synthesis are inceptive events of early estrogen action which precede the acceleration of protein synthesis resulting from such hormone action.

* I am particularly grateful to F. L. Hisaw and H. S. Forrest for useful suggestions and assistance in various ways. I am also much indebted to R. Williamson and Mrs. B. Moore for extensive technical assistance.

This investigation was supported by research grant RG 8871 from the Division of General Medical Sciences, U.S. Public Health Service.

The following abbreviations are used: DNA for desoxyribonucleic acid; RNA for ribonucleic acid; TCA for trichloroacetic acid; AMP for adenosine monophosphate; CMP for cytodine monophosphate

[1] Astwood, E. B., *Endocrinology*, **23**, 25 (1938).

[2] Hisaw, F. L., Jr., *Endocrinology*, **64**, 275 (1959).

[3] Mueller, G. C., in *Biological Activities of Steroids in Relation to Cancer* (New York: Academic Press, 1960), p. 129.

[4] Jensen, E. V., and H. I. Jacobson, *Recent Progr. Hormone Research*, **18**, 387 (1962).

[5] Wilmer, E. N., *Biol. Rev.*, **36**, 368 (1961).

[6] Jensen, E. V., *Perspect. Biol. Med.*, **6**, 47 (1962).

[7] Hamilton, T. H., *Nature*, **193**, 88 (1962).

[8] Villee, C. A., and D. D. Hagerman, *J. Biol. Chem.*, **233**, 42 (1958).

[9] Talalay, P., and H. G. Williams-Ashman, these PROCEEDINGS, **44**, 15 (1958).

[10] Baron, D. N., M. B. P. Gore, and D. C. Williams, *Biochem. J.*, **74**, 20*P* (1960).

[11] Tompkins, G. M., K. L. Yielding, and J. Curran, these PROCEEDINGS, **47**, 270 (1961).

[12] Spaziani, E., and C. M. Szego, *Endocrinoloy*, **63**, 669 (1958).

[13] Hollander, V. P., and M. L. Stephens, *J. Biol. Chem.*, **234**, 1901 (1959).

[14] Engel, L. L., in *Mechanisms of Action of Steroid Hormones* (New York: Pergamon Press, 1961), p. 1.

[15] Telfer, M. A., *Arch. Biochem. Biophys.*, **44**, 111 (1953).

[16] Mueller, G. C., A. M. Herranen, and K. F. Jervell, *Recent Prog. Hormone Research*, **14**, 95 (1958).

[17] Jervell, K. F., C. R. Diniz, and G. C. Mueller, *J. Biol. Chem.*, **231**, 945 (1958).

[18] Mueller, G. C., J. Gorski, and Y. Aizawa, these PROCEEDINGS, **47**, 164 (1961).

[19] Aizawa, Y., and G. C. Mueller, *J. Biol. Chem.*, **236**, 381 (1961).

[20] Hamilton, T. H., unpublished experiments (1962).

[21] Liao, S., and H. G. Williams-Ashman, these PROCEEDINGS, **48**, 1956 (1962).

THE BIOCHEMICAL FUNCTION OF BIOTIN, VI. CHEMICAL STRUCTURE OF THE CARBOXYLATED ACTIVE SITE OF PROPIONYL CARBOXYLASE†*

BY M. DANIEL LANE‡ AND FEODOR LYNEN

MAX-PLANCK-INSTITUT FÜR ZELLCHEMIE, MÜNCHEN, GERMANY

Communicated January 28, 1963

In 1959 Lynen *et al.*[1] reported that β-methylcrotonyl carboxylase catalyzes the carboxylation of free (+)-biotin when it is substituted for the natural substrate, β-methylcrotonyl CoA.[2] The carboxylation product was isolated[3] as its dimethylester and identified as 1′-N-carboxy-(+)-biotin. Their proposed mechanism visualized enzyme-bound biotin in the form of an allophanic acid derivative, 1′-N-carboxy-(+)-biotinyl-enzyme, as the active "carboxyl-transfer" intermediate in

this and other related carboxylation reactions. This view has been strongly supported[4] by β-methylcrotonyl carboxylase-catalyzed exchange reaction studies with various labeled substrates. Recently Knappe *et al.*[5] degraded $C^{14}O_2$-enzyme (β-methylcrotonyl carboxylase) by brief treatment with trypsin, followed by methylation with CH_2N_2, and then hydrolysis with biotinidase. Essentially all of the C^{14}-activity originally present in $C^{14}O_2$-enzyme was recovered as 1′-N-carbomethoxy-(+)-biotin thus confirming the original hypothesis of Lynen *et al.*[1]

Propionyl carboxylase from pig heart and bovine liver mitochondria have been extensively investigated and found[6-9] to possess enzymatic properties similar to those of β-methylcrotonyl carboxylase. The exchange reactions catalyzed,[6, 8, 9] the stoichiometry between biotin content and $HC^{14}O_3^-$ bound to form $C^{14}O_2$-enzyme,[7] and the stability properties of $C^{14}O_2$-enzyme[7] all evidence this similarity. It is likely therefore that the nature of the HCO_3^- binding sites of the two enzyme types are the same.

The purpose of the present investigation was to determine the chemical structure of the carboxylated active site of propionyl carboxylase. Evidence presented in this report has led to the identification of this site as ε-N-(1′-N-carboxy-(+)-biotinyl)-lysyl-enzyme.

Materials and Methods.—Propionyl carboxylase from bovine liver mitochondria was purified through the second ammonium sulfate fractionation (Step 5) according to the method of Lane and Halenz.[10] The specific activity of the carboxylase was 3.2 units per mg of protein. One unit of carboxylase catalyzes the carboxylation of 1.0 μmole of propionyl CoA per min at pH 8.0 and 25° using the spectrophotometric assay.[11]

Biotin was determined by the yeast growth method[12] (*Saccharomyces cerevisiae strain* 139) after hydrolysis of the enzyme preparations with 3.6 *N* H_2SO_4 for 1 hr at 120° in an autoclave.

Biotinidase was assayed and purified from pig kidney according to methods provided by Dr. J. Knappe (Univ. of Heidelberg). The biotinidase preparation (100-fold purified) used in these experiments had a specific activity of 6.2 units per mg of protein. One unit of biotinidase catalyzes the hydrolysis of 1.0 mμmole of N-(+)-biotinyl-p-aminobenzoate per min at pH 6.0 (phosphate buffer) and 37°.

Pronase, a proteolytic enzyme isolated from *Streptomyces griseus*, was obtained from the California Biochemical Corp.

The 1′-N- and 3′-N-carbomethoxy-(+)-biotin methyl esters were prepared by the method of Knappe *et al.*[3] Biocytin and 1′-N-carbomethoxybiocytin were generously provided by Dr. J. Knappe (Univ. of Heidelberg).

Results.—Preparation of $C^{14}O_2$-enzyme and carboxyl transfer to propionyl CoA: $C^{14}O_2$-enzyme was formed by short-term incubation of propionyl carboxylase with $HC^{14}O_3^-$, ATP, and Mg^{++} as described in the legend for Table 1. After addition of EDTA to prevent enzymatic exchange of unlabeled HCO_3^- with $C^{14}O_2$-enzyme in subsequent steps, the reaction mixture was applied to a Sephadex G-50 column and the eluate fractions containing the $C^{14}O_2$-enzyme, free of all other reaction mixture components, were retained for the experiments to be described. The quantity of carboxyl transferred from $C^{14}O_2$-enzyme to propionyl CoA and of biotin in $C^{14}O_2$-enzyme were compared. Aliquots of the $C^{14}O_2$-enzyme preparations were incubated with propionyl CoA; mμmoles of carboxyl transferred to form methylmalonyl

TABLE 1

$C^{14}O_2$-ENZYME: CARBOXYL TRANSFER TO PROPIONYL CoA, BIOTIN CONTENT, AND METHYLATION

Experiment number	Protein (mg)	C^{14}-carboxyl transfer to propionyl CoA (cpm)	($m\mu$moles)	Biotin content* ($m\mu$moles)	Methylated $C^{14}O_2$-enzyme recovered in Pronase digest† (cpm)
1	18.9	256,000 (910)‡	22	30	153,000
2	21.2	390,000 (1,100)‡	33	34	173,000

$C^{14}O_2$-enzyme was prepared by incubating propionyl carboxylase (specific activity, 3.2 units per mg; 24.1 and 25.0 mg in Experiments 1 and 2, respectively) with the following components (in μmoles): Tris·HCl, pH 8.4, 50; GSH, 2.5; ATP, 5; $MgCl_2$, 5; and $KHC^{14}O_3$ (11,800,000 cpm per μmole), 2.8 in a total volume of 3.8 ml at 1°–2° for 4 min. The specific activity of $HC^{14}O_3^-$ was accurately determined from the total radioactivity incorporated into methylmalonyl CoA and the quantity of ADP formed when an aliquot (1.0 ml) of the complete reaction mixture described above was incubated with propionyl CoA (1.8 μmoles) for 3 min at 25°. Specific activity of $HC^{14}O_3^-$ = cpm incorporated into methylmalonyl CoA/μmoles of ADP formed. The total C^{14}-activity incorporated into methylmalonyl CoA was determined from the specific activity of methylmalonic acid recrystallized to constant specific activity after addition of carrier and alkaline hydrolysis. ADP was determined using the coupled pyruvate kinase-lactic dehydrogenase system and following DPNH oxidation spectrophotometrically. The $C^{14}O_2$-enzyme-containing reaction mixture was applied to a 2 × 15 cm Sephadex G-50 column and the column was washed with 0.01 *M* Tris·HCl, pH 8.4. Fractions containing $C^{14}O_2$-enzyme, which were completely resolved from the other reaction mixture components, were pooled (total volume, 10 and 8.5 ml in Experiments 1 and 2, respectively). Aliquots of the $C^{14}O_2$-enzyme solution were taken for counting, for carboxyl-transfer to propionyl CoA, and for 4 *N* H_2SO_4 treatment. Aliquots (0.4 ml) for carboxyl transfer to propionyl CoA were incubated (25° for 4 min and 1°–2° for 4 min in Experiments 1 and 2, respectively) with (in μmoles): Tris·HCl, pH 8.4, 50; GSH, 2; EDTA, 10; and propionyl CoA, 0.6 in a final volume of 0.83 ml. Millimicromoles of C^{14}-carboxyl-transfer were determined from the acid-stable radioactivity and specific activity of the $HC^{14}O_3^-$ used for $C^{14}O_2$-enzyme formation.

* Biotin content of the enzyme was 0.387 μg per mg of protein (determined as described in *Materials and Methods* section).

† Methylation was accomplished with CH_2N_2 as described in test.

‡ Figures in parentheses represent nonvolatile C^{14}-activity after subjecting $C^{14}O_2$-enzyme to 4 *N* H_2SO_4 hydrolysis for 30 min at 100°.

CoA were calculated from acid stable radioactivity and the accurately-determined specific activity of the $HC^{14}O_3^-$ employed (see Table 1 for details). Treatment of aliquots of the $C^{14}O_2$-enzyme solution with 1 *N* acetic acid at 25° for several min or 4 *N* H_2SO_4 at 100° for 30 min (see Table 1) resulted in essentially complete loss of radioactivity. The biotin content of the carboxylase preparation used in these experiments was determined as described in *Materials and Methods*. As shown in Table 1 there is good agreement between the quantity of carboxyl transferred from $C^{14}O_2$-enzyme to propionyl CoA and the quantity of $C^{14}O_2$-enzyme-bound biotin. The higher temperature (25° for 4 min) during carboxyl transfer in Experiment 1 compared to Experiment 2 (1–2° for 4 min) appears to be responsible for the somewhat lower carboxyl transfer relative to biotin content in Experiment 1. This can be attributed[7] to an increased rate of decarboxylation of $C^{14}O_2$-enzyme at the higher temperature and the consequent shift in reaction equilibrium leading to the decarboxylation (loss of C^{14}-label) of methylmalonyl CoA. Hence, apparent carboxyl transfer from $C^{14}O_2$-enzyme to propionyl CoA is somewhat lower than expected. Nevertheless, the results of these experiments indicate that 1.0 mole of transferable carboxyl is bound per mole of enzyme-bound biotin and are in agreement with those of Kaziro and Ochoa.[7]

Methylation of $C^{14}O_2$-enzyme, enzymatic hydrolysis of the methylated protein, and identification of the C^{14}-labeled hydrolysis product: Stabilization of the labile carboxyl group of $C^{14}O_2$-enzyme by methylation with CH_2N_2 was necessary to prevent its loss during the subsequent proteolytic digestion of the C^{14}-labeled enzyme complex. Kosow and Lane[13] had previously found that C^{14}-biotin covalently bonded to native propionyl carboxylase could be quantitatively recovered as biocytin (ϵ-N-(+)-biotinyl-lysine) following digestion of the labeled carboxylase with Pronase (*Streptomyces griseus* protease). It was concluded that biotin is attached to carboxylase through lysyl ϵ-amino groups of the enzyme. In a similar manner the

methylated $C^{14}O_2$-enzyme was subjected to the proteolytic action of Pronase to determine whether the predicted C^{14}-labeled 1'-N-carbomethoxy-biocytin fragment would be released.

A nine milliliter aliquot of the $C^{14}O_2$-enzyme-containing Sephadex G-50 eluate (231,000 cpm, 17 mg of protein; see legend, Table 1) from Experiment 1 was brought to a final methanol concentration of 85 per cent with ice-cold methanol. The solution was vigorously stirred at 0–2° during the rapid addition of a cold ethereal solution of CH_2N_2 (approximately 30 ml of a 1.0 *M* solution). A persisting yellow color was evident. The enzyme protein, which precipitated during methylation, was recovered by high-speed centrifugation. The sedimented protein was resuspended in a final volume of 7.6 ml containing 200 μmoles of potassium phosphate buffer (pH 7.5), 0.35 ml of ethanol, and 4 mg of Pronase. The proteolytic digestion mixture was incubated for 48 hr at 37°. After several hours of digestion the denatured protein had been completely solubilized, therefore aliquots of the digestion mixture were taken and recovery (60% in Experiment 1) of C^{14}-activity was determined (see Table 1).

The Pronase digest was "streaked" directly on two sheets (90 cm) of Whatman 3 MM paper and chromatographed descending using the *n*-butanol-glacial acetic acid-H_2O (80:20:20, v/v) solvent system. All the radioactivity on the developed chromatograms was located in a single narrow band (R_f = 0.35) which corresponded exactly to 1'-N-carbomethoxybiocytin, but which was not sufficiently resolved from biocytin to warrant its definitive identification. The radioactive band on each chromatogram was cut out and eluted with H_2O. Recovery in the eluate of radioactivity originally present in the Pronase digest was 88 per cent.

Definitive chromatographic identification of the C^{14}-labeled compound was accomplished by use of a second solvent system which clearly resolved biocytin and 1'-N-carbomethoxybiocytin. Aliquots (containing 5000 cpm) of the concentrated eluate were applied to strips of Whatman 3 MM paper and cochromatographed descending with authentic biocytin and 1'-N-carbomethoxybiocytin using n-butanol-H_2O-pyridine-glacial acetic acid (30:24:20:6, v/v) as solvent.[14] The biocytin derivatives were located on the chromatograms with ninhydrin spray reagent and radioactivity with a strip counter. Figure 1 shows the exact correspondence of the single radioactive spot on the chromatogram with authentic 1'-N-Carbomethoxybiocytin (R_f = 0.55) which is definitely resolved from biocytin (R_f = 0.46). This indicates that the C^{14}-labeled fragment released by Pronase action on the methylated $C^{14}O_2$-enzyme is in fact N-carbomethoxybiocytin.

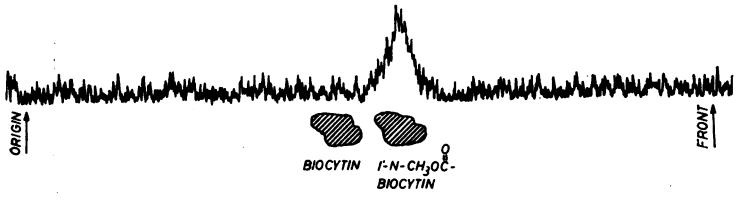


FIG. 1.—Paper chromatographic identification of the C^{14}-labeled compound released by proteolytic digestion of methylated $C^{14}O_2$-enzyme. Shown in the figure are the strip counter plot of radioactivity and locations of cochromatographed authentic compounds (biocytin and 1'-N-carbomethoxybiocytin; R_f values = 0.46 and 0.55, respectively) from the developed chromatogram. See text for details.

Hydrolysis of $C^{14}O_2$-enzyme-derived N-carbomethoxybiocytin by biotinidase and identification of the C^{14}-1′-N-carbomethoxy biotin released: The remaining C^{14}-N-carbomethoxybiocytin-containing eluate, from the initial chromatographic step (n-butanol-glacial acetic acid-H_2O solvent system) described in the preceding section, was subjected to two successive chromatographic purifications prior to incubation with biotinidase. These included: descending chromatography on Whatman 3 MM paper using the n-butanol-H_2O-pyridine-glacial acetic acid solvent system and descending chromatography on water-washed S. and S. No. 2043 *b* paper using the n-butanol-glacial acetic acid-H_2O solvent system. In each case the substances were applied to the paper by "streaking," the chromatogram was developed, and the single radioactive band was eluted. Recoveries in the eluate of applied radioactivity were 80 and 90 per cent, respectively, for the two chromatographic systems.

A portion (20,400 cpm) of the chromatographically-purified C^{14}-labeled N-carbomethoxybiocytin was subjected to enzymatic hydrolysis by biotinidase. The biocytin derivative was incubated for 14 hr at 37° in a final volume of 4.8 ml with 135 μmoles of acetate buffer (pH 6.0), 20 mμmoles of "carrier" 1′-N-carbomethoxybiocytin, 6.0 μmoles of EDTA, and 52 units of purified biotinidase (specific activity, 6.2 units per mg of protein). Additional biotinidase was added during incubation: 26 units at 3 and 6 hr and 30 units at 9 hr. Since biotinidase has a much lower affinity for 1′-N-carbomethoxybiocytin than for the assay substrate, higher enzyme concentrations and addition of carrier 1′-N-carbomethoxybiocytin were necessary for nearly quantitative hydrolysis of the C^{14}-labeled compound. Following incubation, the reaction mixture was applied to a 0.7 × 6.0 cm column of Dowex 50 (×2,200–400 mesh) in the H^+ form after which the column was washed with H_2O. The C^{14}-carbomethoxybiotin liberated during hydrolysis was recovered in 80 per cent yield (19,200 cpm) in the water eluate and the unhydrolyzed C^{14}-N-carbomethoxybiocytin was retained by the resin. After concentration of the water eluate to dryness, the residue was taken up in a small volume of ether, and 5 ml of a 1 *M* ethereal solution of CH_2N_2 was added to methylate the C^{14}-N-carbomethoxy biotin. After concentration to dryness, the methylated product was redissolved in ether saturated with ethylene glycol at 4°. Aliquots containing 3–4,000 cpm were applied to ethylene glycol-impregnated S. and S. No. 2043 *b* paper strips.[3] After application of authentic biotin methylester and 1′-N- and 3′-N-carbomethoxy-(+)-biotin methyl esters, also dissolved in ethylene glycol-saturated ether, the chromatograms were developed for 5 hr at 4° using ether saturated with ethylene glycol as solvent. The authentic compounds were located on the chromatograms with 0.2 per cent $KMnO_4$ spray and radioactivity with a strip counter. As shown in Figure 2 the methylated radioactive fragment formed by biotinidase action on N-carbomethoxybiocytin is chromatographically identical to 1′-N-carbomethoxy-(+)-biotin methylester. Neither biotin methylester nor 3′-N-carbomethoxy-(+)-biotin methylester were labeled. It is apparent therefore that the parent compound hydrolyzed by biotinidase was 1′-N-carbomethoxybiocytin. Since the 1′-N- and 3′-N-isomers were completely resolved and the radioactivity corresponded to the 1′-N-isomer, it can be concluded that carboxylation of propionyl carboxylase occurs at the 1′-N-positions of its biotinyl residues.

Discussion.—The present investigation confirms the earlier finding of Kosow and

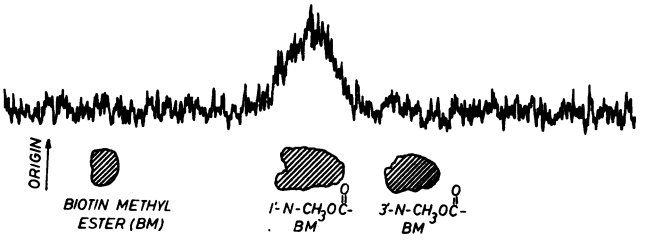
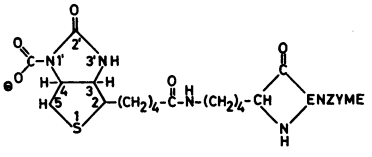


FIG. 2.—Paper chromatographic identification of the C^{14}-labeled product of biotinidase action on $C^{14}O_2$-enzyme-derived N-carbomethoxybiocytin. Shown in the figure are the strip counter plot of radioactivity and locations of cochromatographed authentic compounds (biotin methyl ester and 1'-N- and 3'-N-carbomethoxy-(+)-biotin methyl esters; 3.2, 14.5, and 20 cm from the origin, respectively) from the developed chromatogram. See text for details.

Lane[13] that biotin is bonded to propionyl carboxylase through lysyl ϵ-amino groups of the enzyme. The experiments described show definitively that the active carboxyls of "carboxylated" propionyl carboxylase are attached to the 1'-N-positions of ϵ-N-(+)-biotinyl-lysyl moieties as shown below.



Recently Wakil and Waite[15] proposed that either the ureido carbon atom (2'-position of biotin) of biotinyl-enzyme or the N-carboxy group of enzyme-bound N-carboxy-2-(δ-carboxybutyl)-3,4-diaminothiophane (the diamino carboxylic acid formed by degradation of the urea ring of biotin) serve as the active carboxyl of "carboxylated" acetyl carboxylase. The present investigation with propionyl carboxylase and recent investigations with methylmalonyl-oxalacetic transcarboxylase[16] and β-methylcrotonyl carboxylase[5, 14] are incompatible with this hypothesis. Were the hypothesis applicable to propionyl carboxylase, biocytin, and not N-carbomethoxybiocytin, would have been the C^{14}-labeled fragment obtained when the methylated $C^{14}O_2$-enzyme (propionyl carboxylase) was subjected to proteolytic digestion. As shown in the present investigation N-carbomethoxybiocytin was the only C^{14}-labeled proteolytic digestion product. In addition, degradation of this compound (identified as N-carbomethoxybiocytin) by biotinidase liberated C^{14}-1'-N-carbomethoxy-(+)-biotin and not C^{14}-biotin. When $C^{14}O_2$-enzyme (propionyl carboxylase) was subjected to 4 N H_2SO_4 hydrolysis for 30 min at 100°, essentially no nonvolatile C^{14}-activity remained. Wakil and Waite,[15] on the other hand, found that when $C^{14}O_2$-enzyme (acetyl carboxylase) was subjected to even more drastic hydrolysis conditions (4 N H_2SO_4 for 60 min at 121°) up to 90 per cent of the C^{14}-activity could be recovered in the nonvolatile fraction as free biotin. These discrepancies should be explained. In view of the fact that identical carboxylated active sites have been identified for propionyl carboxylase, methylmalonyl-oxalacetic transcarboxylase, and β-methylcrotonyl carboxylase, it seems unlikely that the site for acetyl carboxylase would be different.

Summary.—$C^{14}O_2$-enzyme was prepared by incubation of bovine liver mitochondrial propionyl carboxylase for several min with $HC^{14}O_3^-$, ATP, and Mg^{++}

and was isolated from the reaction mixture by gel filtration with Sephadex G-50. Carboxyl transfer from $C^{14}O_2$-enzyme to propionyl CoA to form methylmalonyl CoA was stoichiometric with the quantity of biotin present in the $C^{14}O_2$-enzyme (1 mole of carboxyl transferred per mole of biotin). $C^{14}O_2$-enzyme was methylated with CH_2N_2 and subjected to proteolytic digestion with Pronase (*Streptomyces griseus* protease). The fragment released, bearing essentially all of the radioactivity of the original $C^{14}O_2$-enzyme, was identified as N-carbomethoxybiocytin. The C^{14}-labeled N-carbomethoxybiocytin was hydrolyzed by the action of pig kidney biotinidase and the C^{14}-labeled hydrolysis product identified as 1′-N-carbomethoxy-(+)-biotin. Therefore, it is concluded that the carboxylated active site of propionyl carboxylase is ε-N-(1′-N-carboxy-(+)-biotinyl)-lysyl-enzyme.

We wish to thank Dr. J. Knappe for communicating the details for the assay and purification of biotinidase to us prior to their publication and also for providing a generous sample of 1′-N-carbomethoxybiocytin.

* Preceding paper in this series, Himes, R. H., D. L. Young, E. Ringelmann, and F. Lynen, *Biochem. Z.*, in press (1963).

† This paper represents the continuation of a series of investigations on bovine liver mitochondrial propionyl carboxylase. Following are references to the preceding papers in this series: *J. Biol. Chem.*, **235**, 878 (1960); *J. Biol. Chem.*, **235**, 3082 (1960); and *J. Biol. Chem.*, **237**, 2140 (1962).

‡ National Science Foundation Senior Postdoctoral Fellow on leave of absence from the Department of Biochemistry and Nutrition, Virginia Polytechnic Institute, Blacksburg, Virginia.

[1] Lynen, F., J. Knappe, E. Lorch, G. Jütting, and E. Ringelmann, *Angew Chem.*, **71**, 481 (1959).

[2] Abbreviations: CoA, coenzyme A; ATP and ADP, adenosine 5′-triphosphate and diphosphate; Tris, tris (hydroxymethyl) amino methane; GSH, reduced glutathione; EDTA, ethylenediamine tetraacetate; DPNH, reduced diphosphopyridine nucleotide.

[3] Knappe, J., E. Ringelmann, and F. Lynen, *Biochem. Z.*, **335**, 168 (1961).

[4] Lynen, F., J. Knappe, E. Lorch, G. Jütting, E. Ringelmann, and J. LaChance, *Biochem. Z.*, **335**, 123 (1961).

[5] Knappe, J., K. Biederbick, and W. Brümmer, *Angew. Chem.*, **74**, 432 (1962).

[6] Kaziro, Y., L. F. Hass, P. D. Boyer, and S. Ochoa, *J. Biol. Chem.*, **237**, 1460 (1962).

[7] Kaziro, Y., and S. Ochoa, *J. Biol. Chem.*, **236**, 3131 (1961).

[8] Halenz, D. R., and M. D. Lane, *Biochim. Biophys. Acta*, **48**, 426 (1961).

[9] Halenz, D. R., J. Y. Feng, C. S. Hegre, and M. D. Lane, *J. Biol. Chem.*, **237**, 2140 (1962).

[10] Lane, M. D., and D. R. Halenz, in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1962), vol. 5, p. 576.

[11] Tietz, A., and S. Ochoa, *J. Biol. Chem.*, **234**, 1394 (1959).

[12] Lichstein, H. C., *J. Biol. Chem.*, **212**, 217 (1955;; *Fed. Proc.*, **16**, 211 (1957).

[13] Kosow, D. P., and M. D. Lane, *Biochem. Biophys. Res. Comm.*, **7**, 439 (1962).

[14] Knappe, J., B. Wenger, and U. Wiegand, *Biochem. Z.*, in press (1963).

[15] Wakil, S. J., and M. Waite, *Biochem. Biophys. Res. Comm.*, **9**, 18 (1962).

[16] Wood, H. G., and H. Lochmüller, personal communication; *Fed. Proc.*, in press (1963).