadecanoate (iso-C_{16}), (d) (B + 5) peak components (Figure 1b), and (e) (N + 5) peak components (Figure 1f). Symbols used are P, parent peak; C_1, CH_3; C_2, C_2H_5; C_3, C_3H_7, and so on.

When 3-methylpentanoic acid (anteiso-C_6) was used, *B. subtilis* produced three new fatty acids, D, E, and F, appearing after iso-C_{14}, iso-C_{16}, and iso-C_{18} on the chromatogram (Figure 1c). These had equivalent carbon numbers identical with those of anteiso-C_{14}-, anteiso-C_{16}-, and anteiso-C_{18}-fatty acids calculated using the ΔC values for the standard anteiso-C_{15}-fatty acids. Further characterization of components D, E, and F was carried out by mass spectrometry. They were found to be the mass numbers of 242, 270, and 298, indicating C_{14}-, C_{16}-, and C_{18}-fatty acids. These also gave all the expected fragments based on the mass spectrum obtained with the chemical anteiso-C_{15} sample: the characteristic fragments of P-61 and P-79 in an equal intensity and the others such as P-29, P-31, P-43, P-57, P-71, P-85, and P-99 in the expected relative intensities. Thus components D, E, and F are identified as anteiso-C_{14}-, anteiso-C_{16}-, and anteiso-C_{18}-fatty acids.

Similarly, the identification of peak components G, H, and I, produced when 2,2-dimethylbutyric acid was used, and of J, K, and L, when 3,3-dimethylbutyric acid was used, were carried out by comparing ΔC of each component to that of the corresponding standard C_{15}-fatty acid. Thus peak components G, H, I, J, K, and L are identified as 10,10-dimethyldodecanoic, 12,12-dimethyltetradecanoic, 14,14-dimethylhexadecanoic, 11,11-dimethyldodecanoic, 13,13-dimethyltetradecanoic, and 15,15-dimethylhexadecanoic acids. Further identification by mass spectrometry was not carried out for these minor fatty acids which were chromatographically well resolved from other peaks.

When 2-methylpentanoic acid was used, three peaks, A, B, and C (Figure 1b), were produced, whereas when 2-ethylbutyric acid was used, three peaks, M, N, and O (Figure 1f), were produced. They were all with equivalent carbon numbers very similar to those of iso-C_{14}- (A, M), iso-C_{16}- (B, N), or iso-C_{18}- (C, O) fatty acids, and appeared as shoulders of one of the three iso-fatty acids. Since the chemically synthesized standard 11-methyltetradecanoic (related to 2-methylpentanoic) and 11-ethyltridecanoic (related to 2-ethylbutyric) acids have equivalent carbon numbers very similar to iso-C_{15}-fatty acids (Table II), and peak components A, B, and C, and M, N, and O, are produced only by the addition of 2-methylpentanoic and 2-ethylbutyric acid, respectively, to the culture medium, A, B, C, M, N, and O are tentatively identified as 10-methyltridecanoic, 12-methylpentadecanoic, 14-methylheptadecanoic, 10-ethyldodecanoic, 12-ethyltetradecanoic, and 14-ethylhexadecanoic acids.

Further characterization of these six components was carried out by mass spectrometry. Mass spectra of three standard fatty acid methyl esters; iso-C_{16}, 11-methyltetradecanoate, and 11-ethyltridecanoate, and of two bacterial fatty acid samples; mixed peak components of B and 5 (iso-C_{16}), and of N and 5, as methyl esters, are shown in Figure 2. If peak component B is actually 12-methylpentadecanoic acid

as tentatively identified, the mass spectrum of peak (B + 5) should show two series of fragment peaks; one derived from methyl 12-methylpentadecanoate and another from methyl 14-methylpentadecanoate (iso-C_{16}). The standard methyl 11-methyltetradecanoate gave major fragment peaks of P-C_3H_7 (*m/e*, 213), P-C_5H_{11} (*m/e*, 185), P-31, P-75, and P-93[2] (Abrahamsson *et al.*, 1963) (Figure 2a), whereas the standard methyl 14-methylpentadecanoate gave characteristic fragment peaks of P-C_3H_7 (*m/e* 227) and P-31 (Figure 2c), in addition to other fragments. The spectrum of peak (B + 5) (Figure 2d) gave all the expected fragment peaks and the parent mass number was found to be 270 showing peak B is also a C_{16}-fatty acid. Thus peak B is 12-methylpentadecanoic acid. Furthermore, the relative intensities of P-75 and P-93 to P-C_3H_7 suggest that peak B and peak 5 (iso-C_{16}) are produced in almost equal proportions.

Similarly, if peak component N is actually 12-ethyltetradecanoic acid as tentatively identified, the mass spectrum of peak (N + 5) should show two series of fragment peaks; one derived from methyl 12-ethyltetradecanoate and another from methyl 14-methylpentadecanoate (iso-C_{16}). The standard methyl 11-ethyltridecanoate gave major fragment peaks of P-C_2H_5 (*m/e* 227), P-C_5H_{11} (*m/e* 185), P-31 (*m/e* 225), P-61 (*m/e* 195), and P-79 (*m/e* 177)[3] (Figure 2b), whereas the standard methyl 14-methylpentadecanoate, as mentioned above, gave peaks of P-C_3H_7 (*m/e* 227) and P-31 (Figure 2c). The spectrum of peak (N + 5) (Figure 2e) gave all the expected fragment peaks and the parent mass number was found to be 270 showing peak N is also a C_{16}-fatty acid. Thus peak N is 12-ethyltetradecanoic acid. Furthermore, relative intensities of P-61 and P-79 to P-C_3H_7 suggest that peak N and peak 5 (iso-C_{16}) are produced in a ratio of 2 to 1.

The mass spectra of peak components A, C, M, and O, which are not shown here, also confirmed the identification done by gas–liquid chromatography.

Relative Abundance of Fatty Acids. Five C_6-fatty acids among eight C_6 isomers studied have been found to be incorporated into the specific C_{14}-, C_{16}-, and C_{18}-fatty acids structurally related to the added C_6-fatty acid substrates by the growing cells of *B. subtilis*. Each trio of long-chain fatty acids produced from 3-methylpentanoic, 2,2-dimethylbutyric, or 3,3-dimethylbutyric acids were separated by gas–liquid chromatography from the other fatty acids produced under standard conditions. The sums of each group of three new acids were found to be 11.2, 3.9, and 3.4% of the total fatty acids, respectively (Table IV). The long-chain fatty acids produced from 2-methylpentanoic and 2-ethylbutyric acids, however, were over lapped with iso fatty acids with the same carbon atoms on gas–liquid chromatograms even on the 50-ft column, so that their amounts were difficult to estimate accurately. In the cases of 3-methylpentanoic, 2,2-dimethylbutyric, and 3,3-dimethylbutyric acids, the abundance of iso-C_{14}, iso-C_{16}, and iso-C_{18} did not increase, but rather decreased in some cases. Hence it is not unreasonable to assume that this may apply in the case of 2-methylpentanoic and 2-ethylbutyric acids. Thus the relative abundance of the long-chain fatty acids produced from these acid substrates are 15.8 and 24.3% of the total fatty acids, respectively, or perhaps more. It is noteworthy that the abundance of anteiso-C_{15}-fatty acid, most abundant of all, was decreased markedly in the cells of *B. subtilis* grown on the media containing 2-methylpentanoic and 2-ethylbutyric acids. Perhaps anteiso-

TABLE IV: Relative Proportions of Fatty Acids Produced by *B. subtilis*.

Peak No.:[a]	D, G, J[b]	1	2	3	4	E, H, K[b]	5	6	7	8	9	C, F, I,[b] L, O	10	11	
Fatty Acid Substrate Added	Fatty Acid (%)														
	x-C_{14}[d]	*i*-C_{14}	*n*-C_{14}	*i*-C_{15}	*a*-C_{15}	*x*-C_{16}[d]	*i*-C_{16}	*n*-C_{16}	*n*-$C_{16}^{=}$	*i*-C_{17}	*a*-C_{17}	*x*-C_{18}[d]	*i*-C_{18}	*n*-C_{18}	New Acids[e]
None	0	3.9	0.4	10.6	40.4	0	11.8	9.5	1.0	8.6	12.7	0	0.4	1.1	
2-Methylpentanoic	0	2.5[g]	0.3	5.3	27.9	0	23.4[g]	9.7	1.1	10.2	12.8	4.2	0.2	2.2	15.8
3-Methylpentanoic	1.8	1.7	0.5	6.1	33.5	5.8	11.7	7.5	0.3	8.4	13.5	3.6	0.8	3.3	11.2
4-Methylpentanoic	0	3.5	0.4	9.9	34.2	0	12.6	8.0	0	12.5	12.6	0	1.5	2.5	
2,2-Dimethylbutyric	0.1	3.8	0.9	8.5	30.0	2.4	12.9	12.8	0.8	9.2	12.2	1.4	0.5	3.7	3.9
2,3-Dimethylbutyric	0	2.3	1.1	8.4	29.6	0	14.1	15.2	1.0	9.9	13.3	0	1.9	3.4	
3,3-Dimethylbutyric	0.8	1.9	0.2	11.9	36.4	2.3	7.3	5.4	0	13.9	16.8	0.4	0.8	1.7	3.4
2-Ethylbutyric	0	1.5[f]	0.6	4.9	15.1	0	33.5[f]	14.0	0.8	8.4	10.6	2.6	3.8	3.4	24.3
Caproic	0	3.1	0.4	6.7	36.7	0	21.2	4.8	0.3	11.6	12.4	0	1.3	1.8	

[a] See the chromatograms in Figure 1. [b] Additional peak components. See text. [d] *x*-C_{14}, *x*-C_{16}, or *x*-C_{18} represent one of three alphabetically numbered peaks. [e] Peaks 1, 5, and 10 are overlapped with peaks A, B, and C, respectively. [f] Peaks 1, 5, and 10 are overlapped with peaks M, N, and O, respectively.

[2] P-75 and P-93 are P-(C_3H_7 + 32) and P-(C_3H_7 + 32 + 18).

[3] P-61 and P-79 are P-(C_2H_5 + 32) and P-(C_2H_5 + 32 + 18).

C_{15}-fatty acid and these two series of branched-chain fatty acids play a specific and common role in the physiology of *B. subtilis* and their synthesis is controlled to fulfil this function. It is interesting that the abundance of *n*-C_{18}-fatty acid was noticeably increased by the addition of most C_6-fatty acids studied with the exception of 3,3-dimethylbutyric and caproic acids.

Discussion

The eight possible isomers of alkanoic C_6-fatty acids have been studied for the incorporation into long-chain fatty acids by growing cells of *B. subtilis*. The incorporation of the five active fatty acids into long-chain fatty acids may be summarized as follows:

$$RCOOH + n[C_2] \longrightarrow RCH_2(CH_2CH_2)_{n-1}CH_2COOH$$

where R = $CH_3CH_2CH_2CH(CH_3)$, $CH_3CH_2CH(CH_3)CH_2$, $CH_3CH_2C(CH_3)_2$, $(CH_3)_3CCH_2$, or $(CH_3CH_2)_2CH$ and n = 4, 5, or 6.

Work in Bloch's laboratory (Butterworth and Bloch, 1970) and in this laboratory[4] has shown that the system carrying out *de novo* synthesis of long-chain fatty acids in *B. subtilis* is a soluble one and that malonyl-CoA is involved as chain extender to elongate the appropriate short-chain acyl-CoA esters to the corresponding fatty acids with 14–18 carbon atoms. Hence [C_2] in the equation is malonyl-CoA. RCOOH must initially be transformed to its CoA ester before it is incorporated into the related long-chain fatty acids. The order of the five active C_6-fatty acid isomers is shown to be as follows: 2-ethylbutyric $\gg$ 2-methylpentanoic > 3-methylpentanoic > 2,2- or 3,3-dimethylbutyric acids (Table IV). 2,3-Dimethylbutyric acid was inactive as shown by the absence of the expected long-chain isomers. The normal and iso-C_6-acids were judged to be inactive since neither of these changed significantly the amount of the expected long-chain isomers produced. Confirmatory tracer experiments have not yet been done. A similar study in this laboratory done under identical conditions (Kaneda, 1963b) has shown that the order of activity of C_3- to C_5-fatty acid isomers is as follows: isobutyric $\gg$ propionic, butyric > valeric, isovaleric, 2-methylbutyric. These results, taken together with the present observation with C_6-fatty acids suggest that requirements for a short-chain fatty acid to be an active chain initiator in the fatty acid synthesizing system of *B. subtilis* are a chain length of three or four carbon atoms having a methyl or an ethyl side chain at α position of the carbon chain, a β-side chain has distinct but lower activity. An apparent exception to this generalization is 2-methylbutyric acid which should be highly active and its seeming inactivity may be explained as follows: 2-methylbutyrate or its CoA ester is probably as good as the other two but, in *B. subtilis*, 2-methylbutyryl-CoA is supplied endogenously to such an extent (as can be recognized by the fact that anteiso-C_{15}- and anteiso-C_{17}-fatty acids are most abundant of total fatty acids) that additional and exogenous 2-methylbutyric acid is not utilized much by this organism.

Additional short- or medium-chain fatty acids which have been studied for activity as the chain initiator in the fatty acid synthesis are *n*-C_7-, *n*-C_8-, *n*-C_9-, *n*-C_{10}-, *i*-C_7-, and pivalic (neo-C_5)-acids. All these, however, have been found to be inactive.[5] Although data are not yet complete, the following generalization may be possible: to be incorporated into long-chain fatty acids, the chain length of a normal fatty acid should be in a range of two to five carbon atoms and this range for branched-chain series tends to shift toward the longer side.

The addition of 2-methylpentanoic acid caused the synthesis of the three related long-chain fatty acids: 10-methyltridecanoic, 12-methylpentadecanoic, and 14-methylheptadecanoic, whereas the addition of 2-ethylbutyric acid caused the synthesis of three related long-chain fatty acids; 10-ethyldodecanoic, 12-ethyltetradecanoic, and 14-ethylhexadecanoic. These fatty acids were not well resolved from iso-fatty acids with the same carbon atoms; iso-C_{14}, -C_{16}, and -C_{18}, even by the 50-ft EGA SCOT column. If, however, one assumes that the production of iso-C_{16}-fatty acid does not change greatly by the addition of either 2-methylpentanoic or 2-ethylbutyric acid, the ratio of 12-methylpentadecanoic acid to iso-C_{16}-fatty acid in peak (B + 5) is calculated to be about 1, whereas the ratio of 12-ethyltetradecanoic acid to iso-C_{16}-fatty acid in peak (N + 5) is about 2 (Table IV). The same relative proportions are also obtained from the data of mass spectrometry (Figure 2, see text).

The present study has shown that a wide variety of new branched-chain fatty acids can be synthesized by *B. subtilis* if the appropriate short-chain fatty acids, and presumably their CoA esters as well, are exogenously supplied to the organisms. Under the standard culture conditions, acetyl-, isobutyryl-, 2-methylbutyryl-, and isovaleryl-CoA esters produced from related α-keto acids by specific α-keto acid dehydrogenases (Namba *et al.*, 1969) are the only available chain initiators. Hence four sets of fatty acids, normal, iso even numbered, anteiso, and iso odd numbered, are produced. It will be interesting to know the biological role and activity of the new fatty acids in some specific enzyme reactions, *e.g.*, if they have positional specificity in the synthesis of triglycerides or of phospholipids. Work along this line is in progress.

The purified system from epididymal adipose tissue of rat produces the same acid, iso-C_{16}, as the major product when either of the substrates, isobutyryl-CoA or isocaproyl-CoA, is used (Horning *et al.*, 1961). In contrast to the bacterial system described here and the rat system, the purified system from pigeon liver produces a longer chain fatty acid from the chain initiator with longer chain: *n*-C_{16} as the major product from acetyl-CoA, and *n*-C_{18} from butyryl-CoA (Bressler and Wakil, 1961). The mechanism by which the chain length of the major long-chain fatty acid produced, is regulated in the fatty acid synthesis still remains to be understood.

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Cobamides and Ribonucleotide Reduction. VII. Cob(II)alamin as a Sensitive Probe for the Active Center of Ribonucleotide Reductase*

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ABSTRACT: Incubation of deoxyadenosylcobalamin with a molar excess of ribonucleotide reductase from *Lactobacillus leichmannii*, dihydrolipoate, and a nucleoside triphosphate results in the formation of a paramagnetic cobamide. The reaction does not occur if any component of the system is omitted, is relatively slow under optimal conditions, and is believed due to oxidative degradation of a reactive cobamide intermediate. Electron spin resonance spectra of the cobamide product recorded with frozen solutions at low temperature indicate that the cobamide is cob(II)alamin, but the spectra show unique hyperfine and superhyperfine structure. Similar cob(II)alamin electron spin resonance spectra were obtained when cob(II)alamin was formed: (1) from hydroxocobalamin and dihydrolipoate in the presence of reductase, a nucleoside triphosphate and 5′-deoxyadenosine or certain other closely related nucleosides; (2) by photolysis of deoxyadenosylcobalamin in the presence of dihydrolipoate, a nucleoside triphosphate and reductase; or (3) by photolysis of other cobamides in the presence of a nucleoside triphosphate, 5′-deoxyadenosine, dihydrolipoate, and reductase. The details of the spectra, especially in the low-field region, vary significantly with the identity of the nucleoside triphosphate present, the nucleoside present, and the conditions used for recording the spectra. The data are interpreted to mean that when cob(II)alamin is bound to the active center of the reductase the unpaired electron of the cobalt atom is subject to a relatively constant magnetic environment provided a nucleoside triphosphate is bound to the allosteric site of the enzyme and a nucleoside such as 5′-deoxyadenosine is bound to the active center.

It is assumed that this relatively constant magnetic environment depends on the conformation of the active center and of the ligands bound there, and that it is for this reason that the spectrum varies with the particular nucleoside triphosphate at the allosteric site and with the nucleoside at the active center.

In the reduction of ribonucleotides by the reductase of *Lactobacillus leichmannii* deoxyadenosylcobalamin assists hydrogen transfer by a mechanism similar to that for dioldehydrase and various mutases and deaminases (Hogenkamp *et al.*, 1968; Hogenkamp, 1968). These other reactions involve a specific intramolecular hydrogen transfer and also hydrogen transfer from substrate to the Co-bound 5′-methylene of deoxyadenosylcobalamin. The ribonucleotide reductase reaction differs from the others in that the donated hydrogen comes from thiol groups and is, therefore, in equilibrium with water. Consequently the reductase catalyses a unique hydrogen exchange between the 5′-methylene group of deoxyadenosylcobalamin and water. Also, hydrogen transfer is intermolecular in the reductase reaction and intramolecular in the others.

It has been assumed that in these reactions a reactive intermediate is formed by donation of a hydride ion to the 5′-methylene of the cobamide, and the the intermediate is closely related to cob(I)alamin, a powerful nucleophile that attacks water at neutral pH, especially in the presence of buffers (Tackett *et al.*, 1963; Das *et al.*, 1968). Although this postulated intermediate has not been detected directly, the results reported here indicate that cob(II)alamin, a likely product of its degradation, is slowly accumulated in the reductase system.

The cob(II)alamin formed from the coenzyme by ribonucleotide reductase, presumably at its active center, becomes a probe capable of relaying information through changes in its electron spin resonance spectrum related to changes in the conformation of the active center.

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