TABLE I

SPECIFIC ACTIVITY OF THE DEGRADATION PRODUCTS OF C^{14}-SQUALENE

Degradation product	Specific activity, c.p.m. per mg. C
Acetone	306
Carbonyl carbon	10
CHI_3	360
Succinic acid	70
-COOH	30
$-CH_2-$	80
Levulinic acid	150
CHI_3 (carbon 5)	17
-COOH (carbons 1 and 4)	28
$-CH_2-$ (carbons 2 and 3)	310

and it is probable from the data that only one of the methyl carbons of the acetone fragment contained C^{14} (Fig. 1).

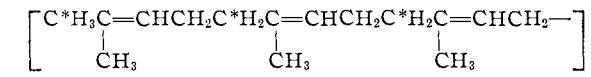

Fig. 1.—Principal distribution of isotopic carbon in biosynthetic squalene.

The distribution of isotope in the squalene suggests that the mevalonic acid was not decarboxylated to yield a five carbon intermediate prior to condensation. If a five carbon intermediate is first produced, the compound would have to react asymmetrically to give the observed isotope distribution. This possibility has not been excluded by the data presented here. Whether mevalonic acid is or is not the biological precursor of squalene and cholesterol, there appears to be little breakdown and re-condensation prior to polymerization. It is likely that decarboxylation occurs during or after polymerization.

Complete details will be presented in a subsequent publication.

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SOME ENZYMATIC REACTIONS OF 6,8-DITHIOLOCTANOIC (DIHYDROLIPOIC) ACID AND ITS ACETOACETIC THIOESTER[1]

Sir:

Evidence has been presented that AcAc[2] synthesis in soluble liver fractions from a source of Ac-S-CoA involves the intermediate formation of AcAc-S-CoA by thiolase, which catalyzes reaction 1, followed by deacylation of AcAc-S-CoA to AcAc.[3-5] However, a direct deacylation of synthetic AcAc-S-CoA is not catalyzed by these enzyme fractions.

$$2\text{Ac-S-CoA} \rightleftharpoons \text{AcAc-S-CoA} + \text{HS-CoA} \quad (1)$$

We find that AcAc synthesis by partly purified ox or chicken liver enzyme preparations from Ac-S-CoA (generated by phosphotransacetylase from acetyl phosphate and CoA-SH[3]) requires the addition of a mono- or dithiol compound. Among active thiols, (±)-DTO[6] has the greatest activity, half maximum activation of AcAc synthesis occurring with 1×10^{-4} *M* (±)-DTO compared to 3.5×10^{-4} *M* BAL and 2×10^{-3} *M* GSH. These liver fractions also catalyze the reactions (2), (3)

$$(\pm)\text{-S-AcAc-DTO} + H_2O \longrightarrow \text{AcAc} + (\pm)\text{-DTO} \quad (2)$$

$$\text{AcAc-S-Pn (or CoA)} + (\pm)\text{-DTO} \rightleftharpoons \text{Ac-S-Pn (or CoA)} + (\pm)\text{-S-Ac-DTO} \quad (3)$$

Mono-(±)-S-AcAc-DTO was synthesized by reacting one equivalent of diketene with (±)-DTO. Presumably it is the 8-ester, since acetic anhydride and DTO have been shown to give 8-S-Ac-DTO.[7] While DTO does not give a positive nitroprusside assay for sulfhydryl,[8,9] we find the monothiol, monothioester form does. Thus, monoacylation of DTO results in the appearance of one sulfhydryl equivalent (by nitroprusside assay), as well as one thioester equivalent (measured with hydroxylamine or optically at 240 mμ). The synthesis and breakdown of mono-S-acyl-DTO compounds can therefore be measured by nitroprusside assay.

The thioesterase catalyzing reaction 2 is assayed optically by following the decrease in light absorption at λ 310 mμ or λ 240 mμ, since (±)-S-AcAc-DTO has an absorption spectrum characteristic of acetoacetyl thioesters.[10,11] It is present in liver but apparently not in other tissues. The enzyme has been purified 40-fold from chicken liver and shown to be different from AcAc-SG thioesterase.[12] It hydrolyzes S-AcAc and S-Ac esters of DTO, MTO and BAL (Table I).

As measured optically at λ 310 mμ, liver and heart enzyme fractions catalyze a disappearance of AcAc-S-Pn and AcAc-S-CoA, provided DTO (or certain mono- or dithiols) is added. With DTO, the decrease in E_{310} due to disappearance of AcAc-SR is accompanied by a simultaneous increase in E_{240}, signifying an increase in total thioester concentration. Balance studies (Table II) show that for each mole of AcAc-S-Pn disappearing, *two* moles of

(1) Supported by grants from the U. S. Public Health Service (No. A-739) and the Williams-Waterman Fund of the Research Corporation.

(2) Abbreviations: 6,8-dithioloctanoic (dihydrolipoic) acid, DTO; monothioloctanoic acid, MTO; coenzyme A (reduced), CoA-SH; pantetheine, Pn-SH; glutathione, GSH; dimercaptopropanol, BAL; thioesters, acyl-S-R; acids: acetic, Ac; acetoacetic, AcAc; *tris*(hydroxymethyl)-aminomethane, Tris; E, optical density; all reference is to sign of rotation (*cf.* footnote 7).

(3) E. R. Stadtman, M. Doudoroff and F. Lipmann, *J. Biol. Chem.*, **191**, 377 (1951).

(4) J. R. Stern, M. J. Coon and A. del Campillo, *Nature*, **171**, 28 (1953).

(5) J. R. Stern, M. J. Coon and A. del Campillo, *J. Biol. Chem.*, **221**, 1 (1956).

(6) DTO, MTO and 6-thioldecanoate were kindly supplied by Dr. J. A. Brockman of Lederle Laboratories.

(7) I. C. Gunsalus, L. S. Barton and W. Gruber, THIS JOURNAL, **78**, 1763 (1956).

(8) I. C. Gunsalus in "The Mechanism of Enzyme Action," the Johns Hopkins Press, Baltimore, Md., 1954, p. 545.

(9) I. Fridovitch and P. Handler, *J. Biol. Chem.*, **223**, 321 (1956).

(10) F. Lynen, *Federation Proc.*, **12**, 683 (1953).

(11) J. R. Stern, *J. Biol. Chem.*, **221**, 33 (1956).

(12) J. R. Stern and G. I. Drummond, *Federation Proc.*, **16**, 363 (1956).

TABLE I

SUBSTRATES FOR THIOESTERASE

15-fold purified chicken liver thioesterase. ~0.5 μM thioester in 0.067 M Tris–HCl buffer pH 7.0. Specific activity = $-\Delta E_{240}$ per minute per mg. protein. Values in parentheses refer to synthetic S-acetyl ester.

	Specific activity
8-S-AcAc-MTO	82
(±)-8-S-AcAc,6-ethyl-MTO	69
(±)-S-AcAc-DTO	56 (6.0)[a]
(±)-6-S-AcAc-MTO	17
(±)-6-S-AcAc-Decanoate	7.5
S-AcAc-BAL	310 (30)

[a] (+)-6-S-Ac-DTO was hydrolyzed at same rate as the synthetic ester.

thioester and *one* mole of thiol are formed. This stoichiometry is consistent with reaction 3 and follows from the fact that AcAc-SR does not assay as thioester by the hydroxylamine method[5] while S-Ac-DTO assays as both thiol and thioester. The Ac-S-Pn and Ac-S-DTO formed were further identified by paper chromatography. It is not yet determined whether 6- or 8-S-Ac-DTO is formed. This mixed thiolysis reaction represents a novel enzymatic synthesis of S-Ac-DTO. It differs from the synthesis of (+)-6-S-Ac-DTO from Ac-S-CoA and (−)-DTO catalyzed by DTO transacetylase[7,8] in that it involves transacetylation with a 4-carbon fragment and utilizes both (−)-DTO and (+)-DTO.[13] The enzyme(s) catalyzing reaction 3 differs from the thiolase[14,15] of the fatty acid cycle (reaction 1) in that (a) AcAc-S-Pn is more reactive than AcAc-S-CoA, (b) it is less sensitive to iodoacetamide, and (c) it is not readily reversible, if at all. Interestingly, other thiol compounds which activate AcAc synthesis (*e.g.*, BAL, GSH, cysteine) can substitute for DTO in reaction 3, yielding the corresponding S-Ac ester.

TABLE II

THIOLYSIS OF AcAc-S-Pn BY DTO

1.50 μM AcAc-S-Pn, 4.0 μM DTO, 150 μM Tris–HCl buffer pH 8.1 and 0.9 mg. ox liver protein. Incubated 13 minutes at 25°. Values in μM.

	Acetyl acceptor		
	(±)-DTO	(+)-DTO	(−)-DTO
Δ AcAc-S-Pn[a]	−1.50	−1.50	−1.50
Δ Sulfhydryl[b]	+1.43	+1.44	+1.71
Δ Hydroxamic acid	+2.99	+2.98	+2.88

[a] Measured optically at 310 mμ. [b] Nitroprusside assay.

(±)-S-AcAc-DTO is reduced by DPNH in the presence of crystalline heart β-hydroxybutyryl-S-CoA dehydrogenase.[16] Liver fractions do not catalyze a thiolysis of (±)-S-AcAc-DTO by DTO, CoA-SH, Pn-SH or GSH. Nor do they convert (+)-6-S-Ac-DTO[7] or (±)-8-S-Ac-DTO to AcAc.

Since thioesterases exist in liver for S-AcAc-DTO and AcAc-SG, the latter are possible intermediates in AcAc synthesis. However, experiments so far have failed to demonstrate their enzymatic formation, *e.g.*, by AcAc transfer from AcAc-S-CoA (or Pn). The precise role of DTO and other thiols in activating AcAc synthesis is being investigated further.

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(13) We are indebted to Dr. K. Folkers of Merck–Sharp and Dohme Research Laboratories for (+) and (−) lipoic acids.

(14) J. R. Stern and S. Ochoa, in "Biochemical Problems of Lipids," Butterworths Publications, London, 1956, p. 162.

(15) F. Lynen, K. Decker, O. Wieland and D. Reinwein, ref. 14, p. 142.

(16) J. R. Stern, unpublished experiments.

A NEW PATHWAY FOR PROPIONATE OXIDATION

Sir:

Oxidation of propionate in animal tissues occurs by a carboxylation pathway through methyl malonate to succinate.[1] Stadtman[2] has observed the formation of β-alanyl-CoA from acryl-CoA extracts of *Clostridium propionicum*. Mahler and Huennekens suggest an α-oxidative pathway.[3] In this communication evidence is presented in support of a β-oxidative pathway in peanut mitochondria.[4]

The oxidation of sodium propionate-1-C^{14} to $C^{14}O_2$ by mitochondria isolated from cotyledons of germinated peanuts[4a] is dependent upon ATP,[5] CoA, DPN, GSH and αKG; TPN and Mn^{++} stimulate the oxidation.

TABLE I

COFACTOR REQUIREMENTS FOR OXIDATION OF PROPIONATE-1-C^{14} TO $C^{14}O_2$

The complete reaction mixture contained 0.1 μmole propionate-1-C^{14} (5500 c.p.m.); 0.5 ml. mitochondria (approximately 20 mg. protein) in 0.2 M Tris- 0.5 M sucrose, pH 7.2, containing about 5×10^{-3} % BAL; 10 μmoles phosphate buffer, pH 7.1; 50 μmoles KCl; 1 μmole ATP; 0.3 μmole CoA; 0.2 μmole DPN; 0.1 μmole TPN; 5 μmoles GSH; 1 μmole αKG; 0.2 ml. 20% KOH in the center well; 0.3 ml. 10 M H_2SO_4 in the sidearm, final volume 1.7 ml. Time of incubation, 2 hr., temperature 25°. % oxidation = $BaC^{14}O_3$ (c.p.m.) × 100/substrate (c.p.m.).

Components	% Oxidation	Components	% Oxidation
Complete	24	−TPN	21
−ATP	1	−GSH	15
−CoA	2	$-MnSO_4$	20
−DPN	11	−αKG	5

Since pools of pyruvate, lactate, succinate and methyl malonate added during propionate-1-C^{14} oxidation do not acquire any label, these compounds do not appear to participate as intermediates. Furthermore, no propionate-dependent fixation of $C^{14}O_2$ can be demonstrated.

To examine the course of oxidation, propionate-1-C^{14}, -2-C^{14} and -3-C^{14}[6] were incubated with a complete reaction mixture for different periods of time and ether-extractable reaction products separated by paper chromatography. In each case a new radioactive spot (R_f 0.25 in ethanol ammonia; propionate R_f 0.42) appeared, which decreased

(1) M. Flavin, P. J. Ortiz and S. Ochoa, *Nature*, **176**, 823 (1955).

(2) E. R. Stadtman, *Federation Proc.*, **15**, 360 (1956).

(3) H. R. Mahler and F. M. Huennekens, *Biochim. et Biophys. Acta*, **11**, 575 (1953).

(4) In a recent personal communication, Dr. M. J. Coon describes a propionate → βHP → β-alanine pathway in animal tissues. In peanut mitochondria no β-alanine accumulates.

(4a) P. K. Stumpf, *Plant Physiology*, **30**, 55 (1955).

(5) Abbreviations: ATP, adenosine triphosphate; CoA, coenzyme A; DPN, diphosphopyridine nucleotide; GSH, glutathione; Mn^{++}, manganese; αKG, α-ketoglutarate; βHP, β-hydroxypropionate.

(6) Kindly donated by Dr. Harland G. Wood.