On the basis of PHILPOT's[10] calculation of the molecular weight and flavin content of xanthine oxidase it would appear that there are two molecules of FAD and one atom of molybdenum per molecule of enzyme with a molecular weight of about 230,000.

REFERENCES

1 H. S. CORRAN, J. G. DEWAN, A. H. GORDON AND D. E. GREEN, *Biochem. J.*, 33 (1939) 1694.
2 E. G. BALL, *J. Biol. Chem.*, 128 (1939) 51.
3 B. L. HORECKER AND L. A. HEPPEL, *J. Biol. Chem.*, 178 (1949) 683.
4 D. B. MORELL, *Biochem. J.*, 51 (1952) 657.
5 E. C. DE RENZO, E. KALEITA, P. HEYTLER, J. J. OLESON, B. L. HUTCHINGS AND J. H. WILLIAMS, *J. Am. Chem. Soc.*, 75 (1953) 753.
6 W. W. WESTERFELD AND D. A. RICHERT, *J. Biol. Chem.*, 184 (1950) 163; 192 (1951) 35.
7 H. R. MAHLER *J. Am. Chem. Soc.*, 75 (1953) 3288.
8 E. B. SANDELL, *Colorimetric Determination of Traces of Metals*, New York (1944).
9 E. DIMANT, D. R. SANADI AND F. M. HUENNEKENS, *J. Am. Chem. Soc.*, 74 (1952) 5440.
10 J. ST. L. PHILPOT, *Biochem. J.*, 33 (1939) 1707.

Received August 10th, 1953

SYNTHESIS OF BUTYRYL-COENZYME A BY REVERSAL OF THE OXIDATIVE PATHWAY*

by

PHILIP G. STANSLY** AND HELMUT BEINERT

Institute for Enzyme Research, University of Wisconsin, Madison, Wis. (U.S.A.)

The individual steps in the enzymatic oxidation of BuCoA*** and their reversibility have recently been established[1,2]. The present communication presents evidence for the overall synthesis of BuCoA from AcCoA in a system of six purified enzymes.

The essential sequence of reactions is: AcCoA $\overset{\text{I}}{\rightleftharpoons}$ AcAcCoA $\overset{\text{II, DPNH}}{\rightleftharpoons}$ β-OH-BuCoA $\overset{\text{III}}{\rightleftharpoons}$ crotonyl CoA $\overset{\text{IV, reduced benzylviologen}}{\rightleftharpoons}$ BuCoA, where I is the AcAcCoA cleavage enzyme[3,4,5], II is the β-OH-acyl CoA dehydrogenase[1,2,5,6], III is the unsaturated acyl CoA hydrase[1,2,5,7] and IV is the BuCoA dehydrogenase[1,8,9]. DPNH was generated from DPN by the oxidation of lactate catalyzed by lactic dehydrogenase (V)[10]. Benzylviologen was maintained in the reduced state by the oxidation of hypoxanthine catalyzed by xanthine oxidase (VI)[11].

The complete system consisted of $MgCl_2$, 2 μM; DPN, 3 μM; $CH_3{}^{14}COCoA$ (150,000 c.p.m. per μM) 2 μM; potassium lactate, 100 μM; benzylviologen, 0.5 mg; hypoxanthine, 1.5 mg; I, 0.04 mg; II and III, 0.2 mg; IV, 2.5 mg; V, 0.6 mg; VI, 2 mg; water to 1 ml. The reaction mixture was adjusted to pH 6† and incubated anaerobically for one hour at 38° C.

After deproteinization with perchloric acid, the CoA derivatives were extracted with phenol-benzyl alcohol[12] and the hydroxamic acids prepared by treatment with an excess of neutral hydroxyl-

* This work has been supported in part by contract AT 11-1-64 between the U.S. Atomic Energy Commission, Division of Biology and Medicine and the University of Wisconsin.
** Fellow, Heart Institute, National Institutes of Health. Additional support from the Helen Hay Whitney Foundation is gratefully acknowledged.
*** The following abbreviations are used: BuCoA (butyryl Coenzyme A thioester), Ac (acetyl), AcAc (acetoacetyl), DPNH (reduced diphosphopyridine nucleotide).
† A low pH was used to favor the reduction of AcAcCoA since this DPNH dependant reaction is markedly influenced by H^+.

amine of low salt content[13]. After freeze-drying, the hydroxamic acids were extracted into alcohol and chromatographed on Whatman no. 1 filter paper with carrier butyrohydroxamic acid in either of two solvent systems, viz. *n*-butanol-3 $N NH_4OH$* or amyl alcohol-acetic acid[14]. After development, the paper was lightly sprayed with alcoholic ferric chloride reagent, the chromatogram cut into 1 × 2 cm rectangles and the radioactivity determined.

The butanol-ammonia system was particularly useful in separating crotono- (R_F 0.44) from butryo- (R_F 0.65) hydroxamic acids. The results obtained in such a system are illustrated in Fig. 1. In this figure, the first area of intense radioactivity represents acet- (R_F 0.24) and β-OH-butyro- (R_F 0.20) hydroxamic acids. The second area of radioactivity is due to butyrohydroxamic acid. It comprises about 15% of the total radioactivity of the chromatogram, indicating synthesis of about 7.5% BuCoA from AcCoA on a molar basis. It is noteworthy that under the experimental conditions, no radioactive crotonohydroxamic acid was detected. Having established the latter point, the amyl alcohol-acetic acid system was more frequently used because of its greater convenience. In this system, the R_F values for acet-, β-OH-butyro-, crotono- and butyrohydroxamic acids were 0.38, 0.37, 0.69 and 0.7 respectively.

Table I shows the percent of total radioactivity present in the butyrohydroxamic acid spot of the developed chromatogram in the latter system and hence the relative synthesis of BuCoA under certain experimental conditions. It is evident that without IV or the reducing systems for II and IV, BuCoA synthesis is abolished or markedly decreased.

TABLE I

Condition	*% of total radioactivity in butyrohydroxamic acid*
1. Complete system, 60 min incubation	15.5
2. Without BuCoA dehydrogenase	0
3. Without the DPNH generating system	4.0
4. Without benzylviologen	0
5. Complete system, approx. 1 min incubation	0.15

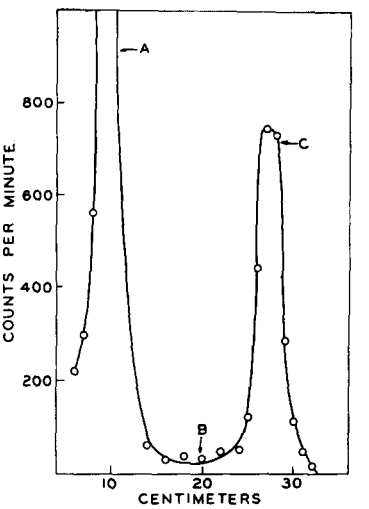

Fig. 1. Enzymatic synthesis of butyryl CoA as shown by radioactivity of chromatographed hydroxamic acids. The abscissa represents distance from origin of the chromatogram. The area under *A* comprises acet- plus β-OH-butyrohydroxamic acids (17,590 c.p.m.). *B* indicates the position of crotonohydroxamic acid. The area under *C* consists of butyrohydroxamic acid (2710 c.p.m.).

REFERENCES

1 H. R. MAHLER, *Federation Proc.*, 12 (in press).
2 H. BEINERT, R. M. BOCK, D. S. GOLDMAN, H. R. MAHLER, S. MII, P. G. STANSLY AND S. J. WAKIL, *J. Am. Chem. Soc.*, in press.
3 D. E. GREEN, D. S. GOLDMAN, S. MII AND H. BEINERT, *J. Biol. Chem.*, 202 (1953) 137.
4 J. R. STERN, M. J. COON AND A. DEL CAMPILLO, *J. Am. Chem. Soc.*, 75 (1953) 1517.
5 F. LYNEN, L. WESSELY, O. WIELAND AND L. RUEFF, *Angew. Chem.*, 64 (1952) 687.
6 S. J. WAKIL, D. E. GREEN, S. MII AND H. R. MAHLER, *J. Biol. Chem.* (in press).
7 J. R. STERN AND A. DEL CAMPILLO, *J. Am. Chem. Soc.*, 75 (1953) 2277.
8 H. R. MAHLER, *J. Am. Chem. Soc.*, 75 (1953) 3288.
9 D. E. GREEN, S. MII, H. R. MAHLER AND R. M. BOCK, *J. Biol. Chem.* (in press).
10 A. KORNBERG AND W. E. PRICER, *J. Biol. Chem.*, 193 (1951) 481.
11 H. S. CORRAN AND D. E. GREEN, *Biochem. J.*, 32 (1938) 2231.
12 D. R. SANADI AND J. W. LITTLEFIELD, *J. Biol. Chem.*, 201 (1953) 103.
13 H. BEINERT, D. E. GREEN, P. HELE, H. HIFT, R. W. VON KORFF AND C. V. RAMAKRISHNAN, *J. Biol. Chem.*, 203 (1953) 35.
14 A. R. THOMPSON, *Aust. J. Sci. Res. Series*, B 4 (1951) 180.

Received August 10th, 1953

* When this system was used, the filter paper was pretreated with saturated oxalic acid, then thoroughly rinsed and dried. This treatment reduced a troublesome forerun of iron-reacting substances and elongation of spots, phenomena discussed by THOMPSON[14].