in the ratio between the hydrolysis velocities of the two isomers obtained under the influence of Co^{++}, or by altering the incubation temperature do not necessarily presuppose the existence of different enzymes. The points of attachment of L- and D-substrates to the same enzyme may be only in part identical and in part not. Changes in pH may affect in a different way the non-identical points of attachment, causing a difference in pH-activity curves. Similarly, the difference in effect of Co^{++} on the hydrolysis of L- and D-asparagine may be explained by difference in its way of reaction with the non-identical points of attachment.

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STUDIES ON THE MECHANISM OF FATTY ACID SYNTHESIS

VII. BIOSYNTHESIS OF FATTY ACIDS FROM MALONYL CoA

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SUMMARY

Malonyl CoA can serve as the starting point for the synthesis of long-chain fatty acids in animal tissues. For any given tissue the rate of formation of fatty acids from malonyl CoA is many times faster than that from acetyl CoA. TPNH and one enzyme fraction (R_2) are required for the synthesis of fatty acids from malonyl CoA. DPNH is much less effective than TPNH in some tissues.

The fatty acids formed from malonyl CoA by the isolated enzyme system of cow mammary gland are similar in chain length distribution to those found in milk.

The following abbreviations are used: ATP, adenosine triphosphate; CoA, coenzyme A; DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; R_1, enzyme fraction that carboxylates acetyl CoA to malonyl CoA; R_2, enzyme fraction that catalyzes the synthesis of fatty acids from malonyl CoA.

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INTRODUCTION

In previous reports from this laboratory[1-9] the various cofactor requirements for the synthesis of long-chain fatty acids from acetate in the pigeon liver system have been described. In a more recent report[10] the requirements for the entire sequence were ultimately narrowed down to malonyl CoA, TPNH and only one fraction (R_{2gc}) of the pigeon-liver preparation. Rapid synthesis of long-chain fatty acids was demonstrated in a reaction mixture consisting of the above components.

In the present paper, data bearing on the distribution in various tissues of two enzyme fractions of the pigeon liver system for synthesis of fatty acids from acetyl CoA (R_1 and R_2*, respectively) are presented. These data suggest that malonyl CoA is widely, if not universally, used by animal tissues as substrate for the rapid synthesis of fatty acids. It is further demonstrated that the synthesis of the fatty acids present in cows' milk takes place in the mammary glands of lactating cows by way of the malonyl CoA pathway.

METHODS AND MATERIALS

Preparation of enzymes

Tissues packed in ice were obtained from freshly killed animals from the slaughter house and were used for the preparation of enzymes with a minimum loss of time.

All tissues were cleaned of extraneous materials, cut into small pieces and homogenized in a Waring blendor for 15 to 20 sec at top speed in 0.005 *M* phosphate buffer, pH 7.0 the ratio of tissue to buffer being 1 : 1.5, w/v. The resulting homogenate was centrifuged for 30 to 40 min at 6,000 × *g*. The precipitate was discarded, and the supernatant was filtered through two layers of cheese cloth. At this step most of the lipides of the mammary gland and adipose tissue preparations were easily removed since during centrifugation at 0° solid lumps of lipide are formed in the centrifuge tubes. The supernatant was then further centrifuged for 90 min at 104,000 × *g* in a Spinco Model L ultracentrifuge. The final supernatant fraction, filtered through glass wool, and the particulate fraction resuspended in the same buffer, were used as the sources of the enzymes. However, the protein concentrations of the extracts obtained from the fatty tissues (both adipose tissues and suprarenal fat) were too low for enzymic studies. The protein in these extracts was concentrated by precipitation with ammonium sulfate (60 % saturation). The precipitate was dissolved in a small volume of 0.005 *M* phosphate buffer, pH 7.0.

All manipulations were carried out between 0 to 5° with maximum speed. The enzyme preparations were tested as soon as possible and when stored were kept at —20°. Most of the activity is lost when the enzyme preparation is frozen and thawed several times. Even at —20°, 20 to 30 % of the activities were lost during storage for two to three weeks.

Assay of the enzyme activities

Determination of R_1 activity: According to the data of Tables I and II, the R_1 fraction is the limiting factor in tissues for the total synthesis of fatty acids from acetyl CoA as the starting material. The amounts of fatty acids synthesized by the

* In this paper the nomenclature for describing the two active fractions required for fatty acid synthesis is the same as the one adopted in previous communications of the series[1,6,8].

tissues from acetyl CoA in the presence of all the cofactors would thus be a measure of the concentration of R_1.

For routine assays[6,7], a mixture of 20 μmoles of potassium phosphate buffer of pH 6.5, 4.0 μmoles of $KHCO_3$, 50 mμmoles of TPNH, 0.3 μmoles of $MnCl_2$, 1.0 μmole of ATP, 30 mμmoles of [1-^{14}C]acetyl CoA (60,000 counts/min), the required amount of enzyme (usually 500 to 1000 μg of protein), and water to 0.4 ml was placed in ground-glass stoppered test tubes and incubated at 38°. The reaction was continued for exactly 10 min and then terminated by the addition of 0.2 ml of 1.0 *N* KOH. The reaction mixture was then heated in a water bath for 15 min, and after acidification with 0.2 ml of 2.0 *N* HCl, was extracted with pentane[1]. The radioactivity in the pentane extract, counted as described by WAKIL *et al.*[1], was taken as a measure of enzyme activity.

Determination of R_2 activity: The reaction mixture[10] here consisted of 20 μmoles of potassium phosphate buffer, pH 6.5, 50 mμmoles of TPNH, 6 mμmoles (12,000 counts/min) of labelled malonyl CoA, limiting amount of enzyme (usually 100 to 300 μg of protein), and water to 0.4 ml. The reaction time was at 38° exactly 10 min, and the subsequent steps were the same as those in the estimation of R_1 activity. In tissues with high R_2 activity, the amount of protein added in the assay was lowered to the point that the total radioactivity in the pentane extract did not exceed 2,000 counts/min.

WAKIL *et al.*[7] have described the method of following fatty acid synthesis spectrophotometrically by measuring the rate of oxidation of TPNH. This method is suitable for tissues containing high specific activity, but in many cases the activity was too low to be followed spectrophotometrically. The measurement of radioactivity in the pentane extracts was sufficiently sensitive to cover the entire gamut of tissue activities. For uniformity of comparison of the specific activities of all the tissues, the radioactivity in the pentane extract was used throughout.

Materials

Labelled malonyl CoA was prepared from [1-^{14}C]acetyl CoA by incubating [1-^{14}C]acetyl CoA with purified R_1 from chicken liver[10] in the presence of potassium phosphate buffer of pH 6.5, ATP, $KHCO_3$, and $MnCl_2$. Malonyl CoA formed in the reaction was purified by chromatography on paper. The preparation of [1-^{14}C]acetyl CoA and of labelled malonyl CoA will be described in more detail in another communication[11].

The sources of the following chemicals are indicated in parentheses: CoA (Pabst Laboratories); DPNH, TPNH and ATP (Sigma Chemical Company); and [1-^{14}C]-acetate (Nuclear Instrument and Chemical Corp.).

RESULTS

Distribution of R_1 and R_2 activities in various tissues

The relative distribution of R_1 and R_2 activities in the microsomal and supernatant fractions of several tissues tested, as summarized in Table I, brings out a few striking features. Compared to beef tissues, the livers of the two avian species are the most active sources of R_2. Both R_1 and R_2 can be sedimented, at least partially, by prolonged centrifugation at high speed—an indication of their particulate nature.

Finally, the specific activity of R_2 is invariably many times higher than that of R_1. This would indicate that the fatty acids are formed at a much faster rate from malonyl CoA than from acetyl CoA and that in any given tissue the former can serve as a better substrate for the rapid synthesis of fatty acids.

TABLE I

R_1 AND R_2 ACTIVITIES OF VARIOUS MAMMALIAN AND AVIAN TISSUES

Activities as mμmoles of labelled acetyl CoA or malonyl CoA incorporated into long-chain fatty acids per mg protein/10 min. Assay conditions for both R_1 and R_2 were as described in METHODS.

Tissue	*Sedimentable fractions*		*Supernatant*	
	R_1	R_2	R_1	R_2
Beef liver	—*	—	0.013	0.15
Beef brain	0.07	0.36	—	1.8
Beef pancreas	—	—	0.008	0.3
Beef lungs	0.05	0.21	0.03	0.23
Beef kidneys	—	—	0.005	0.06
Beef small intestine	—	0.015	—	0.015
Beef mammary gland	0.5	2.0	0.9	5.3
Beef adipose tissue	—	0.95	—	2.3
Beef suprarenal fat	0.24	0.90	0.65	7.0
Beef aorta	0	0	0	0
Chicken liver	0.2	1.6	0.7	14.5
Chicken ovary	—	0.02	—	0.04
Chicken oviduct	—	0	—	0
Pigeon liver	0.98	—	1.0	28.9

* Not tested.

That the formation of malonyl CoA is the limiting step in fatty acid synthesis from acetyl CoA is further demonstrated in Table II. It will be seen here that when extracts of beef liver are supplemented with R_1 from another tissue (chicken liver), the ability to form fatty acids from acetyl CoA is greatly enhanced.

TABLE II

ACCELERATION OF FATTY ACID SYNTHESIS FROM Ac* CoA BY SUPPLEMENTATION OF THE EXTRACT FROM BEEF LIVER WITH PURIFIED R_1 FROM CHICKEN LIVER[10]

The basic system contained all the components described in METHODS for assay of R_1. 3 mg of the beef liver extract were used in the assay. Total volume 0.4 ml; incubation 10 min at 38°. The R_1 of chicken liver (0.3 mg) was a purified preparation.

	Counts/min/assay
Beef liver extract (no supplement)	102
Beef liver extract (plus R_1 supplement)	734

Effect of pH on the R_2 activity of beef liver

The pH curve (Fig. 1) demonstrates sharp optimal activity at pH 6.5, and this seems to agree well with a similar curve obtained by WAKIL *et al.*[1] for the pigeon liver system with acetyl CoA as substrate.

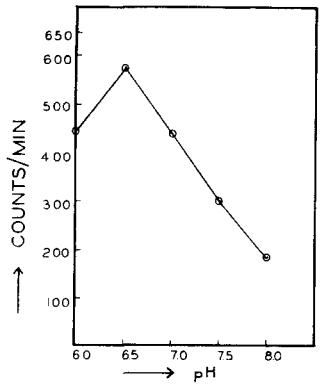

Fig. 1. Effect of pH on the R_2 activity of beef liver. Except for phosphate buffer, the concentrations of components were exactly the same as described in METHODS. Each assay tube contained 170 μg of purified beef liver extract (R_2) and 20 μmoles of potassium phosphate buffer of the pH indicated. Total volume 0.4 ml; incubation time 10 min at 38°.

Requirements for reduced pyridine nucleotides

The requirement for TPNH in the synthesis of fatty acids from malonyl CoA is demonstrated in Table III. In other experiments (not included in Table III) a similar requirement for TPNH in fatty acid synthesis from malonyl CoA was obtained with enzymes prepared from tissues like beef liver, chicken liver, etc. In the system of cow mammary gland, DPNH could replace TPNH with only partial efficiency. It may be recalled that GIBSON *et al.*[6] and WAKIL *et al.*[7] have also observed the equivalence of TPNH and DPNH in the synthesis of long-chain fatty acids from [1-^{14}C]-acetyl CoA by pigeon-liver enzymes. These results, however, differ from those of HELE, POPJÁK AND LAURYSSENS[12], who found an absolute requirement for DPNH in their system prepared from rabbit mammary gland.

TABLE III

THE SPECIFICITY OF THE PYRIDINE NUCLEOTIDE REQUIREMENT IN THE SYNTHESIS OF FATTY ACIDS FROM MALONYL CoA IN THE EXTRACT OF COW MAMMARY GLAND

The basic system contained a mixture of 20 μmoles potassium phosphate buffer pH 6.5; 6 mμmoles of malonyl CoA and extract of cow mammary gland (250 μg protein), and 50 mμmoles of TPNH or DPNH where indicated. Final volume 0.4 ml; incubation for 10 min at 38°.

Nucleotide added	*Radioactivity incorporated into fatty acids from malonyl CoA in 10 min*
None*	0
TPNH	2,015
DPNH	448
TPNH + DPNH	1,653

* The enzyme was stored at — 20° for 3 weeks.

The types of fatty acids formed from malonyl CoA by the cow mammary gland

Cows' milk contains fatty acids of varying chain lengths from C_4 to C_{18}. It was of interest, therefore, to resolve the fatty acids formed from malonyl CoA by the cow mammary gland enzyme system and to compare their relative distribution with that which has been found in cows' milk. At the end of a 10-min incubation period

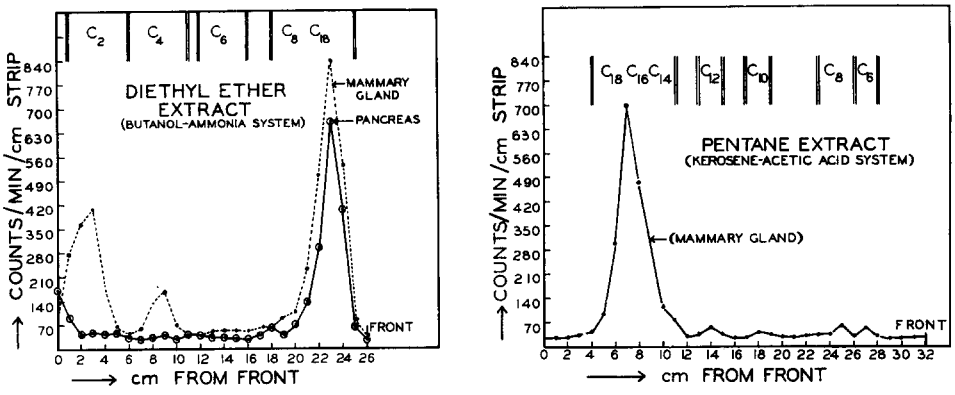

Fig. 2. Distribution of radioactivity in the fatty acids separated on the paper chromatograms of the diethyl ether (a) and pentane (b) extracts of the cow mammary gland system incubated with labelled malonyl CoA.

the fatty acids in the reaction mixture were extracted first with pentane and then with diethyl ether[3]. The short and long-chain fatty acids in the ether and pentane extracts were separated on paper according to REID AND LEDERER[13] and by reverse phase-chromatography according to KAUFMANN AND NITSCH[14].

After the chromatograms had been allowed to run for the desired length of time, the papers were dried, cut into 1-cm strips, and the radioactivity in each 1 cm strip was read in the gas-flow counter. The results, as presented in Fig. 2 (a and b), clearly demonstrate that distinct peaks for C_4 and C_6 fatty acids are obtained in the ether extracts. In this particular system of chromatographic separation (*n*-butanol saturated with 1.5 *N* ammonia), C_8 and higher fatty acids are not resolved, and they appear in one peak[3]. The same applies to Fig. 2(a). The second curve obtained from the corresponding diethyl ether extract of the fatty acids formed by another tissue like beef pancreas shows complete absence of two peaks due to C_4 and C_6 fatty acids. In Fig. 2(b) the resolution of the longer-chain fatty acids formed by the mammary gland extract, as was obtained in the pentane extract, is demonstrated. The values obtained in Fig. 2(a and b) are further analyzed in Table IV. It will be seen that the relative proportions of C_4 and C_6 fatty acids, as compared to the total fatty acids formed by

TABLE IV

THE RELATIVE CHAIN LENGTH DISTRIBUTION OF FATTY ACIDS SYNTHESIZED BY COW MAMMARY GLAND FROM MALONYL CoA

Carbon atoms in fatty acid chain	*Radioactivity*			*% radioactivity in pentane + ether extract*	*% distribution of fatty acid(s) in milk*[15]
	Pentane extract Counts/min	*Ether extract Counts/min*	*Pentane + ether extract Counts/min*		
4	—	312	312	7.0	3.5
6	33	81	114	2.6	1.7
8	57				
10	24				
12	51	2152	3985	90.4	94.8
14					
16	1701				
18					

the cow mammary tissue preparation, closely agree with the analytical values to be found in the literature for the fatty acids of cows' milk[15]. It is rather interesting that in an isolated system from cow mammary tissue malonyl CoA gives rise to the same types of fatty acids as are found in the milk secreted by the same tissue.

Attempts at purification of beef liver R_2

Considerable purification of R_1 and R_2 of both chicken and pigeon liver has been achieved in this laboratory. Even though the specific activity of beef liver R_2 in the initial extract is rather low, the easy availability of large amounts of this tissue encouraged us to attempt the preparation of an enzyme system with greatly enhanced specific activity by the method of WAKIL *et al.*[10] and GIBSON *et al.*[6]. Solid ammonium sulfate was added to the extract of beef liver to bring the saturation to 25 %. The small precipitate formed was discarded. Most of the activity precipitated between 25 and 35 % saturation of $(NH_4)_2SO_4$. This precipitate was redissolved in small amounts of 0.005 *M* potassium phosphate buffer of pH 7.0, and the solution was dialysed for 3 to 4 h against the same buffer. It was then diluted with the same buffer to a protein concentration of 30 mg/ml and treated with calcium phosphate gel[16] in the ratio of 2 mg gel to 1 mg protein. After centrifugation the supernatant was discarded and the gel was eluted 5 to 6 times with 4 volumes of 0.1 *M* potassium phosphate buffer of pH 7.0. The gel eluates were combined and again solid ammonium sulfate added to bring the saturation to 35 %. After these treatments R_1 activity was totally abolished, and a twenty-fold purification of R_2 was achieved. The enzyme (R_2), however, became very unstable at this stage, and further attempts at purification on DEAE ion-exchange cellulose columns were unsuccessful. The steps of purification are summarized in Table V.

TABLE V

PURIFICATION OF BEEF LIVER EXTRACT (R_2)

Stage in purification	*mμmoles malonyl CoA incorporated/mg protein/10 min*
After clarification of the homogenate at 104,000 × *g*	0.15
After precipitation with ammonium sulfate	1.8
After adsorption on and elution from gel	3.0

DISCUSSION

Probably one of the significant aspects of the present study is the demonstration that malonyl CoA can serve as a universal substrate for fatty acid synthesis in animal tissues. The requirements for the synthesis from malonyl CoA are much narrower than those for synthesis from acetyl CoA. Thus, whereas with acetyl CoA as the starting material, R_2, R_1, ATP, Mn^{++}, HCO_3^-, and TPNH are absolute requirements, with malonyl CoA as starting point, R_2 and TPNH would be enough to give total synthesis. Also, in any given tissue, the rate of formation of fatty acids is many times faster with malonyl CoA, as compared to acetyl CoA.

Acetyl CoA must pass through malonyl CoA before it can be incorporated into

fatty acids, but once the latter compound is generated the successive condensations are relatively quicker. The rate of formation of malonyl CoA from acetyl CoA seems to be the limiting step in the synthesis from acetyl CoA. It remains, however, to be seen whether in animal bodies, malonyl CoA can be formed by mechanisms other than that of carboxylation of acetyl CoA.

The distribution of R_1 and R_2 activities reveals some interesting features. Of all tissues studied, the avian liver is the most active source of the system for synthesis of fatty acids. For obvious practical reasons, chicken liver is still the tissue of choice. The mammary gland puts out large amounts of fat in the milk which is continuously secreted, and it is not too surprising to find high activity in this tissue. The high synthesizing activity of both adipose and suprarenal fat is of considerable interest. No doubt the protein concentrations of these tissues are very low, but the fact remains that fat tissues are distributed in all parts of the body in large quantities, and they would add up to considerable amounts of synthetic activities. Attention should be drawn here to the interesting work of HAUSBERGER *et al.*[17,18] who have demonstrated that not only the adipose tissues of the rat synthesize fatty acids from labelled glucose, but the specific activity of these tissues is markedly higher than that of the liver of the same animal. The markedly higher synthetic activity of fat tissues compared to that of bovine liver with both acetyl CoA and malonyl CoA as substrates has also been borne out by the data of the present communication.

Finally, it is probably proper to note the lack of activity of the ovaries and the oviducts of laying hens, in contrast to the high activity in the cow mammary gland. This would imply that whereas at least part of the fat secreted in milk is synthesized by the mammary gland, the fatty acids in egg yolk are synthesized outside the ovaries or oviduct, most probably in the liver.

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THE IDENTIFICATION AND QUANTITATIVE ESTIMATION OF ETHANOLAMINE AND SERINE IN LIPID HYDROLYSATES

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SUMMARY

A paper chromatographic method for the separation and identification of minute amounts (0.01–0.10 μmole) of ethanolamine and serine in lipid hydrolysates is described. The chromatograms are stained directly with a ninhydrin reagent and the resultant coloured spots cut out and extracted for quantitative estimation. The method has been applied to the analysis of several phospholipid preparations. The results are compared with those obtained by a dinitrofluorobenzene method.

INTRODUCTION

The present study was undertaken in an attempt to find a suitable method for the quantitative analysis of phosphatidylethanolamine and phosphatidylserine in phospholipid fractions. It was felt that determination of ethanolamine and serine in hydrolysates of lipids represented the most practical approach to the solution of this problem. Several such assay procedures have been described, and the field was reviewed recently by DITTMER, FEMINELLA AND HANAHAN[1], who found that many of the available methods were inadequate.

A previous paper chromatographic procedure[2] for the micro estimation of nitrogenous phosphatide constituents has been found to give good results with standard ethanolamine and serine solutions, but DITTMER[1] *et al.*[1] and MUNIER[3] have proved that it is unsatisfactory when applied to lipid hydrolysates. The chromatographic method described in this paper is both rapid and convenient, and is suitable for the determination of from 0.01 to 0.10 μmole of each compound in lipid hydrolysates.

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