STUDIES ON THE CYCLOPHORASE SYSTEM

XXV. FATTY ACID OXIDATION IN THE RABBIT LIVER SYSTEM

by

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In a previous study of this series[1], it was shown that the cyclophorase-mitochondrial (C.M.) system of rabbit kidney catalyzed the complete oxidation of even-numbered fatty acids from C_4 to C_{12}. The odd-numbered fatty acids from C_5 to C_{13} were oxidized as far as propionic acid. The non-accumulation of intermediates during fatty acid oxidation by the kidney system made it difficult to gain insight into mechanism of reaction. A few observations on the C.M. system of rabbit liver were also reported, from which it appeared that acetoacetate accumulated during the oxidation of the even-numbered fatty acids, but not of the odd-numbered fatty acids.

The present investigation was aimed at extending these earlier observations on the liver system, with special reference to the factors which determine the accumulation of acetoacetic acid.

Oxidation of β-hydroxy acids

Both the liver and kidney systems contain an enzyme which catalyzes the oxidation of β-hydroxy acids to the corresponding keto acids. GRAFFLIN AND GREEN[1] observed that the yield of acetoacetate, formed during the oxidation of β-hydroxybutyrate in absence of sparker, was variable and usually low in relation to the oxygen uptake. This discrepancy increased with increasing chain length of the hydroxy acid.

When the oxidation of β-hydroxybutyrate is conducted in the presence of 0.033 *M* fluoride, approximately one molecule of acetoacetate is formed for each atom of oxygen consumed (Table I). Thus, whatever the process involved in the further utilization of acetoacetate or in the low yield of acetoacetate, it does not proceed in presence of fluoride. Under these conditions, the oxidation of butyrate (Table I) and other fatty acids is suppressed, while that of the higher hydroxy acids is greatly reduced as compared to the rate in absence of fluoride.

It appears that in absence of fluoride, two oxidizing enzyme systems are involved; whereas in presence of fluoride, only one of these two is active *viz.* the β-hydroxybutyric dehydrogenase which catalyzes the one-step oxidation of β-hydroxybutyrate to acetoacetate. In absence of fluoride, the general fatty acid oxidase system is able to carry out the oxidation of a limited amount of β-hydroxybutyrate essentially to carbon dioxide

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TABLE I

ACETOACETATE FORMATION FROM β-HYDROXYBUTYRATE WITH AND WITHOUT FLUORIDE

Substrate	*μ moles*	*Fluoride μ moles*	*O_2 Uptake μ atoms*	*Acetoacetate formed μ moles*
a. DL-β-hydroxybutyrate	100	—	52.5	29.6
	100	100	42.2	36.5
b. DL-β-hydroxybutyrate	100	—	61.1	21.6
	100	100	30.2	32.4
c. DL-β-hydroxybutyrate	100	—	37.3	20.3
	100	100	32.4	31.8
d. Butyrate	10	—	88.6	2.9
	10	100	7.0	0

1.5 ml of rabbit liver preparation, made to a final vol. of 3.0 ml with 0.9% KCl containing 3 μmoles AMP, 4 μmoles of $MgCl_2$, 20 μmoles of phosphate at pH 7.2, substrate and fluoride as indicated. Gas phase oxygen. Temp. 38°. The experiment was terminated when O_2 uptake ceased (2–3 hours). O_2 uptake in experimental flask corrected for O_2 uptake without substrate.

and water. There is sufficient amount of endogenous substrate in the enzyme preparation to spark this oxidation. The discrepancy between acetoacetate formation and oxygen uptake in absence of fluoride is thus apparently due to the fact that a small and variable amount of β-hydroxybutyrate is being attacked by the fatty acid oxidase. It should also be pointed out that the β-hydroxybutyric dehydrogenase works less effectively on the higher hydroxy acids, whereas the fatty acid oxidase works equally well on hydroxy acids between C_4 and C_{10}.

This clarification of the low yield of acetoacetate during the oxidation of β-hydroxybutyrate points to a significant fact *viz.* that the acetoacetate formed by the action of the β-hydroxybutyrate dehydrogenase is not identical with the acetoacetate which arises during the activity of the fatty acid oxidase. The former is metabolically inert under conditions where the latter is capable of entering the citric acid cycle, and of undergoing oxidation to carbon dioxide and water.

Oxidation of odd and even-numbered fatty acids

There is an extensive body of information on the oxidation of fatty acids by various types of preparations from rat liver (*cf.* [2,3]), but only a few complete balance studies have been reported. The rabbit liver C.M. system has a few experimental advantages (ease of preparation in satisfactory quantity, low blank, high activity and stability) which make it particularly suitable for such balance studies. Depending upon the chain length from 10 to 30 μmoles can be oxidized to completion in one manometer cup containing 1 to 1.5 ml of the rabbit liver preparation (60 to 90 mg dry weight). Manometric and isotopic data could therefore be collected and compared under conditions of complete substrate disappearance. The carbon of the even-numbered fatty acids could be completely accounted for as carbon dioxide and acetoacetate. In the case of the odd-numbered acids, a small amount of propionate may be spared. β-Hydroxybutyrate could not be found among the end products—a result in line with the fact that this substrate is rapidly oxidized by the liver preparation. Such satisfactory analytical conditions eliminate some of the uncertainties which attend the interpretation of many results previously reported on fatty acid oxidation.

Table II shows representative data on the oxidation of even-numbered fatty acids.

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The theoretical oxygen uptake for complete metabolism of the substrate is calculated after taking into account that moiety which accumulates as acetoacetate. From the difference between this theoretical value and the observed value for oxygen uptake (corrected for blank respiration), the percentage of substrate actually metabolized is calculated (column 6). Considering the variety of determinations entering this figure, the agreement between the isotopic and manometric data is satisfactory. This shows that under our experimental conditions, calculations based on manometric data alone are essentially valid, and that no undue error is introduced by assuming that the endogenous respiratory rate is identical whether or not the fatty acid is present in the range of chain lengths listed in the table.

TABLE II

OXIDATION OF EVEN CARBON FATTY ACIDS IN RABBIT LIVER C.M. SYSTEM

Fatty acid added		*Acetoacetate found*	*O_2 Uptake*		*Substrate* metabolized*	*Initial radioactivity recovered in CO_2 and acetoacetate*
Chain length	*μ moles*	*μ moles*	*Theory* μ atoms*	*Observed μ atoms*	*percent*	*percent*
C_4	10	2.5	80	86.5	106.5	
C_4	20	7.8	137.5	122	92.2	
C_4	30	9.9	221	200	93.0	
C_4	30	10.9	213	181	89.3	97.0
C_6	20	15.0	200	222	107.0	
C_6	20	13.2	214	206	97.5	
C_6	25	17.1	263	203	85	94.3
C_8	4	4.3	54	53	98.7	
C_8	10	13.5	112	119	103.0	
C_8	22	18.9	333	301	93.2	
C_8	16	18.4	205	156.5	86.2	86.5
C_{10}	5	6.1	91.5	106	110.0	
C_{10}	10	12.7	178	150	90.0	
C_{10}	12	13.9	225	220	98.3	
C_{12}	5	5.5	126	104	87.0	
C_{14}	3	2.0	104.5	119.5	103.5	

Conditions as in Table I; in addition 5 μmoles of citrate were added per flask.

* O_2 theory = (O_2 for complete oxidation of substrate to CO_2 and H_2O) — (O_2 for complete oxidation of acetoacetate formed).

$$\mu\text{moles substrate metabolized} = (\mu\text{moles substrate put in}) - \frac{(O_2 \text{ theory} - O_2 \text{ observed})}{(O_2 \text{ for complete oxidation of}) \atop 1\ \mu\text{mole substrate}}$$

It is noteworthy that under the conditions of vigorous oxidation, the molar ratio of acetoacetate formed to substrate metabolized approaches a value of 1 at C_6 and generally exceeds 1 at C_8. No further increase in this ratio is observed with increasing chain length.

Table III shows corresponding data for the odd-numbered fatty acids. Two values are given in the column for theoretical oxygen uptake, the higher one assuming complete oxidation of the acid, and the lower one assuming accumulation of propionate. The percentage of substrate metabolized cannot be estimated in this case if propionate is not determined. Since the observed oxygen uptake approaches the higher theoretical value, it would appear that propionate is largely oxidized. Acetoacetate formation is very small in the case of the lower acids, and tends to rise with increase in chain length.

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Thus, for C_9, about 0.5 mole of acetoacetate is formed per mole of fatty acid. There is no increase in that ratio with further increase in the chain length, *e.g.*, C_{11}.

TABLE III

OXIDATION OF ODD CARBON FATTY ACIDS IN RABBIT LIVER C.M. SYSTEM

Fatty acid added		*Acetoacetate found*	*O_2 Uptake*		*Propionate oxidized**	*Initial radioactivity recovered in CO_2 and acetoacetate*
Chain length	*μ moles*	*μ moles*	*Theory*** μ atoms*	*Observed μ atoms*	*μ moles*	*percent*
C_5	20	0.8	253.5* 113.5	221	15.4	
C_5	25	0.6	320 145	152	1.0	
C_5	30	**	390 180	299	17.0	93.4
C_7	10	2.0	174 104	155	7.3	
C_7	20	2.3	361.5 221.5	281	8.5	
C_7	15	**	285 195	237	6.0	99.4
C_9	5	2.6	104 69	110	5.0	
C_9	8	5.2	158.5 102.5	147	6.4	
C_9	12	6.9	245 161	239	11.0	
C_{11}	6	2.4	167 125	148	3.3	
C_{11}	8	4.5	212.5 156.5	201	6.4	

Conditions as in Table II.

* The higher value includes complete oxidation of the terminal propionate residue, the lower value is valid when propionate appears as end product.

$$\text{Propionate oxidized} = \frac{O_2 \text{ observed} - O_2 \text{ theory for propionate spared}}{(O_2 \text{ for complete oxidation of 1 } \mu\text{mole of propionate})}$$

** Not determined, carrier acetoacetate was added at end of experiment.

*** See footnote to Table II for explanation.

Oxidation of odd and even-numbered fatty acids in rat and pigeon liver mitochondrial preparations

Experiments with similar mitochondrial preparations from rat and pigeon liver showed essentially the same picture with respect to acetoacetate production from the even-numbered fatty acids (Table IV). It was surprising, however, that no oxidation of the odd-numbered fatty acids could be observed in the rat liver preparations, except in presence of an even-numbered fatty acid.

Fatty acid oxidation in presence of malonate

The effect of malonate on the oxidation of fatty acids has been studied by several investigators[4–8]. WEINHOUSE *et al.*[6] failed to obtain the preferential conversion of fatty acids to acetoacetate, which was observed by other workers in the presence of malonate. This discrepancy and the variability of results in experiments with malonate, are discussed by GEYER *et al.*[7,8], and by LEHNINGER AND KENNEDY[5], and are attributed to

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TABLE IV

OXIDATION OF ODD AND EVEN CARBON FATTY ACIDS IN RAT AND PIGEON LIVER C.M. SYSTEM

Animal	Fatty acid added		Acetoacetate found	O_2 Uptake		Substrate metabolized*
	Chain length	μ moles	μ moles	Theory* μ atoms	Observed μ atoms	percent
Rat	C_4	30	10.4	216.8	196	93
	C_5	24	0	144**	0	0
	C_6	20	10.7	234.1	206	91
	C_7	15	0	180**	13.2	7
	C_8	10	7.6	159.2	192	115
	C_6	20	4.0	288	250	88
	$C_6 + C_3$	20 + 10	1.8	305.6**	110	
	$C_6 + C_5$	20 + 20	3.6	481**	342	
Pigeon	C_4	30	6.6	247	67	40
	C_5	30	0.7	174.5**	29	19.3
	C_6	20	8.2	254.5	60	39

Conditions as in Table II, with the exception that in the experiments with the C.M. system of rat liver only 1.0 ml of preparation was used and 12 μmoles of ATP were added to each flask.

* *cf.* footnote, Table II.

** Theory is based on the assumption that terminal propionate is not oxidized. In contrast to rabbit liver preparations, rat liver preparations do not oxidize propionate.

differences in the strains of rats used by various investigators. In the rabbit liver C.M. system, malonate at a level of 0.015 *M*, completely inhibited fatty acid oxidation under the standard experimental conditions whereas lower levels of malonate permitted fatty acid oxidation to proceed essentially according to the normal pattern in absence of malonate. It was found, however, that when higher levels of sparker were employed than for routine experiments, *i.e.*, equivalent to or exceeding the amount of fatty acid substrate, fatty acid oxidation in presence of 0.015 *M* malonate proceeded rapidly with an almost quantitative yield of acetoacetate. Table V shows the influence of the level of sparker on the oxidation of caproate, and the efficiencies of different sparkers in a

TABLE V

EFFECT OF LEVEL AND KIND OF SPARKER ON FATTY ACID OXIDATION IN PRESENCE OF MALONATE

Experiment	Sparker added		O_2 Uptake	Acetoacetate found	$\frac{O_2}{\text{Acetoacetate}}$
		μ moles	μ atoms	μ moles	
1.	citrate	0	3	—	—
	citrate	5	15	4.2	3.6
	citrate	15	72	16.6	4.3
	citrate	30	105	20.2	5.2
	citrate	60	115	22.6	5.1
2.	citrate	30	87	20.5	4.25
	pyruvate	60	53	11.6	4.55
	L-proline	30	12	5.9	2.05
	α-ketoglutarate	30	12	4.2	2.85
	L-glutamate	30	4	1.9	2.1
	succinate	60	128	13.6	9.4
	fumarate	30	133	12.4	10.7

Conditions as in Table I, and in addition, 50 μmoles of malonate and 20 μmoles of caproate per flask. All values are corrected for corresponding blanks without fatty acid.

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malonate-blocked system. It is to be noted that citrate and pyruvate are more effective sparkers for acetoacetate formation from caproate in a malonate-blocked system than L-glutamate, L-proline and α-ketoglutarate. The malonate block is less complete when succinate and fumarate are used as sparkers (oxygen: acetoacetate ratio in Table V). The failure of WEINHOUSE *et al.*[6] to obtain fatty acid oxidation in a malonate-blocked system may well be due to the low level of sparker which they used.

In a non-malonate system, the sparker fulfils two functions: 1. that of initiating fatty acid oxidation; and 2. that of supplying the condensing partner for the C_2 unit formed during β-oxidation. In a malonate-blocked system, the second function is suppressed and unambiguous evidence is thus provided for the first function. Since the citric acid cycle is non-operative, a more or less stoichiometric relation exists between the amount of sparker and the amount of fatty acid whose oxidation is sparked or initiated.

Table VI shows the almost stoichiometric conversion of fatty acids into aceto acetate in the presence of malonate, when excess sparker is added.

TABLE VI

FATTY ACID OXIDATION IN PRESENCE OF MALONATE

Fatty acid added		*Acetoacetate found*	*O_2 Uptake*	
Chain length	*μ moles*	*μ moles*	*Theory* μ atoms*	*Observed μ atoms*
C_4	40	6.2	12.4	16.3
C_5	30	3.7	14.8	24.4**
C_6	30	8.9	23.8	26.7
C_{10}	5	4.7	15.0	14.1

Conditions as in Table V, 20 μmoles of citrate per flask was used as sparker.

* Oxygen uptake corresponding to oxidation of fatty acid to the observed amount of acetoacetate.

** The discrepancy between O_2 theory and O_2 observed is probably due to oxidation of propionate.

Oxidation of higher and substituted fatty acids

The difficulties encountered in attempts to oxidize higher fatty acids in C.M. systems which oxidize lower fatty acids, have been discussed by KENNEDY AND LEHNINGER[9]. In the rabbit liver system, fatty acids above C_8—even at relatively low levels—inhibit oxidations of the citric acid cycle, and damage the mitochondrial particles in such wise that all oxidative activity tends to decline rather rapidly. The use of labeled C_{16} and C_{18} fatty acids made it possible to test the rate of oxidation of these acids at levels far below those used in manometric experiments. Thus, when 1.0 and 0.1 μmoles of palmitate and stearate were tested per manometer cup, 0.1 and 0.012 μmoles respectively were found to be oxidized in the course of a three-hour run. While there was confirmation that these acids are indeed oxidized, the evidence was clear that the rate of oxidation of the higher fatty acids is relatively slow and not comparable to the rate for the lower members of the series. Interference with the magnesium level was ruled out by the experiments with isotopic C_{16} and C_{18}, since the fatty acids could be tested at molar levels one-fortieth that of the magnesium level.

The branched chain acids 10-methylundecylate and 11-methyldodecylate, and

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10, 11-dihydroxyundecylate were not oxidized under conditions where the unsubstituted fatty acids of the corresponding chain length were oxidized rapidly. Lipase activity is present in the enzyme preparation; and therefore methyl and ethyl esters of fatty acids could be used as substrates whenever the parent fatty acids were attacked. ω-phenyl-substituted fatty acids were inhibitory. It was observed, however, that phenylvalerate can be oxidized at a good rate if the inhibitory phenyl-substituted end-product is removed by condensation with glycine which was added in excess to the medium (Table VII).

TABLE VII

OXIDATION OF PHENYLVALERATE IN PRESENCE AND ABSENCE OF GLYCINE

Citrate	*Phenylvalerate*	*Glycine*	O_2 *Uptake* μ *atoms*
+	—	—	121
+	—	+	98
+	+	—	94
+	+	+	195*

Conditions as in Table II; 10 μmoles of 5-phenylvalerate and 30 μmoles of glycine were added as indicated. Citrate (5 μmoles) was present in all flasks as sparker.

* The theoretical O_2 uptake for oxidation of 10 μmoles of phenylvalerate to benzoate is 120 μatoms.

Distribution of isotope in acetoacetate

There is an extensive literature dealing with the incorporation of label into acetoacetate from fatty acids labeled in the carboxyl group[2,6,7,10,11-14]. Since the evaluation of some of the current theories of fatty acid oxidation depends upon a precise knowledge of isotope distribution, it may be of value to present some data collected for the rabbit liver C.M. system (Table VIII). In proceeding from C_4 to C_8, it is seen that the ratio of isotope in the carbonyl group to isotope in the carboxyl group of acetoacetate rises from a value of about 0.3 to a value of about 0.8. A similar rise in the ratio is found in the homologous series of the odd-numbered fatty acids. The lowest member of the series shows the highest asymmetry of distribution, and the highest members the lowest asymmetry.

TABLE VIII

DISTRIBUTION OF LABEL IN PRODUCTS OF OXIDATION OF FATTY ACIDS (-^{14}C OOH) BY RABBIT LIVER C.M. SYSTEM*

Fatty acid	*Percent of Radioactivity recovered in*					$\frac{A}{B}$	$\frac{D}{C}$
	Resp. CO_2 *A*	*Acetoacetate B*	*Acetoacetate carboxyl C*	*Acetoacetate carbonyl D*	*Total end products E*		
C_4	67.6	29.4	22.6	6.8	97.0	2.3	0.30
C_5	85.1	8.3	6.35	1.95	93.4	10.3	0.31
C_6	49.8	44.5	28.2	16.3	94.3	1.1	0.58
C_7	85.5	13.9	8.45	5.45	99.4	6.1	0.65
C_8	27.6	58.9	33.7	25.2	86.5	0.47	0.75

* For complementary data from these experiments, which are not related to isotope recovery, see Tables II and III.

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Co-oxidation of fatty acids of different chain lengths

In view of the difference between odd and even-numbered fatty acids, with respect to the yield of acetoacetate, it seemed of interest to determine the metabolic pattern which obtained when an even-numbered fatty acid was co-oxidized with an odd fatty acid.

In this type of experiment one difficulty must be avoided if the results are to be considered valid. Since the fatty acid of greater chain length is oxidized preferentially, the exhaustion of the longer chain acid during the experiment must be avoided. For example, when C_4 is co-oxidized with C_7 (in 2:1 molecular proportion) only 10% of the C_4 which would be oxidized in absence of C_7 is attacked.

Table IX summarizes analytical and isotope data on some representative co-oxidation experiments. Experiment No. 1 shows that the formation of acetoacetate from C_6 co-oxidized with C_7 is less than 10% of normal. A similar effect of the odd acid is evident from Experiment No. 3, for the pair $C_4 + C_5$*. It is as though the even-numbered fatty acid acquired the pattern characteristic of the odd-numbered acid. Experiment No. 3 shows that propionate as such, present at a level corresponding to that reached in Experiment No. 1 by degradation of C_7, has no effect on acetoacetate formation from C_6. It may, however, be argued that a C_3 residue, arising from C_7 at the enzyme surface and not identical with ordinary propionate, may still be responsible for the observed inhibition. The inability of rat liver mitochondrial preparations to oxidize odd-numbered fatty acids, as reported earlier in this paper, may well be due to a more pronounced form of the inhibition observed here. The fact that rat liver preparations, in contrast to those from rabbit liver, are unable to oxidize propionate may have a bearing on these observations.

EXPERIMENTAL

Enzyme preparation

The method of preparation of the rabbit liver mitochondrial suspension was described in a previous communication of this series[15]. This type of preparation has been found to be active consistently towards fatty acids up to C_{10} with a QO_2 of 30 to 40. The endogenous respiration is relatively low. One to 1.5 ml (60 to 90 mg dry weight) was used per Warburg flask in a total volume of 3 ml.

Medium

The enzyme suspension was supplemented with 4 μmoles of magnesium chloride, 3 μmoles of adenosine-5-phosphate (Bischoff), 20 μmoles of phosphate (pH 7.2) and made up to a final volume of 3.0 ml with 0.9% KCl. Citrate (5 μmoles) was usually added as a sparker. The gas phase was oxygen and the temperature 38°. A fast shaking rate had to be maintained. Most experiments were continued until the oxygen uptake declined to a negligible rate. The time of the experiment varied from 2 to 3 hours.

Chemicals

Acetoacetate and β-hydroxybutyrate were prepared from the ethyl esters by hydrolysis with the theoretical amount of sodium hydroxide, followed by the removal of alcohol *in vacuo*. The sources of the higher β-hydroxy acids and phenyl substituted fatty acids have been indicated in an earlier communication of this series[1]. 10-Methylundecylic and 11-methyldodecylic acids were gifts from Dr HARRY SOBOTKA. 10,11-Dihydroxyundecylic acid was prepared according to SWERN *et al.*[16]**.

Labeled fatty acids

Valeric, caproic and heptylic acids labeled with ^{14}C in the carboxyl group were obtained from

* The authors had the privilege of seeing unpublished data of Drs SIDNEY WEINHOUSE and RUTH H. MILLINGTON obtained with rat liver preparations in which similar effects were clearly evident.

** We are indebted to Dr N. K. SARKAR for this preparation.

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TABLE IX

SIMULTANEOUS OXIDATION OF FATTY ACIDS ($-^{14}C$ OOH) OF VARIOUS CHAIN LENGTH

Expt. No.	Duration min.	Fatty acid C_3	C_4	C_5	C_6	C_7	Acetoacetate found	O_2 uptake	Percent of Radioactivity recovered in				$\frac{A}{B}$	$\frac{A}{C}$	$\frac{D}{C}$
		μ moles					μ moles	μ atoms	Resp. CO_2 A	Acetoacetate B	Acetoacetate carboxyl C	Acetoacetate carbonyl D			
1	50				2*			11	54.0	49.0	28.2	20.8	1.10	1.93	0.74
					5*	15		107	33.0	2.6	1.72	0.88	12.7	19.2	0.51
						15*		127	63.8	3.94	2.30	1.64	16.2	27.8	0.71
					5	15*		89	40.7	2.62	1.58	1.05	15.5	25.8	0.66
2	35			20*				65	44.5		1.82			24.4	
			5	20*				64	38.1		1.81			21.0	
			5*					29	68.4		9.70			7.05	
			5*	20				57	11.2		0.46			24.4	
3	50				2*			0	46.0	56.9	33.9	23.0	0.81	1.36	0.68
		10			2*			55	55.5	50.0	29.2	20.8	1.11	1.90	0.71

Conditions as in Table II.

* The substrate bearing the labeled carboxyl group in each particular experiment is indicated by an asterisk in the columns for the μ moles of fatty acid added.

the Bio-organic Group, Radiation Laboratory of the University of California; butyric and stearic acids from the Texas Research Foundation; and palmitic acid from Tracerlab. We are gratified to Dr SIDNEY WEINHOUSE for a gift of carboxyl labeled octanoic acid.

Analytical procedures

Oxygen uptake and carbon dioxide production were corrected for the corresponding blanks with sparker (citrate) and without fatty acid. Respiratory CO_2 was determined in some experiments by the manometric difference between a flask with alkali in the center well and one without alkali. In both flasks the contents were acidified at the end of the experiment by tipping in acid from a sidearm. The experiments on co-oxidation of various fatty acid substrates were terminated after 30 to 50 minutes by tipping in 0.2 ml of 60% perchloric acid from a sidearm and allowing complete absorption of CO_2 into the center well.

Acetoacetic acid was determined in an aliquot of perchloric or trichloracetic acid filtrates by the aniline citrate method of EDSON[17].

For radioactivity determination, the respiratory CO_2 and the CO_2 formed from acetoacetic acid by decarboxylation with aniline citrate were recovered from the alkali papers in the center well of the Warburg flasks and converted to $BaCO_3$. For determination of the radioactivity of the carbonyl group of acetoacetic acid, acetone was distilled from the medium after decarboxylation and addition of a known amount of carrier acetone to the neutralized solution. It was then precipitated and counted as the 2,4-dinitrophenylhydrazone. The samples were mounted on aluminum planchets, counted and the counts were corrected for self-absorption of the particular material plated according to standard methods[18]. The probable error in counting was kept below 3%.

DISCUSSION

Any hypothesis covering fatty acid oxidation in the liver C.M. system must provide an adequate explanation for the following observations:

1. Even-numbered fatty acids from C_6 and above yield at least one mole of acetoacetate per mole of fatty acid metabolized.

2. Lower odd-numbered fatty acids yield very little acetoacetate, whilst the higher members of the series yield up to 0.5 mole of acetoacetate per mole of fatty acid.

3. The label from carboxyl-labeled fatty acids is found in the respiratory CO_2 and acetoacetate.

4. The distribution of label in acetoacetate is not symmetrical, the larger proportion being in the carboxyl group. This is also true for acetoacetate arising from valeric and heptylic acids which would be expected to give rise only to one type of C_2 fragment.

5. No intermediates can be observed between the initial substrate and the final products of oxidation.

The original, unmodified recombination hypothesis[12, 19] is untenable since according to this view the acetoacetate should be labeled symmetrically. The CRANDALL AND CURIN hypothesis[13] of two types of C_2 units has the virtue of providing an explanation for the isotope distribution in acetoacetate formed from the even-numbered fatty acids. Additional assumptions have to be made to fit the results with odd-numbered fatty acids into this scheme, as discussed in part by GEYER *et al.*[8, 14]. To account for the preferential carboxyl labeling of acetoacetate derived from valerate, one has to assume the production of unlabeled C_2 units from the C_3 end piece, or from sources other than the fatty acid substrate which would have a preference for the carbonyl group of acetoacetate.

An alternative explanation can be offered which would account for the observations enumerated above, and the essence of which would be quite unavoidable if the concept of a general pool of C_2 units arising during fatty acid oxidation is abandoned, as suggested by some of our experiments. According to this hypothesis, the enzyme unit of fatty acid oxidation within mitochondria consists of one oxidase enzyme in close juxta-

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position to the condensing enzyme (acetyl CoA + oxalacetate) and the acetoacetate forming enzyme (2 acetyl CoA $\rightleftharpoons$ acetoacetate)*. If we may assume (1) that each unit is self-contained (*i.e.* no C_2 pool between enzyme units), (2) that the cleavage of β-keto acids is reversible**,

$$RCOCH_2COOH \rightleftharpoons RCOOH + CH_3COOH$$

and (3) that the rate of β-oxidation is more rapid than the rate of disposal of C_2 units***, the asymmetric incorporation of carboxyl ^{14}C into acetoacetate can be accounted for as follows. Representing octanoate as consisting of four C_2 units, $ABC\overline{D}$§ where D represents the carboxyl bearing C_2 unit, the following reaction will take place at the β-keto acid stage:

$$\begin{array}{lll} 1.\ ABC\overline{D} & \rightleftharpoons ABC & + \overline{D} \\ 2.\ ABC & \rightleftharpoons AB & + C \\ \quad AB\overline{D} & \rightleftharpoons AB & + \overline{D} \\ 3.\ AB & \rightleftharpoons A & + B \\ \quad A\overline{D} & \rightleftharpoons A & + \overline{D} \rightleftharpoons \overline{D}A \end{array}$$

As the result of β-oxidation proceeding more rapidly than disposal of C_2 units, the label will move down the molecule, each C_2 unit being less labeled than its predecessor, and more labeled than its successor. The equilibration process will not be complete, since the disengagement of acetoacetate or the final disposal of C_2 units places a limit on the extent of the process. The distribution of label in acetoacetate formed by carboxyl-labeled butyrate should provide a measure of the extent and speed of the equilibration process. It is assumed that the terminal C_4 unit of the even-numbered fatty acid, being in effect a preformed molecule of acetoacetate, is the principle source of acetoacetate. Acetoacetate also arises by way of recombination of C_2 units arising along the fatty acid chain. It is reasonable to expect that in this recombination process the first C_2 unit taken up by the enzyme would form the head end of acetoacetate, whilst the second unit would form the tail end. Since according to this hypothesis the order of introduction of C_2 units corresponds to the order of isotope concentration, *i.e.*, successive C_2 units contain less isotope, more label will be found in the carboxyl (head) than in the carbonyl (tail) moiety of acetoacetate.

The above interpretation has been developed in more detail elsewhere (20). This hypothesis obviates the necessity for postulating two different types of C_2 units which, although entering a common pool, would still maintain their identity.

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* More than one molecule of the acetyl-oxalacetate condensing and acetoacetate-forming enzymes would have to be present for each molecule of fatty acid oxidase.

** Studies on fatty acid synthesis and breakdown in extracts of *Cl. kluyverii* suggest that the cleavage of β-ketoacids is indeed reversible[21].

*** The rate of oxidation of fatty acids is as rapid as the rate of oxidation of members of the citric acid cycle. This would suggest that in the liver C.M. system the rate of β-oxidation is not rate limiting.

§ $\overline{D}$ denotes radioactivity in D.

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SUMMARY

A study has been made of fatty acid oxidation in the cyclophorase-mitochondrial system of rabbit liver with particular reference to the mechanism of acetoacetate formation:

1. The oxidation of fatty acids and their β-hydroxy derivatives to CO_2 and H_2O is inhibited by fluoride, whereas the conversion of β-hydroxybutyrate to acetoacetate by β-hydroxybutyric dehydrogenase is not affected.

2. For even-numbered fatty acids the molar ratio of acetoacetate formed to substrate metabolized approaches 1 at C_6 and may exceed 1 at C_8 and higher (upper limit 1.5).

3. For the lower odd-numbered fatty acids acetoacetate formation is very small. For C_9 and C_{11}, about 0.5 mole of acetoacetate is formed per mole of fatty acid oxidized.

4. The ratio of carbonyl to carboxyl label in the acetoacetate produced from the carboxyl-labeled C_4 to C_8 acids is found to increase steadily from 0.3 to 0.8.

5. The oxidation of even-numbered fatty acids proceeds in a similar manner in preparations from rat and pigeon liver. No oxidation of odd-numbered acids was, however, observed with rat liver.

6. The inhibition of fatty acid oxidation by malonate can be overcome by addition of members of the citric acid cycle in relatively high concentration. Under these conditions fatty acids are almost quantitatively converted to acetoacetate.

7. The oxidation of longer chain acids proceeds at a low rate as demonstrated with labeled C_{16} and C_{18}.

8. Odd-numbered fatty acids have an inhibitory effect on acetoacetate formation from even-numbered acids when oxidized simultaneously.

9. Possible mechanisms which explain the mode of acetoacetate formation and the distribution of label in acetoacetate are discussed.

RÉSUMÉ

Le présent travail se rapporte à l'oxydation des acides gras dans le système de cyclophorase provenant des mitochondries du foie de lapin. Une attention toute particulière a été portée au mécanisme de formation de l'acétoacétate.

1. Les fluorures inhibent l'oxydation des acides gras et de leurs dérivés β-hydroxyle; cependant ils sont sans effet sur la transformation du β-hydroxybutyrate en acétoacétate par la déhydrogénase β-hydroxybutyrique.

2. Dans le cas des acides gras à nombre pair d'atomes de carbone le rapport des concentrations molaires d'acétoacétate produit et de substrat métabolisé s'approche de l'unité pour une chaîne en C_6. Ce rapport s'élève au dessus de l'unité pour les chaînes en C_8 ou plus (limite supérieure 1.5).

3. Les acides gras à nombre impair d'atomes de carbone ne produisent que des quantités très minimes d'acétoacétate. L'oxydation d'une molécule d'acide gras en C_9 ou C_{11} donne naissance à 0.5 molécule d'acétoacétate.

4. L'oxydation d'acides gras de C_4 à C_8 marqués dans le groupement carboxylique, donne naissance à de l'acétoacétate doublement étiqueté. Le rapport de la radioactivité des groupements carbonyle/carboxyle croît de 0.3 à 0.8.

5. Des préparations de foie de rat ou de foie de pigeon oxydent également bien les acides gras à nombre pair. Le foie de rat semble cependant incapable d'oxyder les acides gras à nombre impair.

6. Le malonate inhibe l'oxydation des acides gras mais cette inhibition est renversée par l'addition de fortes concentrations de l'un des acides du cycle de l'acide citrique. Dans ces conditions expérimentales les acides gras sont presque entièrement transformés en acétoacétate.

7. A l'aide d'acides gras en C_{16} et C_{18} marqués dans le groupement carboxylique il est démontré que l'oxydation des acides gras à longue chaîne procède avec lenteur.

8. Oxydés simultanément, les acides gras à nombre impair exercent une action inhibitrice sur la formation de l'acétoacétate à partir des acides gras à nombre pair.

9. Les mécanismes de formation de l'acétoacétate ainsi que la distribution de l'isotope dans la molécule d'acétoacétate sont aussi discutés.

ZUSAMMENFASSUNG

Die Fettsäureoxydation im Mitochondrien-Zyklophorase-System von Kaninchenleber wurde untersucht mit besonderer Berücksichtigung des Mechanismus der Azetessigsäurebildung:

1. Fluorid hemmt die Oxydation der Fettsäuren und ihrer β-Oxy-Derivate zu CO_2 und H_2O, während die Umwandlung von β-Oxy-Buttersäure in Azetessigsäure durch die β-Oxy-Buttersäure-dehydrase von Fluorid nicht beeinflusst wird.

2. Im Falle der geradzahligen Fettsäuren nähert sich das molare Verhältnis von Azetessigsäure-

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bildung zu Substratverbrauch dem Wert 1 bei C_6 und überschreitet diesen Wert bei C_8 und höheren Säuren (obere Grenze 1.5).

3. Die niedrigen ungeradzahligen Fettsäuren liefern sehr wenig Azetessigsäure. Bei der Oxydation von 1 Mol C_9 oder C_{11} wird etwa 0.5 Mol Azetessigsäure erhalten.

4. Das Isotopenverhältnis von Carbonyl- zu Carboxyl-Gruppe in der Azetessigsäure, die aus den in der Carboxylgruppe markierten C_1–C_8-Säuren entsteht, wächst stetig von 0.3 bis 0.8 an.

5. Geradzahlige Fettsäuren werden in ähnlicher Weise in Präparaten aus Ratten- und Taubenleber oxydiert.

6. Die Malonsäurehemmung der Fettsäureoxydation kann durch Zusatz von Gliedern des Zitronensäurezyklus in verhältnismässig hoher Konzentration aufgehoben werden. Die Fettsäuren werden dann fast quantitativ zu Azetessigsäure abgebaut.

7. Höhere Fettsäuren werden langsam oxydiert. Dies kann mit den markierten C_{18}- und C_{16} Säuren gezeigt werden.

8. Wenn gleichzeitig der Oxydation unterworfen, haben ungeradzahlige Fettsäuren einen hemmenden Einfluss auf die Azetessigsäurebildung von geradzahligen Säuren.

9. Mögliche Mechanismen zur Erklärung der Bildungsweise der Azetessigsäure und der Isotopenverteilung werden besprochen.

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