Bell, C. L., Victor, T. A., and Danyluk, S. S. (1971), Biophysical Society Abstracts, 15th Annual Meeting, Feb 15–18, New Orleans, La., p 88a.
Berg, H. (1965), *J. Electroanal. Chem. 10*, 371.
Blears, D. J., and Danyluk, S. S. (1967), *J. Amer. Chem. Soc. 89*, 21.
Brahms, J., Michelson, A. M., and Van Holde, K. E. (1966), *J. Mol. Biol. 15*, 467.
Broom, A. D., Schweizer, M. P., and Ts'o, P. O. P. (1967), *J. Amer. Chem. Soc. 89*, 3612.
Cavalieri, L., and Nemchin, R. (1964), *Biochim. Biophys. Acta 87*, 641.
Chan, S. I., Schweizer, M. P., Ts'o, P. O. P., and Helmkamp, G. K. (1964), *J. Amer. Chem. Soc. 86*, 4182.
Crothers, D. M., Sabol, S. L., Ratner, D. I., and Müller, W. (1968), *Biochemistry 5*, 1817.
Danyluk, S. S., and Victor, T. A. (1970), Quantum Aspects of Heterocyclic Compounds in Chemistry and Biochemistry, Pullman, B., and Bergmann, E. D., Ed., Academic Press, p 394.
Gellert, M., Smith, C. E., Neville, D., and Felsenfeld, G. (1965), *J. Mol. Biol. 11*, 445.
Giessner-Prettre, C., and Pullman, P. (1970), *J. Theor. Biol. 27*, 87.
Glasoe, P. K., and Long, F. A. (1960), *J. Phys. Chem. 64*, 188.
Hammonds, C. N., Westmoreland, T. D., and Day, M. C. (1969), *J. Phys. Chem. 73*, 4374.
Jackman, L. M., and Sternhell, S. (1969), Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry, New York, N. Y., Pergamon Press.
Laszlo, P. (1967), Progress in Nuclear Magnetic Resonance Spectroscopy, Emsley, J. W., Feeney, J., and Sutcliffe, L. H., Ed., Vol. 3, New York, N. Y., Pergamon Press, p 231.
Leng, M., and Felsenfeld, G. (1966), *J. Mol. Biol. 15*, 455.
Müller, W., and Crothers, D. M. (1968), *J. Mol. Biol. 35*, 251.
Müller, W., and Emme, I. (1965), *Z. Naturforsch. B 20*, 834.
Nemethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66*, 1773.
Prestegard, J. H., and Chan, S. I. (1969), *J. Amer. Chem. Soc. 91*, 2843.
Reich, E., and Goldberg, I. H. (1964), *Progr. Nucl. Acid Res. 3*, 183.
Sobell, H. M., Jain, S. C., Sakore, T. D., and Nordman, C. E. (1971), *Nature (London) 231*, 200.
Victor, T. A. (1969), Structure and Interactions of Actinomycin D, Ph.D. Thesis, University of Illinois at the Medical Center, Chicago, Ill.
Victor, T. A., Hruska, F. E., Bell, C. L., and Danyluk, S. S. (1969), *Tetrahedron Lett. 53*, 4721.
Worley, J. D., and Klotz, I. M. (1966), *J. Chem. Phys. 45*, 2868.

Biosynthesis of Methylheptadecanes in *Anabaena variabilis*. *In Vitro* Incorporation of *S*-[*methyl*-^{14}C]Adenosylmethionine†

Steven W. G. Fehler and Robley J. Light*

ABSTRACT: Sonication of lysozyme-treated *Anabaena variabilis* cells yielded a cell-free extract which catalyzed the incorporation of the methyl group from *S*-[*methyl*-^{14}C]adenosylmethionine into the branched methylheptadecanes. The apparent K_m for *S*-adenosylmethionine was 1.1×10^{-4} M. The pH optimum was 7.0, and a partial dependence on NADPH could be demonstrated in a short-term dialysis experiment. Activity was inhibited markedly by 10^{-3} M Cu^{2+}, 10^{-3} M Zn^{2+}, 10^{-3} M EDTA, 10^{-3} M dithiothreitol, and by 0.1% solutions of the detergents Triton X-100, sodium deoxycholate, sodium dodecyl sulfate, and celylpyridinium chloride.

In vivo incorporation studies have established that [*methyl*-^{14}C]methionine serves as a radioactive precursor to the methyl-branched alkanes of blue-green algae (Han *et al.*, 1969; Fehler and Light, 1970). Mass spectrometry of the branched alkanes from *Nostoc muscorum* established their structure as a 50:50 mixture of 7- and 8-methylheptadecane by comparison with authentic synthetic material (Han *et al.*, 1968). More recently, gas–liquid chromatography (glc) on a highly efficient 750 ft × 0.02 in. column established the presence of about 10% of 6-methylheptadecane in this mixture (Han and Calvin, 1970). The mass spectrum of the methylheptadecane mixture from *Anabaena variabilis* appears identical with that from *N. muscorum* (Fehler and Light, 1970). *In vivo* incorporation studies with [*methyl*-$^2H_3$]methionine in *A. variabilis* established that the methyl group is incorporated specifically into the branched methyl group of the methylheptadecanes, and that all three deuterium atoms are retained in the transfer.

This report describes a crude cell-free system from *A. variabilis* which catalyzes the incorporation of the methyl group from *S*-[*methyl*-^{14}C]adenosylmethionine into the methylheptadecanes.

Experimental Section

Anabaena variabilis cultures were grown as described previously (Fehler and Light, 1970), except that 4.5 l. of medium was contained in the glass tubes, and cells were harvested at

† From the Department of Chemistry, Florida State University, Tallahassee, Florida 32306. *Received January 20, 1972.* Supported by Grants GB 8325 and GB 26325 from the National Science Foundation and by a Career Development award (to R. J. L.), K3-AM-11,762, from the Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

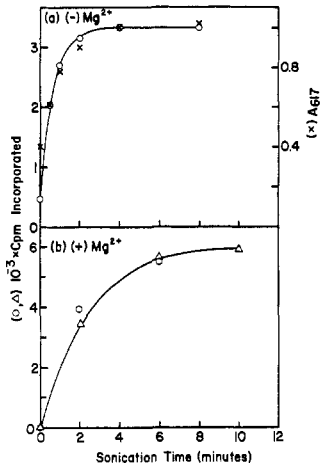

FIGURE 1: Effect of sonication on the incorporation of *S*-[*methyl*-^{14}C]adenosylmethionine into hydrocarbon. Conditions of growth and lysozyme treatment of *A. variabilis* cells and of the assay for radioactive hydrocarbon product are described in the Experimental Section. All incorporation values are averages of duplicate incubations. (a) Cells were harvested from a culture with a Klett reading of 500. The sonication was carried out in the absence of Mg^{2+}, and 0.6-ml aliquots of the total sonicated extract (not centrifuged) were incubated for 30 min with 19.2 nmoles (0.1 μCi) of *S*-[*methyl*-^{14}C]-adenosylmethionine. In a control incubation made with cells prior to lysozyme treatment, the incorporation value was 176 cpm. For estimation of the release of protein, cells were centrifuged at 12,000*g* for 10 min, and the absorbance of the supernatant at 617 mμ was taken as a measure of the phycocyanin proteins. (b) Cells were harvested from a culture with a Klett reading of 560. The sonication was carried out in the presence of 10^{-3} M Mg^{2+}, and aliquots of the sonicated extract were centrifuged for 10 min at 12,000*g* prior to incubation. Aliquots (0.6 ml) of either the uncentrifuged (○) or the supernatant fractions (△) were incubated for 30 min with 19.2 nmoles (0.1 μCi) of *S*-[*methyl*-^{14}C]adenosylmethionine.

Klett readings varying from 400 to 650 (540-mμ filter). Cells were harvested by batch centrifugation for 5 min at 12,000*g*, suspended in distilled water, and recentrifuged, and then resuspended in 40 ml of 0.1 M TES buffer[1] (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid), pH 7.0. In some experiments the buffer also contained 4×10^{-3} M Mg^{2+}. Lysozyme (1.5×10^6 units) from Sigma Chemical Company was added, and the cell suspension was incubated at room temperature for 20 min. The suspension was cooled to 5° and sonicated for 10 min at maximum power with a Model S-110 Branson sonifier. The sonication was carried out in a Rosette cell cooled in an ice–salt bath which maintained the temperature at about 5°. The extract was centrifuged for 10 min at 12,000*g*, and the resulting supernatant was employed for most of the studies described in this paper. The supernatant could be stored frozen for at least 2 weeks without loss of activity.

The assay of methylheptadecane biosynthesis catalyzed by this extract involves the incorporation of ^{14}C from L-*S*-[*methyl*-^{14}C]adenosylmethionine into hydrocarbon. Standard incubations contained 0.6 ml of crude extract, 19.2 nmoles (0.1 μCi) of *S*-[*methyl*-^{14}C]adenosylmethionine, and other additions as indicated in each experiment. Incubations proceeded for 30 min at 27° and were stopped by the addition of 3 ml of methanol. The mixture was extracted three times with 3-ml aliquots of hexane, and the emulsion formed in each extraction was broken by centrifugation. Carrier *n*-octadecane (250 μg) was added to the combined hexane extracts, and the solvent was evaporated under nitrogen in order to remove traces of methanol and water. The residue was redissolved in 0.5 ml of hexane and applied to a column containing 1.0 g of 110° heat-activated neutral Alcoa alumina layered on 0.5 g of silicic acid (Mallinckrodt Silicar CC-4, 100–200 mesh). Hydrocarbons were eluted with 15 ml of hexane. The hexane was evaporated in a scintillator bottle, 15 ml of scintillator solution (4 g of diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. of toluene) was added, and the radioactivity was assayed in a Tri-Carb Model 3214 liquid scintillation counter at settings usually giving 80% efficiency for ^{14}C. Duplicate incubations varied less than 10%, and boiled enzyme controls yielded less than 10 cpm above background.

In order to establish the identity of the radioactive hydrocarbon product, a threefold larger incubation was carried out for 2 hr, and the hexane extract was chromatographed without added carrier. Aliquots of the hexane eluate from the chromatography column were analyzed by glc as described previously (Fehler and Light, 1970). Effluent fractions from the glc column were collected in U tubes cooled in an acetone–Dry Ice bath (92% collection efficiency), rinsed into scintillator bottles with scintillator solution, and assayed for radio activity.

In experiments testing potential precursors for the hydrocarbons in the cell-free extract it was found that L-[*methyl*-^{14}C]methionine was incorporated as well as L-*S*-[*methyl*-^{14}C]-adenosylmethionine. Other potential precursors for the hydrocarbon chain tested were sodium [1-^{14}C]acetate, 28 μCi/μmole; [1-^{14}C]acetyl-coenzyme A, 47 μCi/μmole; [1-^{14}C]palmitylcoenzyme A, 42 μCi/μmole; and [3-^{14}C]serine, 5 μCi/μmole. None of them were incorporated in detectable amounts.

All radioactive precursors were obtained from New England Nuclear. Aliquots were assayed for radioactivity using a dioxane scintillator solution (8 g of diphenyloxazole and 100 g of naphthalene per l. of dioxane) which gave a counting efficiency of 61% for ^{14}C. Scintillation materials were obtained from Packard. TES buffer, dithiothreitol, and carrier *S*-adenosylmethionine iodide were obtained from Calbiochem.

Results

An extract of *A. varabilis* cells which will catalyze the incorporation of *S*-[*methyl*-^{14}C]adenosylmethionine into hydrocarbon can be obtained by sonication of a concentrated, lysozyme-treated, cell suspension. Figure 1 shows that maximum release of activity is obtained by 6–8 min of sonication in the presence of Mg^{2+}, and by 2–4 min in the absence of Mg^{2+}. There is a parallel release of protein as illustrated by the phycocyanin pigments which were measured by their absorbance at 617 mμ (ÓhEocha, 1965). Microscopic examination of the suspension showed that lysozyme treatment was sufficient to convert most of the cells to spheroplasts, and that no cells remained visible after two minutes of sonication.

Glc analysis of the incorporated radioactivity showed that more than 99% of the product is accounted for in the methylheptadecane fraction (Figure 2).

In view of the *in vivo* studies with [*methyl*-^{14}C]methionine and [*methyl*-^{2}H$_3$]methionine (Fehler and Light, 1970), the methylheptadecane product is very likely labeled in the branched methyl group. This would indicate that an endogenous acceptor is present in the crude extract which can accept the methyl group in the process of being converted to hydrocarbon. The extract is not capable of converting pre-

[1] Abbreviation used is: TES buffer, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.0).

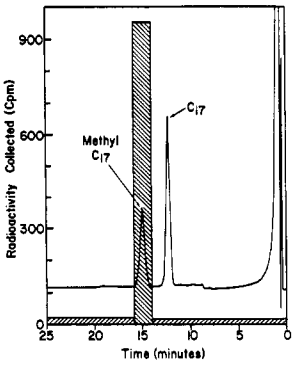

FIGURE 2: Glc of the radioactive hydrocarbon produced from sonicated extracts incubated with *S*-[*methyl*-^{14}C]adenosylmethionine. The extract was prepared and incubated without added Mg^{2+} as described in the Experimental Section. Isolation of the radioactive hydrocarbon product is also described in the Experimental Section. An aliquot of this hydrocarbon product was injected onto a 6 ft × 0.25 in. glass column packed with 4% SE-30 on Gas Chrom Q and operated at 165°. The recorder tracing represents the mass of the endogenous hydrocarbons from the extract, and the bar graph represents the radioactivity collected from the effluent gas. The collection efficiency was about 92%, and hence about 98% of the injected radioactivity was accounted for by that collected in the methylheptadecane region. Triplicate analyses varied only ±2%.

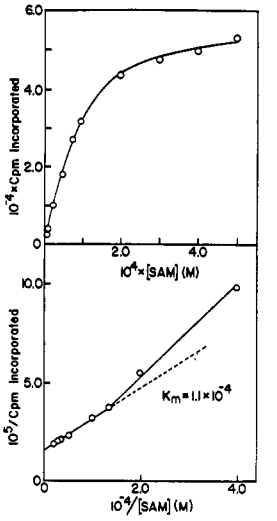

FIGURE 4: Effect of *S*-[*methyl*-^{14}C]adenosylmethionine (SAM) concentration on the incorporation of radioactivity into hydrocarbon. Extracts were prepared as described in the Experimental Section, and 0.5-ml aliquots were incubated for 30 min at 27° with 2.4 μmoles of Mg^{2+}, 72 nmoles of NADPH, and 0.1 ml of 0.1 M TES buffer containing indicated amounts of *S*-[*methyl*-^{14}C]adenosylmethionine (specific activity 52.3 μCi/μmole). The product hydrocarbon was isolated and assayed as described in the Experimental Section.

cursors such as acetate, acetyl-CoA, or palmityl-CoA into either the methyl acceptor or into heptadecane under the conditions of these incubations, however.

Cells from older cultures (Klett readings above 550) yielded somewhat more stable extracts than cells from younger cultures (Klett readings below 475). The former could be stored for 13 hr at 4° or 1.5 hr at room temperature without loss of activity, while the latter lost activity completely after 1.5 hr at 4°.

Figure 3 shows that after a short lag period the reaction is linear for at least 1 hr. Since subsequent experiments were carried out with 30-min incubations, the incorporation values obtained are good approximations of initial velocities.

The effect of *S*-adenosylmethionine concentration is shown in Figure 4. There is a break in the reciprocal plot at concentrations below 10^{-4} M. This break could arise from dilution of the radioactive substrate by endogenous *S*-adenosylmethionine or from competing reactions in the crude extract, and no significance should be attached to it at this stage of purification. The apparent K_m based upon the points at higher substrate concentration is 1.1×10^{-4} M.

The effect of pH on activity is shown in Figure 5. Lowering the pH to 5.0 and immediately readjusting to pH 7.0 led to irreversible loss of activity, hence there is also a pH effect on the stability of the extract. For this reason all experiments were run at pH 7.0.

Upon dialysis of the extract in the absence of Mg^{2+}, lost activity could not be restored by adding Mg^{2+} back to the extracts. The amount of activity loss could be decreased, however, by including Mg^{2+} in the dialyzing buffer (Table I).

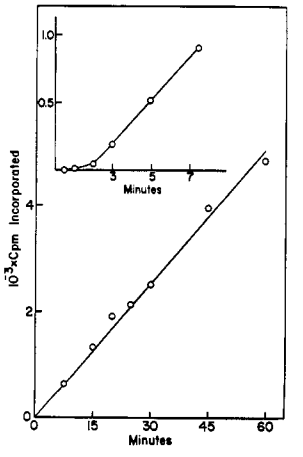

FIGURE 3: Time course of incorporation of *S*-[*methyl*-^{14}C]adenosylmethionine into hydrocarbon catalyzed by *A. variabilis* extract. The inset shows data taken at shorter incubation periods in a separate experiment. Preparation and incubation of the extract without added Mg^{2+}, and isolation and assay of the hydrocarbon product are described in the Experimental Section.

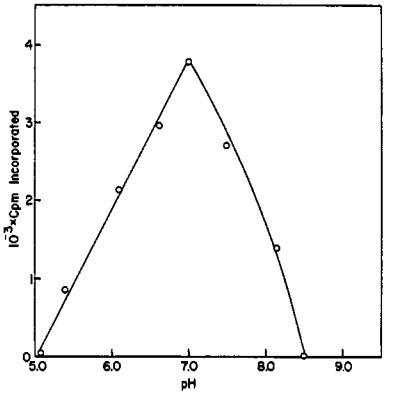

FIGURE 5: Effect of pH on the incorporation of *S*-[*methyl*-^{14}C]-adenosylmethionine into hydrocarbon. Extracts were adjusted to the appropriate pH with dilute HCl or KOH and 0.6-ml aliquots were incubated for 30 min at 27° in the presence of 2.4 μmoles of Mg^{2+}, 72 nmoles of NADPH and 19.2 nmoles (0.1 μCi) of *S*-[*methyl*-^{14}C]-adenosylmethionine. The product hydrocarbon was isolated and assayed as described in the Experimental Section.

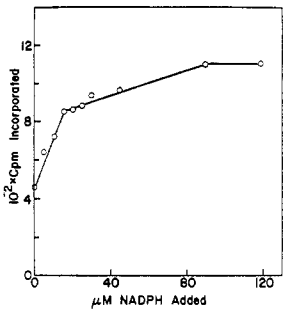

FIGURE 6: NADPH stimulation of the incorporation of *S*-[*methyl*-^{14}C]adenosylmethionine into hydrocarbon catalyzed by a partially dialyzed extract. The extract was prepared with 4×10^{-3} M Mg^{2+} as described in the Experimental Section and dialyzed for 6 hr at 4° against 0.1 M TES buffer (pH 7.0) containing 4×10^{-3} M Mg^{2+}. Incubations were run for 30 min at 27° and contained 0.6-ml aliquots of dialyzed extract, 19.2 nmoles (0.1 μCi) of *S*-[*methyl*-^{14}C]-adenosylmethionine, and the indicated concentration of NADPH. The product hydrocarbon was isolated and assayed as described in the Experimental Section.

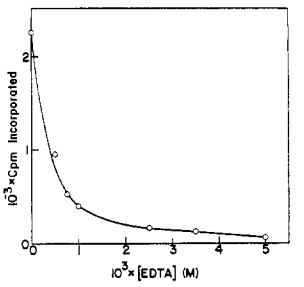

FIGURE 7: Inhibition of *S*-[*methyl*-^{14}C]adenosylmethionine incorporation into hydrocarbon by EDTA. Preparation of the extract and isolation and assay of the product hydrocarbon are described in the Experimental Section. Incubations were run for 30 min at 27° and contained 0.5 ml of sonicated extract, 19.2 nmoles (0.1 μCi) of *S*-[*methyl*-^{14}C]adenosylmethionine, and 0.1 ml of 0.1 M TES buffer containing EDTA to give the final concentration indicated.

When various cofactors were tested in the reaction it was observed that neither ATP nor CoA-SH had any effect if assayed separately, but that together they inhibited the incorporation (Table II). This inhibition was prevented by the addition of NADPH and can be explained as an NADPH requirement, with ATP and CoA-SH stimulating a side reaction which results in the oxidation of endogenous NADPH. In support of the explanation it was observed that addition of ATP and CoA-SH to a 27-fold diluted extract resulted in a rapid decrease in absorbance at 340 mμ. The decrease was equivalent to an endogenous NADPH concentration of 1.2×10^{-4} M in the undiluted extract.

A requirement for some reducing agent such as NADPH might be expected from consideration of possible methylation mechanisms which should involve a nucleophilic displacement reaction of a carbon–carbon double bond on the methyl group of *S*-adenosylmethionine (Lederer, 1969). Any carbonium ion or double bond generated in this process would require reduction to produce a saturated alkane. It was not possible to deplete completely the endogenous NADPH by dialysis or charcoal treatment without causing irreversible loss of activity. A partial dependence on added NADPH could be demonstrated, however, by a short-term dialysis experiment in which the irreversible loss of activity was 36% (Figure 6).

In view of the stabilization by Mg^{2+}, the effects of EDTA and of other metal ions were tested. EDTA inhibits markedly at 10^{-3} M (Figure 7). The divalent cations Cu^{2+} and Zn^{2+} inhibited the reaction completely at 10^{-3} M, while other cations showed only moderate or no inhibition at this concentration (Table III).

Dithiothreitol is an inhibitor of the reaction at concentrations above 10^{-3} M as shown in Table IV. This inhibition can be overcome by preincubation of the dithiothreitol with Fe^{3+}, but not by Fe^{2+} (Figure 8). The stoichiometry of this effect, showing a maximum at a 2:1 ratio of Fe^{3+}:dithiothreitol, is that expected for oxidation of dithiothreitol by Fe^{3+} (Cleland, 1964), and indicates that dithiothreitol must be in the reduced form to be inhibitory. The incomplete protection by Fe^{3+}, and the increasing inhibition shown at Fe^{3+} concentration above

TABLE I: Mg^{2+} Effect on Dialysis of Extract.

Extract Dialyzed			Mg^{2+} Added at Reaction Time[c]	Cpm Incorporated into Hydrocarbon[d]
with Mg^{2+}[a]	without Mg^{2+}	Aged Control[b]		
+	−	−	−	1153
−	+	−	−	572
−	+	−	+	653
−	−	+	−	1581

[a] Mg^{2+} (4 mM) was added to the dialysis solution. Dialysis was for 6 hr at 4°. [b] The aged control was maintained at 4° for 6 hr without dialysis. [c] Mg^{2+} (4 mM) was added to the reaction just prior to the addition of L-*S*-[*methyl*-^{14}C]adenosylmethionine. [d] Average of duplicate incubations run for 30 min at 27°. Incubations contained 0.6 ml of extract, 72 nmoles of NADPH and 19.2 nmoles (0.1 μCi) of *S*-[*methyl*-^{14}C]-adenosylmethionine, and product was isolated and assayed as described in the Experimental Section.

TABLE II: Effect of ATP and Coenzyme A on Incorporation.

ATP[a]	CoA[b]	NADPH[c]	Cpm Incorporated into Hydrocarbon[d]
−	−	−	3074
+	−	−	3117
−	+	−	3024
−	−	+	3169
+	+	−	861
+	+	+	2934

[a] ATP (0.5 mM) was preincubated with sonicated extract for 10 min prior to the addition of 19.2 nmoles (0.1 μCi) of L-*S*-[*methyl*-^{14}C]adenosylmethionine. [b] Coenzyme A (0.5 mM) was preincubated with sonicated extract similarly to ATP. [c] NADPH (1.5 mM) was preincubated with sonicated extract similarly to ATP. [d] Average of duplicate incubations carried out for 30 min at 27°. The extract control (no ATP, CoA, or NADPH) was aged 10 min prior to start of the reaction. Product was isolated and assayed as described in the Experimental Section.

TABLE III: Effect of Metal Ions on Incorporation.[a]

Metal Ion	% of Control[b]
Mg^{2+}	109
Mn^{2+}	116
Ca^{2+}	91
Fe^{2+}	89
Fe^{3+}	76
Co^{2+}	64
Cu^{2+}	0
Zn^{2+}	0

[a] Incubations contained 0.4 ml of sonicated extract, 0.2 ml of 0.1 M TES buffer, pH 7.0, 19.2 nmoles (0.1 μCi) of *S*-[*methyl*-^{14}C]adenosylmethionine and the indicated metal ions at 10^{-3} M concentration. [b] All ions but Fe^{3+} were run in one experiment giving a control incorporation value of 1896 cpm; the control for the Fe^{3+} experiment was 3872 cpm. Product was isolated and assayed as described in the Experimental Section.

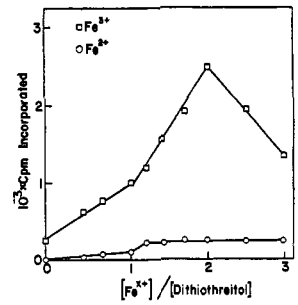

FIGURE 8: Effect of Fe^{3+} (□) and Fe^{2+} (○) on the dithiothreitol inhibition of *S*-[*methyl*-^{14}C]adenosylmethionine incorporation into hydrocarbon. Preparation of the extract and isolation and assay of the product hydrocarbon are described in the Experimental Section. Dithiothreitol (600 nmoles) and the indicated quantity of Fe^{3+} or Fe^{2+} were preincibated for 10 min in 0.1 ml of 0.1 M TES buffer, pH 7.0, prior to the addition of 0.5 ml of extract and 19.2 nmoles (0.1 μCi) of *S*-[*methyl*-^{14}C]adenosylmethionine. Incubations were then run for 30 min at 27°. The Fe^{3+} and Fe^{2+} experiments were done on different enzyme extracts. Control values with no added dithiothreitol or Fe were 3424 cpm (□) and 2461 cpm (○), respectively.

the 2:1 ratio, can be explained on the basis of inhibition shown by Fe^{2+} and Fe^{3+} alone (Table III).

Table V shows that several classes of detergent markedly inhibit the reaction at a concentration of 0.1%.

Preliminary attempts at standard purification procedures have all led to loss of activity. These attempts include centrifugation for 1 hr at 100,000*g*; precipitation with ammonium sulfate, acetone, ethanol, or acid, and gel filtration on Sephadex G-25 or G-50.

Discussion

Albro and Dittmer (1969) have reported a cell-free preparation from the bacterium *Sarcina lutea* which catalyzes the biosynthesis of long-chain hydrocarbons, principally C_{27} and C_{29}, from [2-^{14}C]acetate, [1-^{14}C]palmitate, [16-^{14}C]palmitate, and [16-^{14}C]palmityl-CoA. Characteristics of this system favor a biosynthetic mechanism involving head-to-head condensation of two intermediate chain length fatty acids arising from separate pools (Albro and Dittmer, 1970).

An alternate mechanism, involving chain elongation of fatty acids followed by decarboxylation, may be involved in the biosynthesis of the *n*-C_{29} alkane in leaves from the plant *Brassica oleracea*. Kolattukudy (1970) has summarized evidence, obtained largely in his laboratory, supporting the elongation–decarboxylation pathway in this system.

Hydrocarbon production in blue-green algae is distinguished from either the plant or bacterial systems in that the principle hydrocarbon is *n*-heptadecane (Winters *et al.*, 1969), and branched alkanes other than the iso or anteiso type are found (Han *et al.*, 1968). The data from Han *et al.* (1969) on *in vivo* incorporation of [18-^{14}C]stearate into heptadecane and

TABLE IV: Inhibition of Incorporation by Dithiothreitol.[a]

Dithiothreitol (M)	Cpm Incorporated into Hydrocarbon[b]
None	1791
1×10^{-6}	2080
1×10^{-5}	1834
1×10^{-4}	1879
1×10^{-3}	4.9
1×10^{-2}	1.1

[a] Incubations contained 0.4 ml of sonicated extract, 0.2 ml of 0.1 M TES buffer, pH 7.0, 19.2 nmoles (0.1 μCi) of *S*-[*methyl*-^{14}C]adenosylmethionine and dithiothreitol at the indicated concentration. [b] Product was isolated and assayed as described in the Experimental Section.

TABLE V: Inhibition of Incorporation by Detergents.[a]

Detergent	Concentration (g/100 ml)	Cpm Incorporated into Hydrocarbon[b]
None		1387
Triton X-100	0.001	1348
	0.01	918
	0.1	130
Sodium deoxycholate	0.001	1234
	0.01	1133
	0.1	531
Sodium dodecyl sulfate	0.001	1492
	0.01	889
	0.1	131
Cetylpyridinium Chloride	0.001	1296
	0.01	1025
	0.1	4

[a] Incubationsc ontained 0.5 ml ofsonicated extract, 0.1 ml of 0.1 M TES buffer, pH 7.0, 19.2 nmoles (0.1 μCi) of *S*-[*methyl*-^{14}C]adenosylmethionine, and the detergents at the indicated concentrations. [b] Product was isolated and assayed as described in the Experimental Section.

of [G-^{3}H]vaccenate into the methylheptadecanes of *N. muscorum* support a direct decarboxylation mechanism in blue-green algae as opposed to a head-to-head condensation of shorter chain fatty acids. Their incorporations were low, however, and one could not exclude degradation to acetate or some other simple precursor prior to incorporation.

Although direct decarboxylation of a saturated fatty acid to form a hydrocarbon would appear to be a very simple reaction, its mechanism may not be at all simple. Most decarboxylations appear to involve assistance of a neighboring functional group which can serve as an electron sink.

The reaction catalyzed by the *A. variabilis* extract reported in this paper represents the last stages of biosynthesis of the branched methylheptadecanes. The identification of an endogenous methyl group acceptor or of a methylated intermediate in this reaction should clarify the biosynthetic pathway, and might also provide clues to the mechanism of *n*-heptadecane biosynthesis in this organism.

References

Albro, P. W., and Dittmer, J. C. (1969), *Biochemistry 8*, 3317.
Albro, P. W., and Dittmer, J. C. (1970), *Lipids 5*, 320.
Cleland, W. (1964), *Biochemistry 3*, 480.
Fehler, S. W. G., and Light, R. J. (1970), *Biochemistry 9*, 418.
Han, J., and Calvin, M. (1970), *J. Chem. Soc. D*, 1490.
Han, J., Chan, H. W. S., and Calvin, M. (1969), *J. Amer. Chem. Soc. 91*, 5156.
Han, J., McCarthy, E. D., Calvin, M., and Benn, M. H. (1968), *J. Chem. Soc. C*, 2785.
Kolattukudy, P. E. (1970), *Annu. Rev. Plant. Physiol. 21*, 163.
Lederer, E. (1969), *Quart. Rev., Chem. Soc. 23*, 453.
ÓhEocha, C. (1965), *in* The Chemistry and Biochemistry of Plant Pigments, Goodwin, T. W., Ed., New York, N. Y., Academic Press, p 175.
Winters, K., Parker, P. L., and Van Baalen, C. (1969), *Science 163*, 467.

Studies on the Mutual Influences of Substrates on Bovine α-Keto Acid Metabolism†

Wayne A. Johnson* and Jerald L. Connelly‡

With the Technical Assistance of Martin T. Glynn

ABSTRACT: The metabolic interrelationships among the branched-chain α-keto acids, α-ketoisocaproate, α-keto-β-methylvalerate, α-ketoisovalerate, and effects of these metabolites upon the metabolism of pyruvate and of α-ketoglutarate in bovine liver mitochondria have been investigated. The apparent Michaelis constants, determined for mitochondrial dehydrogenases, are of the order of 4×10^{-4} M, for the branched-chain α-keto acids, while those for pyruvate and α-ketoglutarate are slightly lower. The degree and type of influence exerted among the various α-keto acids was examined directly by following the enzymic liberation of $^{14}CO_2$ from carboxyl-labeled substrate in the presence of a second unlabeled α-keto acid. A mutually inhibitory pattern among α-ketoisocaproate, α-ketoisovalerate, and α-keto-β-methylvalerate was noted. In addition, each of the branched-chain keto acids exerted a marked inhibition of pyruvate and α-ketoglutarate dehydrogenase activity. Inhibition was of the competitive type in all instances except for α-ketoglutarate dehydrogenase activity, which is "mixed." The apparent inhibitor constants were in a range such that a significant mutual influence among the branched-chain α-keto acids could exist *in vivo*, at normal cellular concentrations of these metabolites. Moreover, a significant effect of α-ketoisocaproate on pyruvate dehydrogenase was noticed. The net effect of the inhibitory action of the branched-chain α-keto acids on each other and upon pyruvate dehydrogenase in the normal animal is likely a homeostatic balance, which allows for catabolism of excess branched-chain amino acids, while concurrently influencing the flow of pyruvate to acetyl coenzyme A. These studies indicate that interrelationships among the various α-keto acid metabolisms could constitute a significant physiological regulatory mechanism in the normal animal. These relationships, extrapolated to the branched-chain ketoaciduric condition, are consistent with observed symptoms of the disease.

Since the discovery of branched-chain ketoaciduria (BCKA)[1] in humans, a number of investigators have proposed possible modes of action whereby the elevated levels of the branched-chain amino and keto acids may produce the observed physiological and neurological symptoms. Silberman *et al.* (1961) have suggested that increased keto acid concentration in the developing brain may decrease synthesis of

† From the Guy and Bertha Ireland Research Laboratory, Department of Biochemistry, University of North Dakota, Grand Forks, North Dakota. *Received July 19, 1971.* This work was aided by a grant from the National Science Foundation (GB-12934).

* National Defense Education Act Fellow (NDEA-69-02592). Present address: Department of Nutrition and Food Science, College of Home Economics, South Dakota State University, Brookings, S. Dak. 57006.

‡ This investigation was supported (in part) by the Public Health Service research career program award (1-K3-GM-7028) from the National Institute of General Medical Sciences.

[1] Abbreviations used are: BCKA, branched-chain-ketoaciduria; KIC, α-ketoisocaproate; KIV, α-ketoisovalerate; KMV, α-keto-β-methylvalerate; KB, α-ketobutyrate; KV, α-ketovalerate; KC, α-ketocaproate; NAD^-, nicotinamide-adenine dinucleotide (oxidized form); CoA, reduced coenzyme A.