Since *l*-β-hydroxybutyrate also forms a hydroxamic acid in the presence of ATP, CoA, Mg^{++} and hydroxylamine (*cf.* equation (2)) but causes little if any reduction of DPN (Table I) it would appear that reaction (3) is responsible for the optical specificity of the over-all scheme.

The dehydrogenase catalyzing reaction (3), which has been found to be reversible, may be identical with that recently described by Lynen, *et al.*[9] In view of the present work it would appear probable that the β-hydroxybutyryl-CoA participating in this reaction contains the *d*-isomer. Since reactions (3) and (4) are reversible, *d*-β-hydroxybutyrate may be regarded as a "naturally occurring" metabolite in the form of its CoA derivative and may possibly be an intermediate in the enzymatic oxidation and synthesis of butyric acid.

(9) F. Lynen, L. Wessely, O. Wieland and L. Rueff, *Angew. Chem.*, **64**, 687 (1952).

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RECEIVED FEBRUARY 23, 1953

THE ROLE OF N^{15} GLYCINE, GLUTAMINE, ASPARTATE AND GLUTAMATE IN HYPOXANTHINE SYNTHESIS[1]

Sir:

Previous investigations *in vivo*[2,3,4] have shown that carbon dioxide, formate and glycine are precursors of the carbon atoms of the purine ring, and that glycine also supplies one of the four nitrogen atoms of the ring, nitrogen atom 7. It has also been shown that in an extract of pigeon liver where many of the side reactions complicating quantitative work *in vivo* are negligible, carbon dioxide, formate and glycine combine in the definite molecular proportion of 1:2:1 in the synthesis of hypoxanthine.[5] With the use of this technique of comparing the number of molecules of two different substrates utilized in the synthesis of hypoxanthine by pigeon liver extract, a search has been made for the nitrogen precursors of the three nitrogen atoms of the purine ring not supplied by glycine.[5,6] The N^{15}-labeled substrates were individually incubated with pigeon liver extract synthesizing hypoxanthine from glycine-1-C^{14}. At the termination of the incubation the hypoxanthine formed was converted to uric acid by the action of xanthine oxidase. After the addition of carrier, the uric acid was isolated, purified and analyzed for C^{14} and N^{15} content. The C^{14} of the radioactive glycine and the N^{15} of the other labeled substrates were determined and found to have remained sufficiently constant during the 30-minute incubation. From these analytical values it was possible to calculate the number of atoms of N^{15} which each of the nitrogenous compounds tested contributed to the synthesis of each molecule of hypoxanthine formed *de novo* from radioactive glycine. These ratios are reported in the accompanying table. It may be seen that, although ammonia is readily incorporated into purines *in vivo*, its low incorporation here indicates that it is not an immediate precursor of any one of the hypoxanthine nitrogen atoms. The other nitrogenous substrates, however, contributed significantly to hypoxanthine synthesis. The amide group of glutamine supplied approximately two atoms of nitrogen, and the α-amino group of the other substrates, N^{15} labeled glycine, aspartic and glutamic acids supplied one atom each. These integer ratios suggested that these substrates were contributing to specific nitrogen atoms of the purine ring. Degradation of the uric acid samples was carried out into fractions containing nitrogen atoms 1 and 3 combined, 7 and 9 combined and 7 alone. The analyses from the several experiments revealed considerable variation in the pattern of distribution of the N^{15} in the purine molecule and supported the belief that the substrates were specific donors. In the glycine N^{15} experiment the isotope was primarily in position 7; in the glutamine experiment the N^{15} donated by the amide group was found to be about half in the 9 atom and half in the 1 + 3 fraction. In the aspartic and glutamic acid experiments, almost all of the N^{15} was found in the 1 + 3 fraction, with a small amount in the 7 + 9 fraction. In the 7 + 9 fraction, therefore, the 7 atom is supplied by the known precursor glycine, and the 9 atom by the amide group of one of the two molecules of glutamine used in hypoxanthine synthesis. The 1 + 3 fraction has not been further separated, but the biological similarity of the transaminating dicarboxylic acids favors the probability that their α-amino groups both supply the same one atom of this pair, and that the other nitrogen atom of the pair is donated by the amide group of

TABLE I

LOCATION OF N^{15} IN THE PURINE RING

Expt.	N^{15} labeled substrate	Moles of N^{15} utilized for hypoxanthine synthesis per mole of C^{14} labeled glycine	N^{15} in various N atoms[a] of purine molecule (atom per cent. excess N^{15}) 1 + 3	7 + 9	7	9
1	NH_4Cl	0.27	...	...	...	...
2	Aspartic	1.20	0.091	0.009	...	...
3	Glutamic	1.20	.185	.025	...	...
4	Glutamine (amide N^{15})	1.90	.186	.176	.018	0.334[b]
5	Glycine	1.00	.058	.378	.750	.058

[a] The distribution of N^{15} in the purine ring in individual experiments was determined after further dilution of the original sample with varying amounts of uric acid. Therefore, only the N^{15} values of the nitrogen fraction of uric acid within each experiment are comparable. [b] Estimated from the N^{15} determination of nitrogen atom 7 and nitrogen fraction 7 + 9.

(1) Supported by grants from National Cancer Institute, National Institutes of Health, United States Pulic Health Service and the Damon Runyon Memorial Fund for Cancer Research, Inc. One investigator (J. C. S.) was a fellow of the Jane Coffin Childs Memorial Fund for Medical Research 1950–1952.

(2) J. C. Sonne, J. M. Buchanan and A. M. Delluva, *J. Biol. Chem.*, **166**, 395 (1946).

(3) J. M. Buchanan and J. C. Sonne, *ibid.*, **166**, 781 (1946).

(4) D. Shemin and D. Rittenberg, *ibid.*, **167**, 875 (1947).

(5) M. P. Schulman, J. C. Sonne and J. M. Buchanan, *ibid.*, **196**, 499 (1952).

(6) J. C. Sonne and I. Lin, *Federation Proc.*, **11**, 290 (1952).

the second molecule of glutamine used in hypoxanthine synthesis.

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RECEIVED FEBRUARY 25, 1953

ACETOACETYL COENZYME A AS INTERMEDIATE IN THE ENZYMATIC BREAKDOWN AND SYNTHESIS OF ACETOACETATE[1]

Sir:

In a recent note[2] evidence was presented that enzyme preparations from pig heart catalyze the following reversible reactions[3]

(1) Succinyl-S-CoA + acetoacetate $\rightleftharpoons$ succinate + acetoacetyl-S-CoA

(2) Acetoacetyl-S-CoA + CoA-SH $\rightleftharpoons$ 2 acetyl-S-CoA

(3) 2 Acetyl-S-CoA + 2 oxalacetate $\rightleftharpoons$ 2 citrate + 2 CoA-SH

The CoA transferase catalyzing Reaction 1 is analogous to the acetyl-propionyl CoA transphorase discovered by Stadtman[4] in *C. kluyveri*. Reaction 3 is catalyzed by the citrate condensing enzyme.[5] By various methods, including low temperature ethanol fractionation in the presence of zinc ions, the enzymes catalyzing Reactions 1 and 2 have now been separated and the intermediate acetoacetyl CoA has been isolated.

When succinyl CoA[6] and acetoacetate are incubated with the heart CoA transferase, an acid-stable intermediate accumulates which can be precipitated as a crude, alcohol insoluble barium salt. The intermediate yields no citrate (*i.e.*, no acetyl CoA) in the presence of CoA-SH, oxalacetate and citrate condensing enzyme, unless the acetoacetate condensing enzyme (Reaction 2) from either heart or liver is present. As shown in Table I one molecule of CoA-SH (determined as sulfhydryl) is released for each two molecules of citrate formed (*cf.* Reactions 2 and 3).

Acetoacetyl CoA can be further purified by paper chromatography. In ethanol–acetate[7] its R_F is 0.52 at 24° (R_F of acetoacetate, 0.75). Like acetyl CoA[8] it gives a nitroprusside reaction only after treatment with alkali. The absorption spectrum of acetoacetyl CoA eluates is similar to that of Lynen's analog S-acetoacetyl-N-acetyl thioethanolamine.[9] At *p*H 8.1 the compound shows a strong absorption band in the range 290–320 mμ with a peak at 305 mμ. The absorption is markedly increased by magnesium ions. The formation and disappearance of acetoacetyl CoA, through progress of Reaction 1 or 2 in either direction, can thus be readily followed spectrophotometrically (Fig. 1). By means of the optical method it has been possible to obtain an approximate estimate of the equilibrium constant (*p*H 8.1) of Reaction 2 (K^2_{eq}: (Acetyl-S-CoA)^2/(Acetoacetyl-S-CoA) (CoA-SH) $\simeq 5 \times 10^4$) which greatly favors acetoacetyl CoA cleavage. The equilibrium constant of Reaction 1 (K^1_{eq}: (Succinate) (Acetoacetyl-S-CoA)/(Succinyl-S-CoA) (Acetoacetate)) is about 10^{-2}.

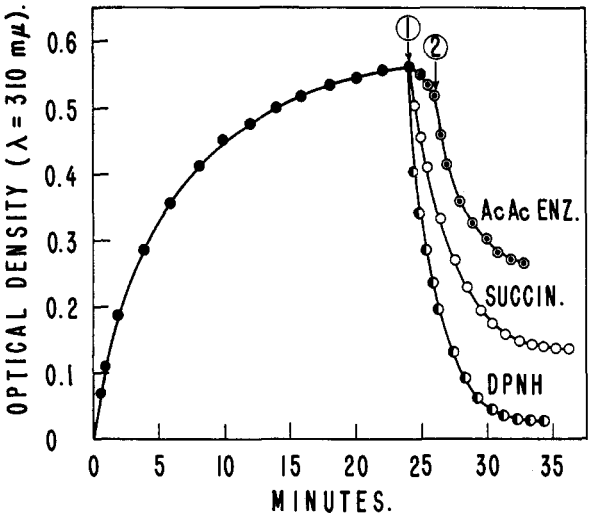

Fig. 1.—Ascending curve represents formation of acetoacetyl CoA on mixing, at *p*H 8.1, 0.4 μM. succinyl CoA and 100 μM. acetoacetate with 0.15 mg. CoA transferase protein (Reaction 1). The descending curves represent three separate experiments: (a) addition of 20 μM. succinate (arrow 1) shifts equilibrium of Reaction 1 to the left. (b) Addition of heart acetoacetate condensing enzyme fraction (0.35 mg. and 1.0 mg. protein at arrows 1 and 2) and CoA–SH causes cleavage of acetoacetyl CoA (Reaction 2). (c) Addition of 0.2 μM. DPNH (arrow 1) causes decrease in extinction through reduction of acetoacetyl CoA to β-hydroxybutyryl CoA.

TABLE I

50 μM. potassium phosphate buffer, *p*H 7.4, 4 μM. $MgCl_2$, 16 μM. potassium oxalacetate, 0.15 μM. CoA–SH (Pabst), potassium salt of intermediate (equivalent to 5.5 mg. of Ba salt), 0.03 mg. crystalline citrate condensing enzyme and, where indicated, ox liver acetoacetate condensing enzyme fraction (free of acetoacetyl CoA deacylase[2]); volume 1.2 ml.; incubation, 30 minutes at 38°.

Acetoacetate enzyme, mg. protein	0	4.3
SH released, μM.	...	0.12[a]
Citrate formed, μM.	0	0.26
SH/Citrate	...	0.46

[a] Corrected for CoA–SH liberated by spontaneous hydrolysis of succinyl CoA present in the acetoacetyl CoA preparation.

The CoA transferase, which has been purified over 100-fold from pig heart extracts, contains as a contaminant the β-hydroxybutyryl CoA dehydrogenase recently described by Lynen, *et al.*[9] DPNH is rapidly oxidized by acetoacetyl CoA (but not by

(1) Supported by grants from The U. S. Public Health Service, The American Cancer Society (recommended by the Committee on Growth, National Research Council), The Williams-Waterman Fund of Research Corporation, and by a contract (N6onr279, T.O. VI) between the Office of Naval Research and New York University College of Medicine.

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

(3) Abbreviations: Coenzyme A (reduced), CoA or CoA-SH; acyl coenzyme A derivatives, acyl CoA or acyl-S-CoA; reduced diphosphopyridine nucleotide, DPNH; μM., micromoles.

(4) E. R. Stadtman, *Federation Proc.*, **11**, 291 (1952).

(5) S. Ochoa, J. R. Stern and M. C. Schneider, *J. Biol. Chem.*, **193**, 691 (1951).

(6) Prepared synthetically from succinic anhydride and CoA-SH by an unpublished method of E. Simon and D. Shemin.

(7) E. R. Stadtman, *J. Biol. Chem.*, **196**, 535 (1952).

(8) F. Lynen, E. Reichert and L. Rueff, *Ann.*, **574**, 1 (1951).

(9) F. Lynen, L. Wessely, O. Wieland and L. Rueff, *Angew. Chem.*, **64**, 687 (1952).