cellules irradiées mais non traitées par cet extrait. Tout se passe comme si la synthèse d'ARN en présence d'extrait de levure dépassait déjà, après 60 minutes, celle des cellules irradiées mais non traitées par cet extrait. L'extrait de levure restaure donc à la fois la formation induite d'enzyme et la synthèse d'ARN chez la levure irradiée.

RÉSUMÉ

La synthèse d'un enzyme adaptatif (catalase), partiellement inhibée par irradiation ultraviolette, est restaurée par l'addition d'un extrait de levure lorsque celui-ci est ajouté immédiatement après l'irradiation. La restauration de la synthèse adaptative ne commence cependant qu'après 120 minutes d'incubation environ. Elle est précédée de la restauration de la synthèse d'ARN dans les cellules irradiées.

SUMMARY

The synthesis of an adaptive enzyme (catalase) is partly suppressed by ultraviolet irradiation but it is restored by yeast extract when the addition follows immediately after irradiation. However, restoration of the adaptive synthesis does not set in before an incubation period of approximately 120 minutes. It is preceded by restoration of RNA synthesis in the irradiated cells.

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STUDIES ON THE MECHANISM OF FATTY ACID SYNTHESIS

IV. BIOSYNTHESIS OF LONG-CHAIN FATTY ACIDS IN A RECONSTRUCTED SYSTEM OF SOLUBLE ENZYMES FROM CHICKEN LIVER*

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In previous communications of this series the preparation and properties of a reconstructed system of soluble enzyme fractions from pigeon liver capable of synthesizing

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long-chain fatty acids from acetate were described[1,2,3]. It was apparent, however, that this system, although of uniformly high activity, did not lend itself to preparations on an adequately large scale and hence to the isolation and purification of the individual enzymes participating in this process. Therefore, the possibility of preparing a similar system from a more suitable starting material was investigated. In this communication the preparation and some of the properties of a system of soluble enzyme fractions from chicken liver is described.

MATERIALS AND METHODS

Preparation of the soluble enzyme system

Livers removed from laying hens were homogenized in a Waring blendor with 1.5 volumes (w/v) of ice-cold potassium phosphate-potassium bicarbonate buffer[4] for 1 minute. The brei was centrifuged for 30 min at 2000 × *g* to remove intact cells, cell debris and nuclei. The supernatant solution was filtered through 4 layers of gauze (this removed most of the fat which accumulated on top) and was further centrifuged in the Spinco preparative centrifuge (rotor No. 30, 78,000 × *g*) for 1.5 hours. The clear, red supernatant solution was filtered through 4 layers of gauze to remove additional fat which accumulated on centrifugation. The supernatant solution, which will be referred to as CLS, could be kept frozen at — 20° with no loss of activity even after long periods of storage.

Ammonium sulfate fractionation of CLS

CLS was fractionated by successive additions of solid ammonium sulfate[5]. The following fractions were collected: (1) 0 to 29 %, (2) 29 to 40 %, (3) 40 to 50 % and (4) 50 to 65 % saturation of ammonium sulfate. All precipitates were taken up in 0.02 *M* potassium phosphate buffer (pH 7.0) containing $5 \cdot 10^{-3} M$ reduced glutathione and the resulting solutions were dialyzed for 4 hours against a large excess of a mixture of potassium phosphate and potassium bicarbonate buffer of pH 7.0 (the concentration of each component being $5 \cdot 10^{-3} M$). The dialyzed fractions, designated R_1, R_2, R_3 and R_4, respectively, were kept frozen at — 20° until used. There was no loss in activity under these conditions. It will be shown in the text (*cf.* Table I) that a combination of R_1, R_2 and R_4 is required for fatty acid synthesis from acetate.

Protamine sulfate purification of R_1

R_1 was further purified by precipitation with protamine sulfate followed by a second ammonium sulfate precipitation. The precipitate which appeared on thawing the frozen solution of R_1 was spun off and the supernatant solution was diluted with 0.04 *M* potassium bicarbonate so as to attain a final protein concentration of 20 per ml. Protamine sulfate (1 %) was added in sufficient amount to bring the ratio mg of protein: mg of protamine sulfate to 40. The precipitate that formed was immediately spun off and the active protein which remained in the supernatant solution was precipitated by addition of ammonium sulfate at 30 % saturation. The precipitate was taken up in 0.02 *M* potassium phosphate buffer (pH 7.0) containing $5 \cdot 10^{-3} M$ glutathione, and the solution (designated R_{11}) was used without further dialysis. R_{11} at this stage could be kept frozen at — 20° but lost activity on repeated freezing and thawing.

Purification of R_2 by ethanol precipitation

R_2 was purified by precipitation with ethanol. The most active fraction was obtained between 15 and 23 % ethanol (v/v) at — 5°. The precipitate was taken up in 0.02 *M* potassium phosphate buffer (pH 7.0) containing $5 \cdot 10^{-3} M$ glutathione and dialyzed for 2.5 h against a large excess of $5 \cdot 10^{-3} M$ potassium phosphate-potassium bicarbonate buffer of pH 7.0. This fraction (R_{22}) is stable and can be kept frozen at — 20°.

Fractionation of R_4 by ethanol precipitation

By fractionation with ethanol R_4 was separated into 2 distinct active fractions: the first, R_{42}, was precipitated at 16 to 30 % ethanol concentration at — 5° and was deep red in color; the second, R_{44}, was precipitated at 40 to 50 % ethanol at — 5° and was almost colorless. The precipitates were dissolved and dialyzed as described for R_{22}. At this stage both enzymes (R_{42} and R_{44}) are stable and can be kept frozen at — 20°.

Enzymic assay

The method of assay for synthesis of fatty acids was carried out as previously described[2]. Acetate-1-^{14}C was incubated with cofactors and enzyme fractions for two hours at 38° under air. The reaction was stopped by addition of 10% alcoholic KOH and the mixture was saponified. Following

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acidification the long-chain fatty acids were extracted into pentane. Aliquots were taken from the pentane extracts for plating and counting. To recover the short-chain acids the aqueous phase was further extracted with ethyl ether. The ether extract was made alkaline with ammonia to avoid losses of the volatile fatty acids. The ether was removed by evaporation and the residual ammonium salts were dissolved in a small volume of water. An aliquot was applied directly on stainless steel plates for counting. Acetate utilization is expressed as μmoles of acetate incorporated into fatty acids during 2 h incubation at 38°. Specific enzyme activity is calculated as μmoles of acetate incorporated per 2 h per mg of protein.

Resolution of fatty acids by chromatography

The long-chain fatty acids were resolved by the paper chromatographic system of KAUFMANN AND NITSCH[6] as previously described[7]. By this method all the saturated N-fatty acids containing an even number of carbon atoms from 10 to 18 are separated as distinct spots (oleic acid has the same R_F as palmitic acid). The paper chromatogram was subdivided into small segments and the radioactivity associated with each fatty acid was determined by direct counting of the paper strips[7].

In one experiment a pooled sample of long-chain fatty acids was mixed with 20 μmoles each of lauric, myristic, palmitic and stearic acid and separated into the individual acids by the reversed-phase partition chromatography technique of HOWARD AND MARTIN[8] as employed by POPJÁK AND TIETZ[9]. To separate the oleic acid which is eluted together with palmitic acid, the acids eluted with 65% acetone were recovered, mixed with an additional 20 μmoles oleic acid and subjected to hydrogenation* in ethyl acetate[9]. The fatty acids were then re-chromatographed, and the stearic acid thus obtained represented the oleic acid in the original mixture. When this column was used for the separation of fatty acids, the amount of each acid eluted from the column was determined by titration with NaOH. The sodium salts were then converted to the free acids, plated, and counted as before.

Materials

The following substances were obtained from commercial sources: crystalline ATP** (Sigma Chemical Company); DPN, DPNH, TPN and TPNH (Sigma Chemical Company); CoA (Pabst Laboratories, about 70% pure); *iso*citric acid lactone, allo-free (California Foundation for Biochemical Research, 85% pure); and glucose-1-phosphate (Schwartz Laboratories). Acetate-1-^{14}C was obtained from Nuclear Instrument and Chemical Corp., Chicago, Illinois.

RESULTS

Fatty acid synthesis by CLS and subfractions

When chicken liver supernatant (CLS) was incubated with acetate-1-^{14}C in the presence of potassium phosphate buffer, ATP, glucose-1-phosphate, *iso*citrate and $MnCl_2$, 0.22 to 0.46 μmoles of acetate were converted into long-chain fatty acids per 10 mg protein during 2 hours. The full homogenate, (supernatant + mitochondria + microsomes), under similar conditions was inactive. The optimal pH for this reaction is 7.0. At this pH, 0.46 μmoles of acetate were incorporated into fatty acids per 10 mg of protein, while at pH 6.5, 0.33 μmoles, and at 7.5, 0.17 μmoles, were incorporated. The optimal temperature for fatty acid synthesis from acetate-1-^{14}C is 38°. Only traces of acetate were incorporated at 25°. 0.14 μMoles and 0.44 μmoles were incorporated into fatty acids per 10 mg of protein at 30° and 38°, respectively.

The CLS was fractionated with ammonium sulfate, as described under METHODS. As can be seen from the results in Table I, 3 fractions, *viz.* R_1, R_2 and R_4, were necessary for fatty acid synthesis from acetate. None of these fractions showed any activity when incubated alone. R_3 is not required and seems to be inhibitory in the overall reaction.

* The author is indebted to the Department of Chemistry, University of Wisconsin, for carrying out all hydrogenations.

** The following abbreviations are used: adenosine-5'-mono-, di- and triphosphate, AMP, ADP, and ATP; oxidized and reduced diphosphopyridine nucleotide, DPN and DPNH; oxidized and reduced triphosphopyridine nucleotide, TPN and TPNH; Coenzyme A, CoA or CoASH; and reduced glutathione, GSH.

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TABLE I

FATTY ACID SYNTHESIS BY R_1, R_2 AND R_4

Fraction incubated	*μmoles of acetate incorporated*
CLS	0.46
R_1	0.03
R_2	< 0.01
R_4	< 0.01
R_1R_2	0.06
R_1R_4	0.06
$R_1R_2R_4$	0.60
$R_1R_2R_3R_4$	0.48

The incubation mixture contained: 25.0 μmoles potassium phosphate buffer pH 7.0; 4.0 μmoles acetate (250,000 c.p.m. acetate-1-^{14}C); 10.0 μmoles *iso*citrate; 20.0 μmoles glucose-1-phosphate; 4.0 μmoles ATP; 15.0 μmoles GSH; 0.2 μmoles CoASH; 0.25 μmoles DPN; 0.3 μmoles TPN; 0.5 μmoles $MgCl_2$; and 0.5 μmoles $MnCl_2$; 10 mg of protein of CLS or 2 mg of R_1, 1.2 mg of R_2 and 2.5 mg of R_4 in a total volume of 0.5 ml.

A study undertaken to determine the amounts of R_1, R_2 and R_4 required for maximal synthesis showed that the relative concentrations of each of these fractions must be kept constant. When R_2 and R_4 were kept constant and R_1 was varied, fatty acid synthesis increased linearly with the amount of R_1 added until a maximum was reached. In contrast to R_1, R_2 seems inhibitory at high concentrations. From this study the following relative amounts of R_1, R_2 and R_4 were chosen: R_1, 2 mg of protein; R_2, 1.2 mg of protein; and R_4, 2.5 mg of protein. Under these conditions the reconstructed system has a specific activity which is 2 to 3 times greater than that of the original CLS.

Further attempts were made to isolate and purify the enzymes participating in synthesis. R_1 was purified 2 to 3 times by precipitation with protamine sulfate. A 2- to 3-fold purification of R_2 was obtained by ethanol precipitation. Fractionation of R_4 with ethanol showed that R_4 was resolved into two active fractions, R_{42} and R_{44}. Neither of these fractions alone could replace R_4 when added to $R_{11} + R_{22}$. When added together, however, complete reactivation of the system was obtained (Table II). The specific activity of the system employing a combination of the crude fractions R_1, R_2, and R_4, was 0.08 μmoles acetate incorporated into fatty acid per mg of protein in 2 h. The specific activity of the combined purified fractions (R_{11}, R_{22}, R_{42},

TABLE II

FATTY ACID SYNTHESIS BY PURIFIED R_1, R_2 AND R_4

Enzymic fractions added	*μmoles of acetate incorporated*
$R_{11}R_{22}$	0.04
$R_{11}R_{22}R_4$	0.50
$R_{11}R_{22}R_{42}$	0.09
$R_{11}R_{22}R_{44}$	0.04
$R_{11}R_{22}R_{42}R_{44}$	0.53

The incubation mixture contained: 25.0 μmoles potassium phosphate buffer, pH 7.0: 4.0 μmoles acetate (250,000 c.p.m. acetate-1-^{14}C); 5.0 μmoles *iso*citrate; 10.0 μmoles glucose-1-phosphate; 3.0 μmoles ATP; 15.0 μmoles GSH: 0.2 μmoles CoASH; 0.25 μmoles DPN; 0.3 μmoles TPN; 0.5 μmoles $MnCl_2$; 0.5 μmoles $MgCl_2$; 1.0 mg of protein of R_{11}; 0.5 mg of R_{22}; 2.5 mg of R_4; 1.8 mg of R_{42}; and 1.2 mg of R_{44} in a total volume of 0.5 ml.

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and R_{44}) was increased to 0.21 μmole acetate per mg of protein, which amounts to a 10-fold purification over the initial CLS.

In the purified system a linear relationship manifested by a constant specific activity between the amounts of protein added (keeping the relative concentrations of R_{11}, R_{22}, R_{42}, and R_{44}, constant) and fatty acid synthesis was observed when the amount of protein added was below 5 mg. At higher concentrations of protein the specific activity of the system decreased. With the purified system, as also with the crude system, fatty acid synthesis was linear with respect to time after an initial lag period of 10 to 20 minutes. Whereas DPN, TPN and CoA were not essential when CLS was used, these cofactors were required in the fractionated system (see next paragraph).

Substrate and cofactor requirement of the fractionated CLS

A detailed study of the substrate and cofactor requirements of the ammonium sulfate fractionated CLS system was undertaken.

Acetate. The system was saturated at a concentration of $8 \cdot 10^{-3} M$ substrate (*cf.* Fig. 1).

Iso*citrate.* The system showed an absolute requirement for *iso*citrate. Maximum activation was obtained in the range 0.01 to 0.02 M. At higher concentrations of *iso*citrate, inhibition of fatty acid synthesis occurred. *Iso*citrate could be replaced by citrate. This is presumably due to the action of aconitase* which is present in R_{44}.

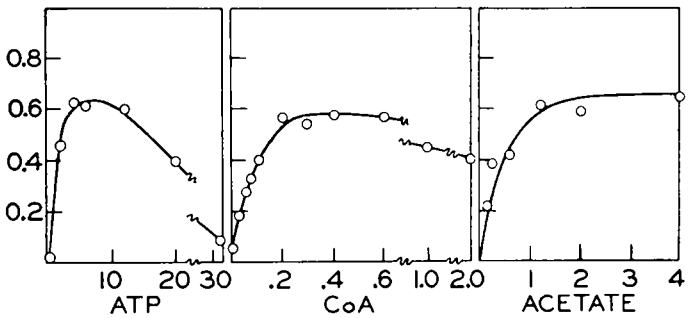

Fig. 1. Amount of fatty acid synthesis as a function of the concentration of ATP, CoA and acetate. The ordinates represent the μmoles of ^{14}C-acetate incorporated into fatty acids; the abscissae, the concentrations of ATP (μmoles per ml), CoA (μmoles per ml) and acetate (μmoles per 0.5 ml). Other additions were made as described in the legend for Table I.

α-Ketoglutarate, succinate and malate were without effect. Although R_4 and also R_{42} and R_{44} contain TPN-linked *iso*citric dehydrogenase*, *iso*citrate could not be replaced with TPNH.

Glucose-1-phosphate. This component markedly stimulated fatty acid synthesis. Maximum stimulation was obtained at a concentration of 0.020 to 0.040 M. In the purified system glucose-1-phosphate and DPN could be completely replaced by DPNH and ATP (Fig. 2). In the presence of 0.006 M ATP, DPNH partially replaced glucose-1-phosphate. When the concentration of ATP was increased to 0.012 M, DPNH completely replaced glucose-1-phosphate. At high concentrations of ATP (in

* *Iso*citric dehydrogenase was assayed as described by OCHOA[10] and aconitase was analyzed spectrophotometrically in a system containing *iso*citric dehydrogenase, TPN and citrate.

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the absence of glucose-1-phosphate and DPNH) some stimulation of fatty acid synthesis was also obtained with DPN.

ATP. The system has an absolute requirement for ATP. Maximum activation of fatty acid synthesis was obtained at 0.004 to 0.012M concentration. At higher concentrations ATP completely inhibits the incorporation of acetate into fatty acid (Fig. 1). In the crude system ADP partially replaced ATP. AMP was without effect.

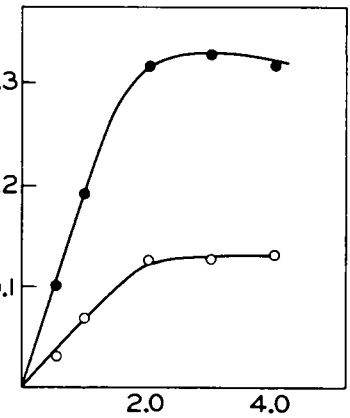

Fig. 2. Amount of fatty acid synthesis as a function of the concentration of DPNH. The ordinate represents the μmoles of ^{14}C-acetate incorporated into fatty acids; the abscissa, DPNH concentration (as the values indicated $\times 10^{-3}M$) at two concentrations of ATP: $6\cdot10^{-3}M$ (open circles) and $12\cdot10^{-3}M$ (closed circles). The reaction mixture did not contain glucose-1-phosphate or DPN. Other additions were: 25.0 μmoles potassium phosphate; 4.0 μmoles acetate (250,000 c.p.m. acetate-1-^{14}C); 5.0 μmoles *iso*citrate; 15.0 μmoles GSH; 0.2 μmoles CoA; 0.3 μmoles TPN; 0.5 μmoles $MgCl_2$; 0.5 μmoles $MnCl_2$; R_{11}, 1.0 mg; R_{22}, 0.5 mg; R_{42}, 1.8 mg; and R_{44}, 1.2 mg of protein in a total volume of 0.5 ml.

CoA. CoA is also required for fatty acid synthesis from acetate. A linear relationship between the amounts of CoA added and fatty acid synthesis is obtained at low concentrations of CoA (Fig. 1). Maximum activation was obtained in the range 0.2 to $0.06\cdot10^{-3}M$. At higher concentrations, inhibition occurred.

DPN and TPN. Both DPN and TPN are required for fatty acid synthesis. Synthesis is abolished if DPN is omitted from the complete system, and is reduced to 40% in the absence of TPN (Table III). High levels of TPN (in absence of DPN) partially restore activity which may indicate a conversion of TPN to DPN. When

TABLE III

SUBSTRATE AND COFACTOR REQUIREMENTS OF THE FRACTIONATED CHICKEN LIVER ENZYMES

	μmoles acetate incorporated into fatty acids
Complete system	0.63
minus *iso*citrate	0.02
minus glucose-1-phosphate	0.21
minus ATP	0.00
minus CoASH	0.01
minus DPN	0.01
minus TPN	0.28
minus $MgCl_2$	0.66
minus $MnCl_2$	0.09
plus 0.15 μmoles α-lipoic acid	0.59
plus 0.15 μmoles thiamine pyrophosphate	0.64

* The complete system contained: 25.0 μmoles potassium phosphate buffer, pH 7.0; 4.0 μmoles acetate (250,000 c.p.m. acetate-1-^{14}C); 10.0 μmoles *iso*citrate; 20.0 μmoles glucose-1-phosphate; 4.0 μmoles ATP; 15.0 μmoles GSH; 0.2 μmoles CoASH; 0.25 μmoles DPN; 0.3 μmoles TPN; 1.0 μmoles $MgCl_2$; 0.5 μmoles $MnCl_2$; 2.0 mg of protein of R_1; 1.2 mg of R_2; and 2.5 mg of R_4 in a total volume of 0.5 ml.

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DPN and TPN were added together to the deficient system, complete restoration of fatty acid synthesis was obtained.

Metal requirement. The presence of Mn^{++} is essential for fatty acid synthesis. Maximum activation was obtained at a concentration level of 0.001 M. $MgCl_2$ could replace Mn^{++} but much larger amounts of $MgCl_2$ were required (*cf.* Table III).

Other reagents

No further stimulation of the incorporation of acetate into fatty acids is obtained by addition of α-lipoic acid or thiamine pyrophosphate to the system of cofactors already present. Although no study of glutathione was made with the chicken liver enzymes, this reagent was routinely added at a concentration of 0.03 M. Glutathione has been demonstrated as an essential component in the pigeon liver system[2].

Type of fatty acids synthesized from acetate-1-^{14}C

Fatty acids which were synthesized by enzymes at three different levels of purity (Table IV) were resolved into individual acids by paper chromatography as described under METHODS. With the CLS or the crude fractions three distinct spots corresponding to stearic, palmitic, and myristic acid, were detected when the chromatograms were developed with copper acetate and potassium ferrocyanide[5]. The ^{14}C distribution in the chromatograms is shown in Table IV. Most of the ^{14}C was incorporated into palmitic acid, but appreciable amounts of ^{14}C were also found in

TABLE IV

CHROMATOGRAPHIC SEPARATION[6] FATTY ACIDS SYNTHESIZED FROM ACETATE-1-^{14}C

Fatty acid	*^{14}C Recovered in fraction as % of total*		
	*CLS**	*$R_1R_2R_4$**	*$R_{11}R_{22}R_{42}R_{44}$***
Ethyl ether extract	—***	traces	—***
Octanoic and shorter (solvent front on chromatogram)	1	< 1	< 1
Decanoic	2	1	< 1
Dodecanoic (lauric)	7	3	3
Tetradecanoic (myristic)	31	15	17
Hexadecanoic (palmitic)	58	80	80
Octadecanoic (stearic)	< 1	< 1	< 1

* Incubation conditions are described in Table I
** Glucose-1-phosphate and DPN were replaced by DPNH.
*** Not estimated.

myristic and lauric acid. Only traces of ^{14}C were detected in stearic acid. Similar results were obtained when a pooled sample of ^{14}C-labeled fatty acids originating from experiments in which a combination of R_1, R_2 and R_4 was chromatographed by the reversed phase chromatography method of HOWARD AND MARTIN[8] as described under METHODS (Table V). In this study oleic acid was separated from palmitic acid after conversion of oleic acid to stearic acid by hydrogenation. Although stearic and oleic acids are contained in the endogenous fat of the enzyme mixture, these acids are synthesized only in very small amounts from acetate-1-^{14}C by the chicken liver fractions under present experimental conditions.

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No appreciable amounts of ^{14}C could be detected in the short-chain fatty acids (ethyl ether extract) even after very short incubation periods (15 min), nor were any acids trapped by addition of unlabeled butyrate, hexanoate or octanoate to the incubation mixture. This is in contrast to observations with soluble preparations of rat and rabbit mammary gland[11,12] in which short chain acids have been found to accumulate.

TABLE V

SEPARATION OF A POOLED SAMPLE OF ^{14}C-LABELED FATTY ACIDS INTO INDIVIDUAL ACIDS

Fatty acid	*^{14}C recovered in fraction*	
	c.p.m.	*% of total*
Decanoic (and shorter acids)	21,100	5.6
Dodecanoic (lauric)	22,700	6.0
Tetradecanoic (myristic)	54,000	14.2
Hexadecanoic (palmitic)	268,500	70.5
Octadecanoic (stearic)	8,700	2.3
Octadec-9-enoic (oleic)	4,900	1.4

The reaction mixture was described in the legend for Table I. The enzymes used were R_1, R_2 and R_4.

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SUMMARY

The preparation and properties of an enzyme system from chicken liver, capable of synthesizing long-chain fatty acids from acetate-1-^{14}C, were described. A soluble extract of liver was resolved into 4 distinct enzymic fractions the combination of which was essential for synthesis. A requirement for ATP, CoA, DPNH, TPN, *iso*citrate and Mn^{++} was established. Under these conditions the major product of the synthesis was palmitic acid but appreciable amounts of ^{14}C were also found in lauric and myristic acids.

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