The Biochemistry of Long-Chain, Nonisoprenoid Hydrocarbons. IV. Characteristics of Synthesis by a Cell-Free Preparation of *Sarcina lutea**

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ABSTRACT: Treatment of *Sarcina lutea* with lysozyme followed by sonication yields a cell-free preparation capable of incorporating fatty acids into hydrocarbons. Coenzyme A, Mg^{2+}, ATP, NADPH, and either pyridoxal phosphate or pyridoxamine phosphate were required for maximum incorporation of palmitate into hydrocarbon. At concentrations of 1.5×10^{-5} M, pyridoxamine phosphate gave twice the incorporation obtained with pyridoxal phosphate. The participation of the coenzyme A derivative of the fatty acid was consistent with the requirement for the first three cofactors, and in agreement with this, palmitoyl coenzyme A was incorporated into hydrocarbons at over 20 times the rate of the free acid in the absence of coenzyme A. The carboxyl carbon of palmitoyl coenzyme A was incorporated into hydrocarbons to only a limited extent, and the pyridoxal derivative appeared to function as a cofactor in the incorporation of fatty acids or their derivatives by a mode not involving decarboxylation. The relevance of these findings to a proposed mechanism of hydrocarbon biosynthesis in which the aliphatic group of a vinyl ether is transferred to the α-carbon of a fatty acid derivative which undergoes decarboxylation is discussed. Optimum incorporation of fatty acids into hydrocarbons was obtained at pH 8. The time course for incorporation and the dependence of the rate of incorporation on substrate and cell preparation concentrations are reported. Data on inhibitor studies of the system that further define cofactor requirements and suggest relationships to the synthesis of hydrocarbons in plant systems are presented and discussed.

In previous papers of this series (Albro and Dittmer, 1969a–c), we have presented evidence that hydrocarbon biosynthesis in *Sarcina lutea* occurs *in vivo* by the condensation of two fatty acids to yield a monounsaturated intermediate as follows

$$RCH_2COOH + R'CH_2COOH \longrightarrow CO_2 + RCH{=}CHCH_2R'$$

The monounsaturated hydrocarbons are reduced to form the fully saturated analog. The fact that ketones were not converted into hydrocarbons as efficiently as fatty acids has led us to postulate intermediates of such a condensation mechanism different from the ketones and secondary alcohols of the classical pathway proposed by Chibnall and Piper (1934). We have now determined and will subsequently report in detail that the aliphatic portion of the alk-1-enyl ether of a neutral plasmalogen is incorporated into hydrocarbons intact and presumably with the formation of a monounsaturated hydrocarbon directly. The characteristics of a cell-free preparation of *S. lutea* that is capable of incorporating various ^{14}C-labeled compounds into hydrocarbons are described here. More extensive studies of this system to be subsequently reported permitted the elucidation of the biosynthetic pathway outlined above. This is the first published description of a cell-free preparation capable of synthesizing long-chain, nonisoprenoid hydrocarbons.

Methods and Materials

Culture conditions for *S. lutea*, the extraction and fractionation of lipids, and assay of radioactivity in lipid fractions have been described previously (Albro and Dittmer, 1969a,b). In addition to the ^{14}C-labeled precursors used in previous studies, palmitoyl-1-^{14}C-CoA (New England Nuclear) and palmitoyl-16-^{14}C-CoA were used. The latter was synthesized by allowing the palmitic-16-^{14}C acid to exchange with an excess of unlabeled palmitoyl chloride (Borgström and Krabisch, 1963) and the reaction product was used to synthesize the CoA derivative as described by Seubert (1960).

Cell-Free Preparation. Freshly harvested or lyophilized cells of *S. lutea* from 48-hr cultures were suspended in 0.1 M phosphate, Tris-HCl, or Tris-phosphate buffer between pH 7 and 8. Routinely, potassium phosphate buffer, pH 8.0, containing 10^{-4} M disodium EDTA and 2×10^{-3} potassium thioglycollate was used since this was the buffer used in the assay procedure. A concentration of 2 g dry weight of cells/100 ml was used. Crystalline lysozyme (Calbiochem, A grade from egg white) was added to give a final concentration of 0.15 mg/ml and the mixture was stirred for 45 min at room temperature. A 3:1 mixture by weight of L-glutamic acid and L-aspartic acid was then added in a total concentration of 1 mg/ml to the resulting gel and the mixture was sonicated for 30 sec (Branson Sonifier at 6 A). Alternatively, the gel was dissipated by a 30-min incubation with 40 μg/ml of beef DNase (Salton and Freer, 1965) but more active preparations were obtained by sonication.

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Standard Assay Conditions. Routinely, 50 ml of the cell-free preparation was added to a 250-ml erlenmeyer flask to which 1 ml of a solution of coenzymes and the ^{14}C-labeled precursor had already been added. Labeled fatty acids were routinely added as a bovine serum albumin complex that was prepared by adding the labeled acid to bovine serum albumin (Sigma, fraction V) in pH 8 phosphate buffer (320 mg of albumin/7.4 ml of buffer). In the hydrocarbon synthesis assay, the flasks were lightly plugged with paper tissue and gently swirled at room temperature at 2 revolutions/sec on a rotary shaker. After 2.5-hr incubation, 5 ml of 6 N HCl and 100 ml of methanol were added and the mixture was transferred to a Waring Blendor. Chloroform was added in two 100-ml portions. The mixture was blended after each addition and finally transferred to a 500-ml graduated cylinder. After the two phases had separated the upper phase was drawn off by suction. The lower phase was filtered through a pad of glass wool and evaporated to dryness at 40–50° *in vacuo*. The lipid residue was applied to a Florisil column in hexane. The equivalent of 7 g of Florisil/g of cell-free preparation solids initially incubated or approximately 0.2 g of Florisil/mg of lipid was used. With a 7-g column, the hydrocarbons were eluted with 50 ml of hexane. The eluate was reduced in volume and then evaporated to dryness on tared planchets. The residue was weighed and assayed for radioactivity. When fatty acids were assayed, the lipids were transesterified with methanolic H_2SO_4 before fractionation on Florisil.

Usually labeled precursor with approximately 1 μCi of activity was used in the assay. The 50 ml of cell-free preparation used in the assay contained approximately 3.5 mg of hydrocarbon. Unless otherwise specified, the activities of the system are given in the units: disintegrations per minute of precursor incorporated per milligram of hydrocarbon per hour per gram dry weight of cells per microcurie of precursor.

Results

General Properties of Cell-Free Preparations. Preparations contained no viable cells as established by plate counts. All capacity to synthesize hydrocarbons was lost after storage at −20° for 2 months, but the activity was restored by dialysis against thioglycollate and augmentation with the cofactors described below. Three slow freeze–thaw cycles also reversibly inactivated the preparations. Lyophilized samples of the preparation stored in tightly closed containers retained activity for up to 4 months. The most satisfactory arrangement, however, was to lyophilize the intact cells and make up the cell-free preparation as needed. Preparations from cells stored up to 6 months showed no diminution of activity. The system was capable of incorporating ^{14}C-labeled acetate and isoleucine into fatty acids and hydrocarbons and palmitic acid into hydrocarbons. Whether the cell-free preparation was made up with Tris or phosphate buffer made no difference in the capacity to incorporate palmitate into hydrocarbons.

β Oxidation of Fatty Acids and Acrylate Inhibition. As reported earlier (Albro and Dittmer, 1969b,c) little or no β oxidation of fatty acids occurs in *S. lutea in vivo*. When palmitate-16-^{14}C was incubated with *S. lutea* cell-free preparations, 81% of the label recovered in the C-15 + C-16 fatty acids was found in the C-15 branched-chain acids. This redistribution was consistent with complete β oxidation of the labeled palmitate to acetate and utilization of the acetate in the synthesis of the branched-chain C-15 acid. When acrylate was added to the preparation in a concentration of 4×10^{-5} M, only 1.5% of the label recovered in the C-16 + C-15 fatty acids was found in the C-15 acids and no further decrease was achieved with a 2.5-fold increase in the concentration of acrylate. The incorporation of label from palmitate into hydrocarbon was stimulated 7.3 and 25.4% with the two concentrations of acrylate. The stimulation could have been due to an increase in the palmitate available by elimination of the loss by β oxidation or to a decrease in dilution of the palmitate pool by endogenous fatty acid synthesis. That is, the acrylate inhibition is consistent with inhibition of β oxidation or fatty acid synthesis. However, no effect of 5×10^{-4} M acrylate on the incorporation of acetate-2-^{14}C into fatty acids or hydrocarbons was observed. Presumably the effect of the acrylate was to inhibit β oxidation, and unless otherwise specified, acrylate was added to the reaction medium in all of the subsequent experiments described here.

Cofactor Requirements. Cell-free preparations were dialyzed at 4° in succession for 3 hr against 5×10^{-3} mM phosphate buffer (pH 8) and overnight against two changes of 5×10^{-4} M sodium thioglycollate–10^{-5} M EDTA in pH 8 phosphate buffer. The dialyzed preparation was lyophilized and stored over silica gel. Portions of the diffusate were treated with acid-washed Norit and this material and the remainder of the diffusate were freeze dried. The dried, nondiffusible material was reconstituted by resuspending it in pH 8 0.05 M potassium phosphate buffer–8.7 mM sodium thioglycollate with the aid of a Teflon pestle and glass homogenizer to give a final concentration of 0.7 g/50 ml. This operation was carried out at 4° and the reconstituted preparation was stored at this temperature until used. In these particular experiments, 0.237 mg of a mixture of L-valine, L-leucine, and L-isoleucine in the proportions by weight of 1:1:2 were added along with the glutamic and aspartic acid to the preparation just prior to sonication. In subsequent experiments, the addition of the three aliphatic amino acids was shown to have no effect on the incorporation of palmitate into hydrocarbons.

The diffusible material from the preparation had absorption maxima at ~260, 327, and 377 (aqueous solution). There was no detectable absorption at 340 or 450 mμ. Coenzyme A, pyridoxamine phosphate, and possibly NAD and/or NADP were therefore suspected of being present. NAD(P)H and flavin were probably absent or in very low concentrations. Charcoal treatment lowered the 260-mμ absorbance to one-fourth its initial value.

The results of experiments in which the various cell-free preparations supplemented with mixtures of cofactors were compared for their capacity to incorporate palmitate-^{14}C into hydrocarbons are given in Table I. Standard assay conditions were used with the addition of 1×10^{-4} M acrylate. The cofactors tested were selected on the basis of their relationship to the metabolism of known precursors and proposed intermediates of synthesis of hydrocarbons as established by studies of *S. lutea* and published reports on plant systems.

As shown in the first part of Table I, dialysis reduced the total activity by 46% and both dialyzed and undialyzed preparations were stimulated by the addition of cofactors. The activity of the dialyzed preparation was restored by the addition of an equivalent amount of the diffusate or approximately half restored by the addition of 0.5 equiv of diffusate. Char-

TABLE I: Cofactor Requirements for the Incorporation of Palmitate into Hydrocarbons *in Vitro*.[a]

Cell Preparation and Augmentation	Act.	Rel Act.[b]
Standard cell-free preparation		
+ No cofactors	1140	100
+ All cofactors[c]	2770	243
Dialyzed cell-free preparation		
+ No cofactors	640	56
+ All cofactors	3250	285
Dialyzed cell-free preparation		
+ No cofactors	644	100
+ All cofactors	3290	511
+ Equivalent of lyophilized diffusate	2800	435
+ 0.5 equiv of diffusate	1430	222
+ Equivalent charcoal-treated diffusate	2490	387
Dialyzed cell-free preparation		
+ All cofactors	3290	100
+ FAD (7.6×10^{-5} M)	3300	100
+ Thiamine hydrochloride (1.8×10^{-4} M)	3300	100
+ Thiamine pyrophosphate (1.3×10^{-4} M)	2720	83
+ EDTA (10^{-3} M)	3080	94
+ $MnSO_4$ (1.4×10^{-4} M)	2720	83
− $MgCl_2$	2630	80
− NADH	3490	106
− NADH and NADPH	2370	72
− ATP	2050	62
− Coenzyme A	1680	51
− Coenzyme A and ATP	1600	49
− Biotin and glutathione	3270	99
− Biotin, $MgCl_2$, and pyridoxal phosphate	970	29
− Pyridoxal phosphate	1840	56
− Biotin, $MgCl_2$, pyridoxal phosphate, NADH, and NADPH	1160	35

[a] The standard cell-free preparations were used except for the inclusion of aliphatic amino acids as indicated in the text. Conditions of dialysis are also given in the text. [b] Relative activities are presented as the per cent of the activity obtained with the initial conditions given in each section of the table. [c] When "all cofactors" were used to supplement the preparation the following compounds were added to give the concentration indicated: ATP, 3.2×10^{-4} M; coenzyme A, 5×10^{-5} M; NADH, 9×10^{-5} M; NADPH, 8×10^{-5} M; glutathione, 6.5×10^{-4} M; pyridoxal phosphate, 2×10^{-4} M; biotin, 4×10^{-6} M; and $MgCl_2$, 5×10^{-5} M.

coal treatment of the diffusate lowered its capacity to restore activity by only 11%.

Thiamine did not stimulate the otherwise supplemented system and thiamine pyrophosphate inhibited it. Presumably neither of these cofactors are required. The inhibitory effect of NADH indicated by the experiment reported in Table I was confirmed with two different lots of the coenzyme. $MnSO_4$ also appeared to inhibit the system, but this was not confirmed in subsequent experiments when the effect of Mn^{2+} in the presence of Mg^{2+} was tested. Glutathione showed no effect in this experiment, but in independent studies it and cysteine hydrochloride were found to have a stimulatory effect if thioglycollate was omitted when working up the preparation. Specific requirements for Mg^{2+}, ATP, coenzyme A, NADPH, and pyridoxal phosphate were indicated. No requirements for cations other than Mg^{2+}, biotin, NADH, or FAD were demonstrated. Doubling the concentrations of all of the cofactors in the complete system minus NADH did not increase the hydrocarbon-synthesizing capac-

TABLE II: Efficiency of Various Palmitate-1-^{14}C Preparations as Hydrocarbon Precursors.[a]

Substrate Preparation	Act.	Rel Act.[b]
Bovine serum albumin complex	2660	100
Free acid	560	21
Free acid in 50 mg of Triton-X 100	0	0
Free acid in 1 ml of 2% dimethyl sulfoxide	1380	52
Free acid in 1 ml of 2% aqueous sodium deoxycholate	2450	92
Potassium salt	505	19
Methyl ester in 0.5 ml of dimethyl sulfoxide	1380	52
Methyl ester in 1 ml of 2% aqueous sodium deoxycholate	2690	101

[a] Assayed with standard system supplemented with the following cofactors in the concentrations indicated in Table I: coenzyme A, ATP, $MgCl_2$, NADPH, and pyridoxamine phosphate. [b] Calculated as percentage of activity obtained with bovine serum albumin complex of palmitate-16-^{14}C substrate.

TABLE III: Distribution of Radioactivity in Hydrocarbons after Incubation of Cell-Free Preparation with ^{14}C-Labeled Acetate and Fatty Acids.

No. of Carbons in Hydrocarbon	% Distribution of Radioactivity			
	Acetate-2-^{14}C	Palmitate-16-^{14}C	Palmitate-1-^{14}C	Myristate-1-^{14}C
<25	0.8	0.4	21.8	21.1
25	4.6	2.8	12.7	21.6
26	2.3	8.7	22.1	12.9
27	17.9	29.9	10.1	17.3
28	7.4	35.0	9.6	18.8
29	65.2	20.2	33.2	8.3
30	1.6	2.7	0.5	0

TABLE IV: The Relative Effectiveness of Pyridoxal and Pyridoxamine Phosphate as Cofactors and the Relationship to Glutamate Requirement.[a]

Expt	Augmentation: Pyridoxal Phosphate, 0.15 mM	Pyridoxamine Phosphate, 0.15 mM	Glutamate, 7.7 mM	α-Ketoglutarate, 8.6 mM	Act.	Rel Act.
1	+	−	+	−	2700	100
2	−	+	+	−	5700	211
3	+	−	−	−	1590	59
4	+	−	−	+	4270	158
5	−	+	−	−	3350	124

[a] Standard cell-free preparations prepared with or without glutamate and supplemented with the cofactors indicated. In addition, ATP, coenzyme A, NADPH, and $MgCl_2$ were added in the concentrations given in Table I.

ity, and it was therefore concluded that none of the cofactors were limiting at the concentrations used.

Optimum Form of Fatty Acyl Precursor for Incorporation. The selection of bovine albumin complex of palmitate-^{14}C as a precursor for most of the experiments reported here was based on the data given in Table II and on the fact that this was the most stable of the preparations tried that gave maximum activity.

Distribution of Radioactivity in Hydrocarbons of Different Chain Lengths after Incubation with ^{14}C-Labeled Acetate and Fatty Acids. Table III gives the distribution of ^{14}C in hydrocarbons of different chain length after incubation of the cell-free preparation with acetate-2-^{14}C, palmitate-16-^{14}C, -1-^{14}C, and myristate-1-^{14}C. Cell-free preparation supplemented with cofactors were incubated with the indicated precursor. The hydrocarbons were isolated and chromatographed on a preparative W-98 column, and the individual peaks were collected (Albro and Dittmer, 1969a). The collected samples were assayed for radioactivity. The total recovery of ^{14}C in the collected samples averaged 95% of that injected onto the column.

It was clear from the distribution of radioactivity that the hydrocarbons synthesized *in vitro* have the same general structures as the hydrocarbons found *in vivo*. Indeed, the distribution of the radioactivity from acetate directly reflects the mass distribution of the hydrocarbons reported earlier (Albro and Dittmer, 1969a). This was expected since approximately equal amounts of acetate would be found in each of the major fatty acids. The differences in the distribution of label from palmitate-1-^{14}C and -16-^{14}C may be attributed to the fact that only those hydrocarbons synthesized without decarboxylation of the palmitate would be labeled with the former. A shift of the distribution of label to hydrocarbons with shorter chains when labeled with palmitate-1-^{14}C was exactly what would be expected. Also, the significantly lower amount of radioactivity in the C-29 hydrocarbons found with myristate-1-^{14}C was consistent with synthesis occurring *in vitro* by the same mechanism determined for synthesis *in vivo* (Albro and Dittmer, 1969c).

Pyridoxamine Phosphate vs. Pyridoxal Phosphate as a Cofactor and the Possible Relationship to Stimulation by Glutamic Acid. When pyridoxamine phosphate was tested as a cofactor in place of pyridoxal phosphate, twice as great a stimulation of incorporation of palmitate into hydrocarbon was obtained with concentrations of 1.5×10^{-4} M. When pyridoxal phosphate was added to the system supplemented with this concentration of the amine, no further stimulation was obtained. Glutamic acid was originally incorporated into the system as a substrate for the generation of ATP and reduced pyridine nucleotide; however, the relatively greater stimulatory effect of pyridoxamine suggested an alternative role. To determine whether the glutamic acid was serving primarily in the conversion of pyridoxal phosphate into pyridoxamine phosphate, incubation mixtures were prepared as in the fully augmented system except pyridoxal derivatives and glutamic acid were added in prescribed combinations (Table IV). The results indicated that glutamic acid stimulation of the system was not primarily involved in conversion of pyridoxal phosphate to pyridoxamine phosphate.

The Intermediary Role of Fatty Acyl-CoA in Hydrocarbon Synthesis. The ATP, CoA, and Mg^{2+} requirement for incorporation of palmitate into hydrocarbon was consistent with fatty acyl-CoA as an intermediate. This was tested in the cell-free system with palmitoyl-16-^{14}C-CoA (Table V), and it was found to be on the order of 20 times as effective a precursor as palmitate in the absence of CoA. Pyridoxamine or pyridoxal phosphate were still required, but the greater stimulatory effect of the amine as observed with palmitate was not found with the CoA derivative. The implications of this and of the data with palmitoyl-1-^{14}C-CoA are considered in the discussion.

Linearity of Hydrocarbon Synthesis with Time. A 200-ml incubation mixture was prepared as for the standard assay and augmented with cofactors. A tracer amount of palmitate-16-^{14}C (0.21 μmole) was added and 33-ml aliquots were removed at 30-min intervals for assay of hydrocarbon radioactivity. The results (Figure 1) indicate that hydrocarbon biosynthesis was linear for at least 4 hr and that the endogenous pools of precursors had not become limiting over this period. In accord with this, assays were run for no more than 3 hr.

Linear Dependence of Incorporation of Palmitate into Hydrocarbon on the Amount of Cell-Free Preparation. Mixtures augmented with cofactors and containing varying amounts of lyophilized cell-free preparation were incubated under stan-

TABLE V: Relative Effectiveness of Palmitoyl-CoA and Palmitate in Hydrocarbon Biosynthesis.[a]

Substrate	Cofactor	Act.	Rel Act.
Palmitate-16-^{14}C	Pyridoxamine phosphate	1,340	100
Palmitate-1-^{14}C	Pyridoxamine phosphate	940	70
Palmitoyl-16-^{14}C-CoA	Pyridoxamine phosphate	31,700	2370
Palmitoyl-1-^{14}C-CoA	Pyridoxamine phosphate	130	10
Palmitoyl-16-^{14}C-CoA	Pyridoxal phosphate	28,800	2150
Palmitoyl-1-^{14}C-CoA	Pyridoxal phosphate	0	0

[a] Standard cell-free preparations supplemented with ATP, NADPH, glutathione, and $MgCl_2$ in the concentrations indicated in Table I were used in all the experiments. In addition, pyridoxal or pyridoxamine phosphate in a concentration of 1.5×10^{-4} M were used as indicated.

dard conditions with palmitate-16-^{14}C. The amount of radioactivity incorporated into hydrocarbon gave a linear relationship with the amount of cell-free preparation (Figure 2).

Dependence of Hydrocarbon Biosynthesis upon Palmitate Concentration. Incorporation of palmitate-16-^{14}C into hydrocarbon was determined with various concentrations of palmitic acid supplied to the system as bovine serum albumin complex. Despite the very crude nature of the preparation, it showed typical saturation kinetics with respect to palmitate (Figure 3).

Optimum pH Conditions. Aliquots of the standard cell-free preparation supplemented with 10^{-4} M acrylate and cofactors were adjusted to various pH values between 5.7 and 8.8. The capacities of these systems to incorporate the label from acetate-2-^{14}C into fatty acids and hydrocarbons and the methyl esters of *S. lutea* fatty acids synthesized *in vivo* from isoleucine-U-^{14}C, methyl palmitate-16-^{14}C, and palmitate-16-^{14}C into hydrocarbon were determined. Preparations coagulated below pH 5.7, and incorporation data for below this value were not obtained. When the data for the various precursors were plotted as the percentage of activity obtained at pH 6.8, the curves for hydrocarbon biosynthesis coincided over most of the pH range studied and it is in this form that the data are presented in Figure 4. Optimum pH for hydrocarbon biosynthesis from fatty acids was in all cases close to pH 8.0. An increase in activity observed with acetate below pH 6 appeared to be related to an optimum for incorporation of acetate into fatty acids in this pH range.

Inhibitor Studies. A variety of inhibitors have been studied in attempts to define cofactor requirements of the system and to relate the properties of the system to those described in plants. Data on the inhibition of palmitate-16-^{14}C incorporation into hydrocarbons are given in Table VI.

Discussion

Redistribution of label from palmitate-16-^{14}C into other fatty acids in the cell-free preparation of *S. lutea* was consistent with the occurrence of β oxidation of fatty acids. The redistribution was 98% inhibited when acrylate was added to the system. Acrylate has been reported to inhibit β oxidation of fatty acids *in vivo* in a strain of *Pseudomonas* (Thijsse, 1964) and fatty acid synthesis in rat organ preparations (Robinson *et al.*, 1963). Either observation could explain the inhibition

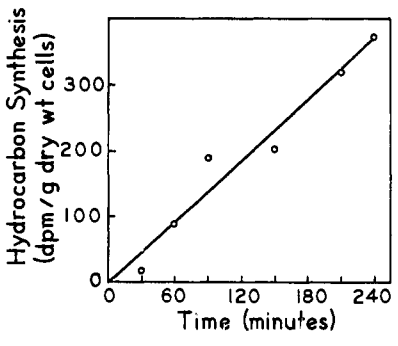

FIGURE 1: The incorporation of radioactivity from palmitate-16-^{14}C into hydrocarbons with time. The standard cell-free preparation was supplemented with ATP, 1.7×10^{-4} M; coenzyme A, 9.0×10^{-6} M; NADPH, 1.8×10^{-5} M; pyridoxal phosphate, 8.2×10^{-5} M; and glutathione, 3.8×10^{-6} M. The calculated regression line is dpm/g dry wt of cell preparation = 1.54 min^{-1} and was characterized by a correlation coefficient $r = 0.941$, $t = 6.218$, and $p < 0.01$ that the linear correlation was due to chance.

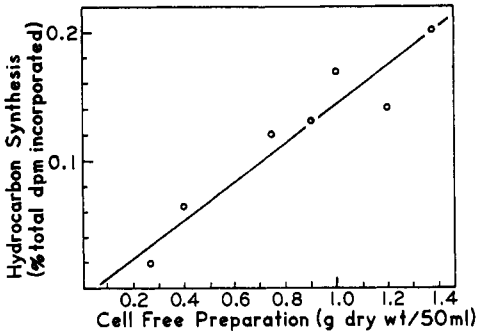

FIGURE 2: Relationship of hydrocarbon synthesis to the concentration of the cell-free preparation. Lyophilized cell-free preparation in the amounts indicated was made to 50 ml with 0.1 M, pH 8 phosphate buffer, and supplemented with the cofactors indicated in Figure 1. Each mixture was incubated with 0.5 mg of palmitate-16-^{14}C (1.0 μCi) on 10 mg of bovine serum albumin. The data fit the regression line $Y = 0.151X - 0.006$. The correlation coefficient was $r = 0.956$, $t = 7.285$, and $p < 0.001$ that there was only chance correlation with a straight line.

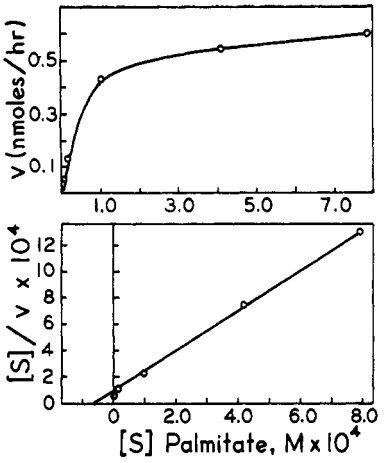

FIGURE 3: Dependence of incorporation of radioactivity from palmitate-16-^{14}C into hydrocarbon on palmitate concentration. Standard cell-free preparation with 10^{-4} M sodium acrylate, 10^{-4} M Na_2 EDTA, 2×10^{-4} M $MgCl_2$, and the cofactors given in Figure 1 except at approximately twice the concentration was used. A bovine serum albumin complex of the labeled palmitate was used as substrate in amounts to give the concentrations of palmitate shown. The palmitate content of the albumin and cell-free preparation was taken into account in the calculation of the final concentrations. The apparent K_m for palmitate in the system was approximately 7.0×10^{-5} and V_{max} was approximately 0.6 nmole of palmitate incorporated/hr per g of cell-free preparation dry wt.

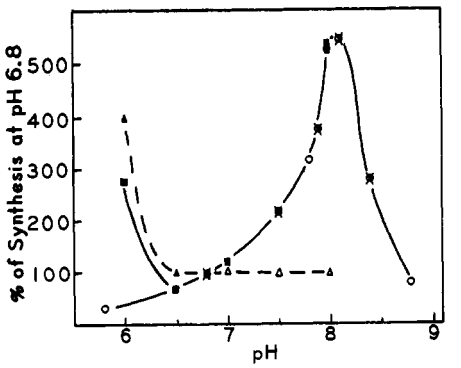

FIGURE 4: Dependence of incorporation of label from fatty acids and acetate into hydrocarbons upon pH. Standard cell-free preparations were supplemented with 10^{-4} M acrylate; ATP, coenzyme A, NADPH, and $MgCl_2$ in the concentrations given in Table I; pyridoxamine phosphate, 1.5×10^{-5} M; and cysteine, 1.2×10^{-3} M. Data are plotted as the per cent of activity obtained at pH 6.8 with various precursors incorporated into hydrocarbon as follows: (●) *S. lutea* fatty acid methyl esters synthesized *in vivo* with isoleucine-^{14}C; (○) *S. lutea* fatty acids synthesized *in vivo* with acetate-^{14}C; (×) methyl palmitate-16-^{14}C; (◑) palmitate-16-^{14}C; and (■) acetate-^{14}C. Incorporation of acetate-^{14}C into fatty acids is shown with the points marked Δ.

of the redistribution of label from palmitate-^{14}C. However, since no effect of acrylate on the incorporation of acetate into fatty acids was observed, the effect of the acrylate in the *S.lutea* system appears to be on β oxidation of the fatty acid.

When undialyzed cell-free preparations of *S. lutea* were augmented with cofactors, a lower level of palmitate incorporation into hydrocarbons was obtained than when the equivalent dialyzed preparation was augmented. The dialysis treatment itself does not account for the difference since recombining the dialyzed preparation with an equivalent amount of the diffusate restores activity only to the level of an undialyzed preparation. Apparently a diffusible inhibitory factor is present in the undialyzed preparations.

The effect of omitting both coenzyme A and ATP from dialyzed preparations was not greatly different than when either was omitted, or in other words the two cofactors were apparently required for the same or closely linked reactions. In contrast, the omission of Mg^{2+} and pyridoxal phosphate together had an effect equal to the omission of each separately, thus suggesting that these cofactors were required for independent steps. By the same type of reasoning, NADPH appeared to be used in a reaction linked to that involving pyridoxal phosphate.

The cofactor requirements, to the extent they were established, were not surprising except for the pyridoxal or pyridoxamine phosphate. The activation of fatty acids by the formation of the CoA derivative in either or both of the two modes of entry into hydrocarbon was not unexpected. If the formation of the CoA derivative occurs by the usual thiokinase reaction, this explains the requirement for ATP, CoA, and Mg^{2+}. An independent requirement for Mg^{2+} as well cannot be ruled out. Whatever the detailed mechanism of incorporation of fatty acids into hydrocarbons, the requirement for a reduced pyridine nucleotide was also not unexpected.

In most of the experiments reported in this paper, the labeled precursor was introduced as a serum albumin complex. This procedure was adopted in part because of the convenience due to the stability of such complexes as compared with emulsions but also because of the established effectiveness. The necessity of dispersing the fatty acid precursor was obvious and normally in a system at pH 8 dispersal as the salt or soap would be expected to be adequate. Actually, such a procedure generally gave low levels of incorporation. Apparently, ionization of the fatty acid is not a factor since methyl palmitate was found to be equally as good a precursor as the free acid when both were dispersed with deoxycholate. Specificity, however, in the mode by which free acids and esters are handled by the system to be reported in the next paper of this series prohibits such an oversimplified interpretation.

Since there are evidently two modes by which fatty acids are incorporated into hydrocarbons, one problem yet to be resolved is that of which cofactors are involved in each mode of entry. The primary difference in the two modes is whether or not the fatty acid is decarboxylated; thus one approach to the problem is the comparison of the effect of cofactors on the incorporation of palmitate labeled in the 1 position with that labeled in the 16 position. With the former, only the mode of entry not involving decarboxylation will be followed while with the latter the combined effect of both modes is measured. Data for one such experiment are given in Table V in the comparison of the incorporation of palmitoyl-1-^{14}C-CoA and palmitate-1- and -16-^{14}C. The ratio of incorporation of label from 1-μCi tracer doses of each was 1:7:10, respectively. That is, the free acid was seven times better a precursor than the CoA derivative in the nondecarboxylation

mode of entry, and under these conditions, 70% of the free acid was incorporated *via* the nondecarboxylation mode of entry. It appeared from this that the CoA derivative functions primarily in the mode of entry involving decarboxylation. This interpretation is supported by a comparison of the incorporation of palmitoyl-16-^{14}C-CoA with palmitoyl-1-^{14}C-CoA. In this case the incorporation of label from the latter was relatively negligible, or virtually 100% of the CoA derivative was decarboxylated.

The data in Table IV also give some indication of what mode of entry the pyridoxal or pyridoxamine phosphate acts. With the amine, incorporation of label from the free acid was doubled while only a slight stimulation was obtained with the CoA derivative. It follows that the pyridoxal derivative probably functions in the nondecarboxylation mode of entry.

The pH optimum of 8 established for hydrocarbon biosynthesis was of interest relative to the pH changes reported earlier that occur during different growth phases in TSB and M73b medium (Albro and Dittmer, 1969a). The pH decreases from 7.2 to 6 during log phase and then increases to 8 during the transition from log to stationary phase. It is during this latter period that the hydrocarbon content of the cells begins to rise, that is, the accumulation of hydrocarbon coincides with a change of pH of the medium to that of the optimum established for the *in vitro* system. Whether the changes are necessarily a consequence of the growth cycle or obligatory for hydrocarbon biosynthesis *in vivo* has not yet been established.

Gholson *et al.* (1963) reported that hydroxyquinoline inhibited hydrocarbon oxidation and either ferrous or ferric ions were required for catabolism. In the *S. lutea* system hydroxyquinoline increased the accumulation of label from palmitate in hydrocarbon and ferrous sulfate caused a net decrease in incorporation. Either hydroxyquinoline stimulation and ferrous sulfate inhibition of biosynthesis or the opposite effect on catabolism may be involved.

The lack of inhibition by avidin was consistent with our failure to show a requirement for biotin and probably rules out participation of biotin covalently bound to its apoenzyme. The apparent stimulation of incorporation of palmitate-^{14}C into hydrocarbon by avidin was apparently not due to general inhibition of fatty acid synthesis and a diminution of dilution of the labeled precursor because avidin did not inhibit acetate incorporation into fatty acids more than 2%. The majority of fatty acids in *S. lutea* are, however, synthesized by the addition of acetate to C-4 and C-5 acids derived from amino acids. If this synthesis does not require biotin, the inhibition of the synthesis of the relatively small amount of normal acid by avidin may have gone undetected. The uninhibited synthesis of normal acids could cause a significant dilution of the palmitate pool.

Isoniazid (isonicotinylhydrazine) reportedly reacts with pyridoxal phosphate and renders it inactive as a coenzyme (*e.g.*, Davison, 1956). We found that isoniazid did not inhibit the residual activity *in vitro* of the system supplemented with all cofactors except pyridoxal phosphate derivatives but did inhibit when they were also added to the system. This suggests that either the interaction of the coenzyme and apoenzyme was inhibited or a isoniazid–pyridoxal phosphate derivative was the actual inhibitor. Pyridoxal phosphate isonicotinylhydrazone has been reported to activate many enzymes that utilize pyridoxal phosphate as a cofactor (*e.g.*, Gonnard and Nguyen-Phillippon, 1961; Bonavita and Scardi, 1959).

TABLE VI: Inhibitor Studies.[a]

Inhibitor	Concentration	% Inhib[b]
8-Hydroxyquinoline	5.0×10^{-4} M	−15
$FeSO_4$	3.8×10^{-4} M	31
Triton X-100	0.01%	100
Avidin[c]	10 μg/50 ml	0
	100 μg/50 ml	−17
	1 mg/50 ml	−54
Isoniazid[d]	1.0×10^{-4} M	13
Isoniazid[e]	1.0×10^{-4} M	0
Nicotinamide	2.0×10^{-3} M	14
Trichloroacetic acid	4.0×10^{-4} M	29
Arsenite[f]	1.0×10^{-4} M	54
Imidazole	1.0×10^{-4} M	0

[a] Assays were carried out with the standard system supplemented with cofactors as in Figure 1 except as indicated in footnotes *e* and *d*. Palmitate-16-^{14}C was used as precursor. [b] The per cent inhibition was calculated as follows: (dpm/mg of hydrocarbon in control − dpm/mg of hydrocarbon in test system) × (100)/(dpm/mg of hydrocarbon in control). [c] Avidin in a concentration of 1 mg/50 ml inhibited the incorporation of acetate-^{14}C approximately 2% into fatty acids and showed no effect on the incorporation of acetate into hydrocarbons. [d] Essentially the same results were obtained whether pyridoxal or pyridoxamine phosphate were used in a concentration of 2×10^{-4} M. [e] No pyridoxal phosphate was used in this assay. [f] Not reversed by adding 1.5×10^{-3} M cysteine.

Imidazole inhibits α oxidation of fatty acids in plants (Martin and Stumpf, 1959), and in an elongation–decarboxylation pathway for conversion of fatty acids into hydrocarbons as proposed to occur in plants (*e.g.*, Kolattukudy, 1967, 1968), α oxidation followed by decarboxylation and reduction could be possible terminating steps of the synthesis (Wanless *et al.*, 1955; Stumpf, 1965). With broccoli leaves, imidazole caused a 30–100% stimulation of the incorporation of acetate into hydrocarbon (Kolattukudy, 1966) which might be explained by the inhibition of the metabolism of fatty acids by α oxidation with the concomitant diversion of fatty acids into hydrocarbons. If this were the case, the lack of effect of imidazole on the incorporation of palmitate into hydrocarbon in *S. lutea* was not unexpected or inconsistent.

Kolattukudy (1965, 1967) has proposed that trichloroacetic acid inhibits the incorporation of acetate and fatty acids into hydrocarbons in plants by acting on the system for elongation of fatty acids. We questioned the validity of the primary evidence against a head-to-head condensation mechanism for hydrocarbon biosynthesis in plants on the grounds that exogenously supplied fatty acids may be specifically incorporated into hydrocarbons by such a mechanism without decarboxylation as occurs under certain conditions with *S. lutea in vivo* (Albro and Dittmer, 1969c). Fully consistent with this criticism, trichloroacetic acid inhibits the incorporation of palmitate into hydrocarbons in *S. lutea in vitro*. This inde-

pendent inhibition of hydrocarbon biosynthesis may explain why trichloroacetic acid inhibits the incorporation of acetate into hydrocarbons two to three times as effectively as it inhibits incorporation into fatty acids in plants (Kolattukudy, 1965). In general, the effects of imidazole and trichloroacetic acid on the *S. lutea* and plant systems are consistent with a head-to-head condensation mechanism functioning in plants as well as in *S. lutea*.

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