SUMMARY

The synthesis of long-chain fatty acids from acetate by the reconstituted fractions of pigeon liver supernatant requires the following cofactors: ATP, CoA, GSH, DPNH, Mn^{++}, *iso*citrate and TPN. Glucose-1-phosphate is needed in crude systems but this component can be replaced by DPNH in the more purified system.

*Iso*citrate cannot be replaced by TPNH or a TPNH-generating system such as glucose-6-phosphate dehydrogenase system plus substrate. However, it can be replaced by a combination of α-ketoglutarate, CO_2, and a TPNH generating system.

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STUDIES ON THE MECHANISM OF FATTY ACID SYNTHESIS*

III. PRODUCTS OF ENZYMIC SYNTHESIS OF FATTY ACIDS

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Considerable experimental evidence has been obtained which supports the concept that fatty acids are synthesized by successive head-to-tail condensations of two carbon (acetate) units[3,4,5]. Much of this information has originated from studies with

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whole animals or tissue slices. In recent years the problem of fatty acid synthesis has been examined in soluble enzyme systems[6,7,8]. Identification and analysis of the fatty acid products synthesized in these systems have substantiated the findings in the earlier studies.

STADTMAN AND BARKER[6], employing a soluble extract of *Clostridium kluyveri*, identified the products of synthesis in this system as butyric and caproic acids by partition chromatography and Duclaux distillation. BRADY AND GURIN[7] separated the long-chain fatty acids ($> C_{10}$) synthesized by a soluble pigeon liver preparation by the copper-lime precipitation method[9] and they showed that the acids formed were unesterified. On decarboxylation of the fatty acids (synthesized from ^{14}C-1-acetate), the content of ^{14}C in the terminal carbon atom of the fatty acids was found to be only slightly above that expected on the basis of successive condensations of acetate units. POPJÁK AND TIETZ[10] separated the fatty acids synthesized from ^{14}C-acetate by homogenates of mammary gland by employing reversed-phase chromatography. Even-numbered, *n*-saturated acids from C_6 to C_{18} were identified, as well as oleic acid. Most of the radioactivity was distributed among the C_6, C_{14}, C_{16} and oleic acids. Later, HELE AND POPJÁK[11], employing a purified, soluble enzyme system from mammary gland, identified the hydroxamates of β-hydroxybutyrate, butyrate, β-hydroxyoctanoate, and octanoate, after treating the incubation mixture with hydroxylamine.

The present paper is concerned with the separation and identification of the fatty acid products synthesized from acetate by an enzyme system reconstructed from soluble fractions of pigeon liver[12,13]. The demonstration of net synthesis of fatty acids by this system and data on the decarboxylation of the synthesized fatty acids are also presented.

METHODS

Incubation and extraction

Fatty acids were synthesized from carboxyl-labeled ^{14}C-acetate by the pigeon liver enzyme system under conditions detailed in previous communications of this series[12,13]. At the end of the incubation period one volume of 10% alcoholic KOH was added and the samples were saponified for one hour in a water bath (90–100°). In early experiments nonsaponifiable compounds were extracted with ethyl ether. Since very little ^{14}C appeared in this ether fraction, samples were routinely acidified (after saponification) to pH 1–2 with HCl and extracted with *n*-pentane or ethyl ether (three extractions with 10 volumes of solvent). The *n*-pentane extracts were then reduced in volume, aliquots were plated as infinitely thin samples and the radioactivity was measured under a thin (1.5 mg/cm^2) end-window Geiger-Mueller tube.

Chromatography

Two paper chromatographic systems were employed to separate the synthesized fatty acids. In the first, that of REID AND LEDERER[14], fatty acids in the ether extract were converted to ammonium salts by adding 100 μl of concentrated ammonium hydroxide. After evaporation of the solvent the ammonium salts were applied to Whatman No. 1 filter paper that had been previously exposed to ammonia vapors. The fatty acid salts were separated after 12–16 hours by ascending movement of the solvent (*n*-butanol saturated with 1.5 *N* ammonia). The paper was dried in air, vertical strips were cut from the paper and subdivided at 1 cm intervals. The radioactivity in each section of paper was measured as before and the distribution of radioactivity was compared with the location of known fatty acids chromatographed on the same paper sheet. The butanol–ammonia system separates fatty acids of chain length C_2 to C_8. Long-chain fatty acids ($> C_{10}$) migrate together and cannot be resolved by this method.

Fatty acids of chain length greater than C_{10} were resolved by the reversed-phase paper chromatographic technique described by KAUFMAN AND NITSCH[15]. Ether solutions of the fatty acids, concentrated to a small volume, were applied to Whatman No. 4 filter paper sheets that had been impregnated with kerosene (Standard Oil Company). Separation of the fatty acids was

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achieved by descending movement of the mobile solvent (85 % acetic acid saturated with kerosene). All of the saturated *n*-fatty acids of even carbon number from C_{10} to C_{18} are separated by this method. Oleic acid which has the same R_F as palmitic acid in this system could be determined as stearic acid after hydrogenation of the fatty acid mixture. Samples of fatty acids were dissolved in ethyl acetate and hydrogenated* overnight, as described by POPJÁK AND TIETZ[10].

Long-chain fatty acids were also separated by the reversed-phase chromatography method of HOWARD AND MARTIN[16]. Before chromatography, unlabeled myristic, palmitic and stearic acids were added to the mixture of synthesized, radioactive fatty acids. The first solvent applied to the column was 60 % (v/v) aqueous acetone. Lauric and shorter chain acids move with the solvent front and emerge from the column in a sharp band. The myristic acid band follows. Palmitic acid (plus oleic acid) is eluted with 65 % acetone and stearic acid with 75 % acetone. Oleic acid is determined as stearic acid after hydrogenation of the palmitic acid fraction followed by a second chromatography. Palmitic acid for decarboxylation studies was prepared in this fashion (see below). The amount of fatty acid eluted from the column was determined by titration with alcoholic NaOH under nitrogen. Sodium salts of the fatty acids were then plated and the radioactivity measured.

Hydroxamates

A study was made of the distribution of ^{14}C in the products obtained after treatment of the enzyme reaction mixture with hydroxylamine. Incubation samples were treated with an excess (approximately 1 m*M*) of neutral hydroxylamine for 30 minutes at 38°. After lyophilization the hydroxamates were extracted with absolute alcohol and then chromatographed in butanol saturated with 3 *N* NH_4OH[17]. Controls with ^{14}C palmitate and ^{14}C palmityl CoA were treated in the same manner.

Separation of free and esterified acids

Fatty acids in the incubation mixture were separated into free and esterified acids by two procedures. In *procedure 1*, the incubation mixtures were acidified to pH 2 with *N* HCl. An equal volume of ethanol was then added and the free acids were extracted three times with *n*-pentane. After saponification of the water-alcohol phase and reacidification the fatty acids extractable with *n*-pentane were defined as esterified acids. In *procedure 2*, the pH of the reaction mixture was adjusted to 2 with perchloric acid and the suspended material was centrifuged down. The precipitate was washed twice with a 1:1 mixture of ethanol and ether. The ethanol-ether phase was diluted with an equal volume of water and then extracted twice with *n*-pentane to separate the free acids. The washed perchloric acid precipitate was extracted with a mixture of pyridine, *iso*propanol and water (1:1:1)[18]. The extract was lyophilized and dissolved in a minimum of water at neutral pH. This fraction is designated as the esterified fatty acid fraction.

Decarboxylation

Decarboxylation of fatty acids was performed by the method of PHARES[19]. Samples were decarboxylated at 75° for a period of 45 minutes. $^{14}CO_2$ was recovered as $Ba^{14}CO_3$, and, after drying *in vacuo* over sulfuric acid, the product was weighed. The sample was then suspended in water and aliquots were plated and radioactivity determined. Corrections in counting were made for self-absorption. Samples of synthesized fatty acids used for decarboxylation studies were prepared by two methods. The first utilized the method of lead salt precipitation as recommended by HILDITCH[20]. A mixture of unlabeled fatty acids extracted from pigeon liver was added as carrier to the radioactive fatty acids obtained by incubation of the pigeon liver system with carboxyl-labeled ^{14}C-acetate. After evaporation of the petroleum ether solution, 15 ml of hot ethanol and 35 ml of lead acetate (750 mg) were added to 800 mg of the fatty acid mixture. The solution was cooled slowly and stored at 5° for 18 hours. The lead precipitate was washed, redissolved and recrystallized from a solution containing 0.25 ml of glacial acetic acid in 50 ml of 95 % ethanol. The second method employed for preparing samples of fatty acid was the reversed-phase chromatographic procedure of HOWARD AND MARTIN[16] (see before). The palmitic acid fraction (eluted with 65 % acetone) was mixed with carrier oleic acid, hydrogenated, and the acids chromatographed a second time by the same procedure.

Net synthesis

For the demonstration of net synthesis of fatty acids, carboxyl-labeled ^{14}C-acetate was incubated with pigeon liver enzyme fractions in a large scale experiment (Table IV). After saponification nonsaponifiable compounds were carefully extracted with pentane in non-greased separatory funnels. After acidification of the residual solution, fatty acids were extracted with pentane, the

* The authors are indebted to the Department of Chemistry, University of Wisconsin, for conducting the hydrogenations.

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pentane phase carefully washed with water and evaporated to dryness. The fatty acids were stored over sulfuric acid in a vacuum desiccator until constant weight was obtained. Samples were then dissolved in pentane and aliquots were plated and counted in the usual way. The remainder of the solution was evaporated to dryness, dissolved in 2.0 ml of ethanol and titrated with 0.01 *N* NaOH in ethanol under nitrogen with brom thymol blue as indicator.

RESULTS

Resolution of synthesized fatty acids

In Fig. 1 a comparison is made between the fatty acids extracted by pentane and diethyl ether from the acidified saponification mixture. The ammonium salts of the fatty acids obtained in each solvent extraction were resolved by the method of REID AND LEDERER[14]. In the pentane extract only fatty acids of chain length C_{10} to C_{18} (R_F 0.6–0.9) were present, whereas in the diethyl ether extract there was, in addition

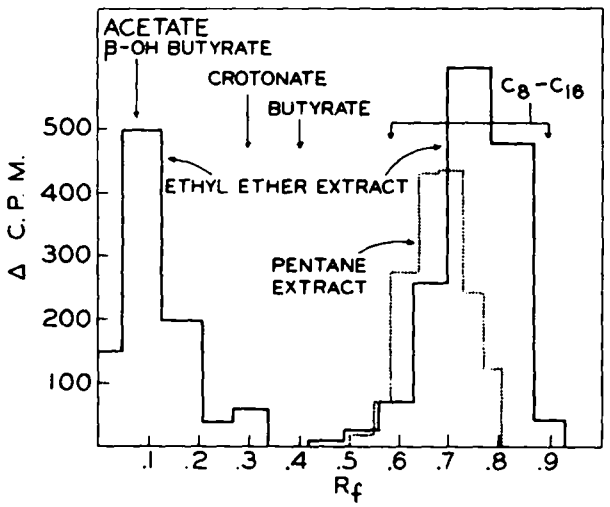

Fig. 1. Distribution of ^{14}C on chromatograms of ethyl ether (solid lines) and pentane (dotted lines) extracts of fatty acids synthesized by the pigeon liver system.

to long-chain acids, radioactivity at the origin, in the region of acetate and β-hydroxybutyrate (R_F 0.1) and crotonate (R_F 0.3). No ^{14}C-labeled products were found in the regions corresponding to butyrate, hexanoate or octanoate. The radioactive component at the origin of several chromatograms of the diethyl ether extract was eluted from the paper and rechromatographed by the method of RABINOWITZ AND GURIN[21]. Practically all of the radioactivity appeared at the same R_F (0.40) as that of an authentic sample of HMG*.

The long-chain fatty acids in the pentane extracts were resolved by reversed-phase chromatography on paper by the method of KAUFMANN AND NITSCH[15]. The results of a typical chromatogram are shown in Fig. 2. Palmitic acid was synthesized to the greatest extent. Progressively smaller quantities of myristic, lauric and decanoic acids were also formed.

* The following abbreviations are used: ATP, adenosine triphosphate; CoA, coenzyme A GSH, glutathione; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; HMG, β-hydroxy, β-methyl glutaric acid; PLS, pigeon liver supernatant solution and PLAPE, pigeon liver acetone powder extract.

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In Table I is presented the distribution of long-chain fatty acids (extracted with pentane) which were synthesized under various incubation conditions and with different preparations of pigeon liver enzymes. The distribution of acids was essentially the same in these experiments. Several fatty acid samples were hydrogenated before chromatography. The small increment in radioactivity in stearic acid appearing after hydrogenation thus represents the oleic acid originally present in the sample. Neither stearic nor oleic acid were synthesized in more than trace amounts by the pigeon liver system.

A diethyl ether extract of fatty acids was also resolved by the reversed-phase chromatographic procedure of HOWARD AND MARTIN[16]. The first band eluted from the column (lauric and shorter-chain acids) contained 21% of the radioactivity of the original extract. The myristic acid fraction contained 30% and the palmitic acid fraction 49%.

In a number of experiments carboxyl-labeled ^{14}C-acetate was incubated with the pigeon liver system, following which neutral hydroxylamine was added and the mixture incubated further for

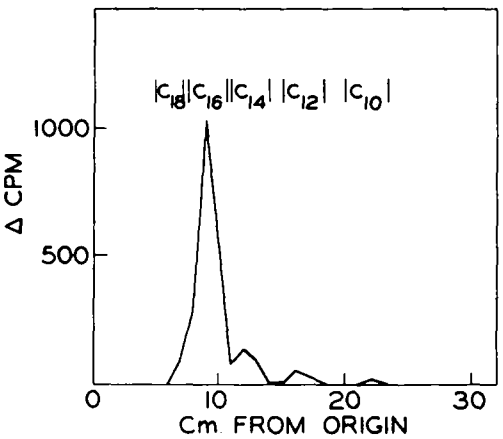

Fig. 2. Distribution of synthesized long-chain fatty acids in the paper chromatographic system of KAUFMANN AND NITSCH[15].

TABLE I

FATTY ACIDS SYNTHESIZED BY PIGEON LIVER SYSTEMS*

Fatty acid	*^{14}C recovered in fraction as per cent of total*			
	1	2	3	4
Capric (decanoic)	2	2	1	1
Lauric (dodecanoic)	11	8	5	12
Myristic (tetradecanoic)	31	25	13	22
Palmitic (hexadecanoic)	51	60	81	65
Stearic (octadecanoic)	2	2	0	0
Oleic** (octadeca-9-enoic)	3	3	—	—

* Components, in μmoles: 25.0 K phosphate buffer, pH 6.5, 2.5 K acetate (250,000 c.p.m.), 3.0 Na *iso*citrate, 6.0 glucose-1-phosphate, 2.0 ATP, 8.0 GSH, 0.01 CoA, 0.15 lipoic acid, 0.25 DPN, 0.25 TPN, 1.0 $MgCl_2$ and 0.2 $MnCl_2$. In experiments 1 and 3 enzyme fractions R_1, R_2 and R_4 prepared from PLS were employed; in experiment 2, R_1, R_2 and R_4 from PLAPE and in experiment 4, R_1 (gel-treated), R_2 and R_4. Also in experiment 4, 3.0 μmoles of DPNH were used in place of glucose-1-phosphate and DPN.

** Determined as difference between the amount of stearic acid before and after hydrogenation.

30 minutes. The hydroxamates so obtained were chromatographed on paper[17] and the distribution of radioactivity determined (see Fig. 3). Only small amounts of radioactivity were detected in the regions corresponding to the hydroxamates of short-chain fatty acids (R_F 0.2–0.8). Replacement of ^{14}C-acetate with ^{14}C-acetyl CoA resulted in a considerable increase in the counting distributed over this region. When samples of radioactive palmityl CoA and palmitate were incubated separately with hydroxyl-

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amine and the incubation mixture, extracted and chromatographed as before, the radioactivity recovered from palmityl CoA was more than 15 times the amount recovered from palmitate.

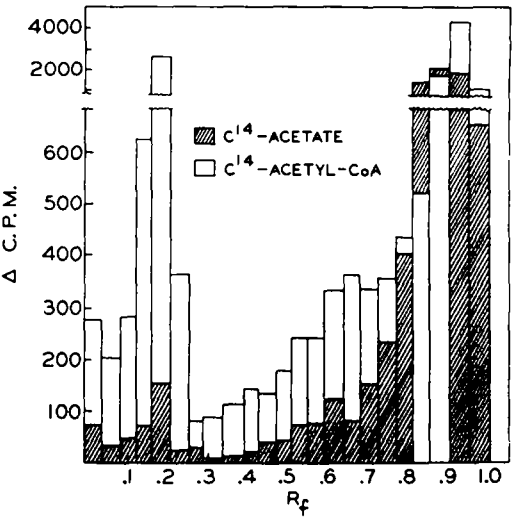

Fig. 3. Chromatograms of ^{14}C hydroxamates obtained after incubation of the enzyme system with ^{14}C-acetate or ^{14}C-acetyl CoA. The following components were added in each experiment (μmoles): 25 K phosphate buffer, pH 7.0, 5.0 *iso*citrate, 18.0 glucose-1-phosphate, 2.5 ATP, 8.0 GSH, 0.2 lipoic acid, 0.2 $MnCl_2$, 0.3 DPN, 0.3 TPN, 1.0 $MgCl_2$, 3.5 mg R_1, 3.0 mg R_2 and 3.5 mg R_4. In the first experiment 0.25 μmoles ($1.4 \cdot 10^5$ c.p.m.) of acetate and 0.012 μmoles of CoA were added, in the second, 0.25 μmoles ($1.4 \cdot 10^5$ c.p.m.) of acetyl CoA. The final volume was 1.0 ml. Samples were incubated for one hour at 38° under nitrogen, and then an additional 30 min after adding 2 mmoles of neutral hydroxylamine.

Separation of free and esterified acids

Saponification of the enzyme incubation mixture, followed by acidification to pH 2, renders all of the long-chain fatty acids extractable with pentane (total acids). If the incubation mixture is directly acidified by either of two procedures (see METHODS) the amount of radioactivity extractable with pentane (free acids) is slightly less. The remainder of the radioactive fatty acids (esterified acids) is extractable following saponification and acidification. Table II presents a balance sheet of the synthesized fatty acids between the free and esterified forms. Good agreement was found between the quantity of total acids and the sum of the free and esterified acids. The distribution of radioactivity in long-chain fatty acids (resolved by paper chromatography[15]) from the free acid fraction was indistinguishable from the distribution of a mixed sample of free acids and total acids.

TABLE II

END-PRODUCTS OF FATTY ACID SYNTHESIS

	c.p.m./aliquot	
Experiment 1		
Total acids	1995	
Free acids*	1250	1895
Esterified acids	645	
Experiment 2		
Total acids	4100	
Free acids**	3700	4200
Esterified acids**	500	

* *Procedure 1* was used in extracting free acids.
** *Procedure 2* was used in extracting free and esterified acids.

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The esterified fatty acid fraction was purified further and identified. The radioactivity of the perchloric acid precipitate remaining after removal of the free acids (see *procedure 2* in METHODS) is extractable with the pyridine-*iso*propanol solvent[18]. By ascending chromatography on paper (water, *iso*propanol and pyridine, 2:1:1) the radioactivity in the extract migrated with the same R_F (0.70) as an authentic sample of ^{14}C palmityl CoA. (KORNBERG AND PRICER[18] have reported an R_F value of 0.67 for this system.) After chromatography[15] of the acids released following saponification of the esterified acid fraction, 85% of the radioactivity was found in palmitic acid and 15% in myristic acid. A sample of the esterified acids was tested as a substrate in the purified palmityl CoA dehydrogenase system (Y′ enzyme) of BEINERT *et al.*[22] which specifically oxidizes long-chain acyl CoA derivatives. The sample of esterified fatty acid was oxidized in this system.

Decarboxylation of long-chain fatty acids

A comparison of the specific radioactivity of the carboxyl carbon of fatty acids (synthesized from carboxyl-labeled ^{14}C-acetate) with the specific activity of the entire carbon chain provides information on the probable distribution of ^{14}C along the carbon chain of the fatty acid[3]. The specific activity of the terminal carbon atom of the fatty acid is expected to be twice that of the complete carbon chain if fatty acids are synthesized by head-to-tail condensations of acetate molecules. In Table III are presented the results of radioactivity measurements of the CO_2 released on decarboxylation of synthesized, saturated, long-chain fatty acids (separated by the lead salt procedure) and of synthesized palmitic acid (separated by reversed-phase chromatography[16]). The specific radioactivity of the terminal carbon atom was 1.7 times the value for all the carbon atoms of the fatty acid chain.

TABLE III

^{14}C CONTENT OF $BaCO_3$ DERIVED FROM THE CARBOXYL CARBON OF SYNTHESIZED FATTY ACIDS

	c.p.m./μatom of carbon	*Ratio*
1. *Saturated fatty acids**		
a. Fatty acids**	10.9	
$BaCO_3$ – expected**	21.8	
$BaCO_3$ – obtained	14.6	1.33
b. Fatty acids**	43.0	
$BaCO_3$ – expected**	86.0	
$BaCO_3$ – obtained	72.5	1.69
2. *Palmitic acid****		
Palmitic acid	22.5	
$BaCO_3$ – expected	45.0	
$BaCO_3$ – obtained	36.6	1.63

* Acids prepared by the lead salt precipitation method. Palmitic acid was the principal acid of these samples.

** Calculated on the basis that all of the ^{14}C of the acid sample was in palmitic acid.

*** Isolated by the method of HOWARD AND MARTIN[12].

Net synthesis of fatty acids

Although the enzyme fractions employed in these studies in fatty acid synthesis con-

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tained endogenous fatty acids it was possible to demonstrate *de novo* synthesis of long-chain fatty acids from acetate. In two large-scale experiments the fatty acids obtained after incubation with acetate were weighed and compared with the weight before incubation. A net increase in the weight of the fatty acid fraction was observed (Table IV). In the second experiment the μmoles of fatty acids synthesized as determined on a weight basis corresponded very closely to the net increase in acid groups (in the pentane extracts) and to the μmoles of fatty acid synthesized as determined by radioactivity measurements.

DISCUSSION

The principal fatty acid synthesized by the pigeon liver system is palmitic acid. Smaller quantities of myristic, lauric and decanoic acids are also formed. This distribution of acids is similar to that reported by POPJÁK AND TIETZ[10] with a homogenate of mammary gland, except that in their system short-chain stearic and oleic acids were also synthesized.

The fatty acids obtained in the present study were predominantly unesterified, a finding in agreement with the results of BRADY AND GURIN[7]. The esterified fatty acid fraction has the properties of palmityl CoA on chromatography and by enzymic assay in the palmityl CoA dehydrogenase system. It has also been demonstrated[23] that the pigeon liver system contains an enzyme that deacylates palmityl CoA.

TABLE IV

NET SYNTHESIS OF FATTY ACIDS

Tube	*Wt., initially*	*Wt., after 3 h*	*Net increase*
*Experiment 1**			
1	6.1	7.1	1.0**
2	6.0	6.9	0.9**
3	6.1	7.4	1.3**
	Initial	*After 3 h*	*Net in μmoles*
*Experiment 2****			
Radioactivity	0	$5.6 \cdot 10^6$ c.p.m.	8.72§
Weight, mg	12.00	14.25	8.79§
Carboxyl groups, μmoles	43.33	51.29	7.96

* Components in μmoles: 30 Na *iso*citrate, 100 phosphate-bicarbonate buffer, pH 7.0, 30 $MgCl_2$, 20 K acetate ($6 \cdot 10^5$ c.p.m.), 5.0 ATP, 5.0 DPNH, 5.0 TPN, 2.0 lipoic acid, and 40 mg glucose-1-phosphate and 3.0 ml dialyzed PLS. Incubation was for 3 h at 38° under nitrogen.

** In these experiments with the unfractionated PLS the net increase in mg of fatty acid is two-fold greater than the amount anticipated, based on ^{14}C incorporation into the fatty acids.

*** Four lots of 9 tubes each (zero time and 3 h incubation) contained the following components, in μmoles per tube: 150 phosphate buffer, pH 6.5, 60 GSH, 0.15 CoA, 16.5 ATP, 3.1 TPN, 40 *iso*-citrate, 75 glucose-1-PO_4, 2.5 DPN, 1.0 $MnCl_2$ and 16.25 K acetate ($1.31 \cdot 10^6$ c.p.m.). Each tube also contained 18.0 mg R_1, 6.5 mg R_2 and 3.5 mg R_4. The final volume in each tube was 2.5 ml. Incubations were for 3 h at 38° under nitrogen. The results represent the *average* of two lots of 9 tubes each at zero time and of two lots after 3 h incubation.

§ Converted to μmoles on the assumption that all of the newly synthesized fatty acid was palmitic acid.

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Short-chain fatty acids are formed only in negligible amounts under routine conditions of incubation. If, however, ^{14}C-acetyl CoA is used in place of acetate during incubation, hydroxamates of short-chain acids may be demonstrated.

Analysis of diethyl ether extracts revealed that HMG is synthesized during routine incubations. In unpublished experiments it has been shown that the pigeon liver enzyme fraction precipitating between 50 and 65% saturation with ammonium sulfate synthesizes HMG from acetyl CoA. If DPNH is added to the system the quantity of HMG formed is markedly reduced and a component with the properties of β-hydroxybutyric acid is increased in amount. Since DPNH functions as an absolute cofactor in fatty acid synthesis[11,13], it is possible that DPNH may be a controlling agent in the utilization of acetate for HMG[21,24] or for fatty acid synthesis.

The demonstration of net synthesis of fatty acids and the results of the decarboxylation experiments indicate that the purified pigeon liver system synthesizes fatty acids *de novo* by successive condensations of acetate units.

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SUMMARY

The long-chain fatty acids synthesized from acetate by an enzyme system reconstructed fom soluble fractions of pigeon liver have been separated and identified by a variety of techniques. Free palmitic acid and progressively smaller amounts of free myristic, lauric and decanoic acids are principally formed in this system. Small quantities of esterified long-chain acids with the properties of palmityl CoA were also found. Short-chain fatty acids do not accumulate, but small amounts of the hydroxamates of short-chain acids can be formed if acetyl CoA is used as substrate in place of acetate. HMG has been identified in the incubation mixture.

Net synthesis of fatty acids was demonstrated by direct weighing, titration and radioactivity measurements. The specific radioactivity of the carboxyl carbon of fatty acids synthesized from carboxyl-labeled acetate was 1.7 times that of the average carbon atom in the entire chain. This finding is in agreement with the concept of fatty acid synthesis via head-to-tail condensations of acetate units.

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THE CLEAVAGE AND SYNTHESIS OF CYSTATHIONINE IN WILD TYPE AND MUTANT STRAINS OF *NEUROSPORA CRASSA**

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Studies on the methionineless mutants of *Neurospora* have established that methionine synthesis goes over the pathway: cysteine + homoserine → cystathionine → homocysteine → methionine[1-3]. In this paper, experiments on the enzymic cleavage of cystathionine and on the reversibility of the above pathway are reported, and a paper chromatographic method for the determination of cystathionine is described.

MATERIALS AND METHODS

Cultures for enzyme preparations were grown under aeration in carboys containing 8–16 liters of the nutrient fluid described by HOROWITZ AND BEADLE[4]. Two methods were used to extract the enzymes (Method A and Method B). The preparations were kept cold throughout the operations.

Method A: The mycelium was extracted twice with 1 ml distilled H_2O per g wet weight of mycelium. After grinding in a mortar with sand, the material was centrifuged at 8,000 *g*. The supernatant was decanted and then brought to 50% of saturation with $(NH_4)_2SO_4$. After 20 minutes the precipitate was collected by centrifugation and dialyzed with stirring for 3–6 hours against 20 volumes of 4 m*M* tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.6. The buffer was renewed and dialysis repeated two times. The dialysate was next frozen, then thawed and the resulting precipitate removed by centrifugation. The supernatant was used as a source of the enzyme.

Method B: Mycelium to be extracted was frozen with dry ice and the cell walls broken in a blendor. This material was centrifuged at 19,000 *g* for 1 hour and the precipitate discarded. The supernatant was dialyzed for 2 hours against 20 volumes of 4 m*M* Tris buffer at pH 8.6. The buffer was renewed and the dialysis repeated two times. The dialysate was used as the source of the enzyme.

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