References

Betheil, J. J., Wiebelhaus, V. D., and Lardy, H. A. (1947), *J. Nutr. 34*, 431.
Bhaduri, A., and Srere, P. A. (1963), *Biochim. Biophys. Acta 70*, 221.
Chareonchai, A., and Johnson, R. M. (1963), *J. Nutr. 81*, 387.
Cohn, C., and Joseph, D. (1959), *Am. J. Physiol. 197*, 1347.
Coore, H. G., Randle, P. J., Simon, E., Kraicer, P. F., and Shelesnyak, M. C. (1963), *Nature 197*, 1264.
Flatt, J. P., and Ball, E. G. (1964), *J. Biol. Chem. 239*, 675.
Haas, E., Horecker, B. L., and Hogness, T. R. (1940), *J. Biol. Chem. 136*, 747.
Hutchens, T. T., Van Bruggen, J. T., Cockburn, R. M., and West, E. S. (1954), *J. Biol. Chem. 208*, 115.
Kallen, R. G., and Lowenstein, J. M. (1962), *Arch. Biochem. Biophys. 96*, 188.
Kornacker, M. S., and Lowenstein, J. M. (1964), *Science 144*, 1027.
Kornberg, A., and Horecker, B. L. (1957), *Methods Enzymol. 3*, 323.
Lardy, H. A., Foster, D. O., Shrago, E., and Ray, P. D. (1964a), *in* Advances in Enzyme Regulation Vol. II, Oxford, Pergamon Press, p. 39.
Lardy, H. A., Shrago, E., Young, J. W., and Paetkau, V. (1964b), *Science 144*, 564.
Layne, E. (1957), *Methods Enzymol. 3*, 450.
Lohmann, K., and Meyerhof, O. (1934), *Biochem. Z. 273*, 60.
Lyon, I., Masri, M. S., and Chaikoff, I. L. (1952), *J. Biol. Chem. 196*, 25.
McComb, R. B., and Yushok, W. D. (1964), *Cancer Res. 24*, 193.
Magasanik, B. (1962), *Cold Spring Harbor Symp. Quant. Biol. 26*, 249.
Medes, G., Thomas, A., and Weinhouse, S. (1952), *J. Biol. Chem. 197*, 181.
Monod, J. (1947), *Growth 11*, 223.
Niemeyer, H., Clark-Turri, L., Garces, E., and Vergara, F. E. (1962), *Arch. Biochem. Biophys. 98*, 77.
Nordlie, R. C., and Lardy, H. A. (1963), *J. Biol. Chem. 238*, 2259.
Ochoa, S. (1955a), *Methods Enzymol. 1*, 699.
Ochoa, S. (1955b), *Methods Enzymol. 1*, 739.
Ochoa, S., Mehler, A. H., and Kornberg, A. (1948), *J. Biol. Chem. 174*, 979.
Phillips, P. H., and Hart, E. B. (1935), *J. Biol. Chem. 109*, 657.
Potter, V. R., and Ono, T. (1962), *Cold Spring Harbor Symp. Quant. Biol. 26*, 355.
Rutter, W. J., and Lardy, H. A. (1958), *J. Biol. Chem. 233*, 374.
Shrago, E., Lardy, H. A., Nordlie, R. C., and Foster, D. O. (1963), *J. Biol. Chem. 238*, 3188.
Snedecor, G. W. (1956), Statistical Methods, 5th ed., Ames, Iowa, Iowa State College Press, p. 90.
Sumner, J. B. (1944), *Science 100*, 413.
Tepperman, H. M., and Tepperman, J. (1958), *Diabetes 7*, 478.
Van Bruggen, J. T., Hutchens, T. T., Claycomb, C. K., Cathey, W. J., and West, E. S. (1952), *J. Biol. Chem. 196*, 389.
Vaughan, D. A., and Winders, R. L. (1964), *Am. J. Physiol. 206*, 1081.
Weber, G. (1963), *in* Advances in Enzyme Regulation Vol. I, Oxford, Pergamon Press, p. 1.

Enzymatic Synthesis of Benzoylornithines*

FINLEY D. MARSHALL† AND OWEN J. KOEPPE

From the Department of Biochemistry, University of Missouri, Columbia

Received May 25, 1964; revised August 13, 1964

An enzyme system has been prepared from chicken kidney which is capable of catalyzing the synthesis of monobenzoylornithine and ornithuric acid in the presence of benzoic acid, ornithine, adenosine triphosphate, and coenzyme A. Evidence has been obtained for the participation of benzoyladenylic acid and benzoyl-coenzyme A as intermediates in the reaction. The principal monobenzoylornithine synthesized is 5-*N*-benzoylornithine, though both the 2-*N*- and 5-*N*-benzoyl derivatives serve as substrates for ornithuric acid synthesis. Preliminary fractionation studies suggest the involvement of at least three enzymes in the overall reaction.

It has long been known that chickens fed benzoic acid will conjugate it to ornithine and excrete the dibenzoyl derivative, ornithuric acid (Jaffe, 1877). More recently McGilvery and Cohen (1950) described the acylation of 2-*N*- and 5-*N*-benzoylornithines by *p*-aminobenzoic acid catalyzed by the 2000-*g*-sedimentable fraction of chicken kidney homogenates. There has been a very brief report of the formation of acetyl-, *n*-valeryl-, and benzoyl-L-ornithines catalyzed by extracts of acetone powders of chicken and duck kidneys (Schachter *et al.*, 1955). The present paper describes the preparation and partial characterization of an enzyme system from chicken kidney which catalyzes the synthesis of mono- and dibenzoylornithines.

* Supported by a U. S. Public Health Service research grant (GM-05475).

† Present address: Department of Biochemistry, State University of South Dakota, Vermillion.

Experimental

Materials.—L-Ornithuric acid was synthesized by the benzoylation of L-ornithine with benzoyl chloride according to a general acylation method (Schulze and Winterstein, 1898). The product had a melting point of 187–188°, which is in agreement with the value reported for ornithuric acid (Sörensen, 1905). 5-*N*-Benzoyl-L-ornithine was synthesized by the benzoylation of the copper chelate of L-ornithine (McGilvery and Cohen, 1950). The product melted at 243–246°, corresponding closely to the 243–245° previously reported (Baldwin *et al.*, 1960). 2-*N*-Benzoyl-L-ornithine was prepared by the benzoylation of 5-*N*-carbobenzoxy-L-ornithine, followed by removal of the carbobenzoxy group by catalytic hydrogenation. The method used was similar to that of Baldwin *et al.* (1960). The product melted at 238–241°, in good agreement with the 239° previously reported (Baldwin *et al.*, 1960). The 2-*N*-benzoyl-L-ornithine could

be separated from 5-*N*-benzoyl-L-ornithine by prolonged paper chromatography using methyl ethyl ketone–2 N NH_4OH (1:1). Treatment of the chromatogram with ninhydrin resulted in a purple color with a 5-*N*-benzoyl-L-ornithine after about 10 minutes at room temperature; however it required about 2 hours for color formation with the 2-*N*-benzoyl-L-ornithine. Benzoyl-AMP and benzoyl-CoA were prepared by methods described by Moldave and Meister (1957).

[1-^{14}C]Benzoic acid was obtained from Nuclear Chicago Corp. DL-[2-^{14}C]Ornithine was obtained from Tracerlab. ATP, AMP, and CoA were purchased from Sigma Chemical Co. L-Ornithine was obtained from Mann Research Laboratories. 5-*N*-Carbobenzoxy-L-ornithine was purchased from Schwarz BioResearch, Inc. Inorganic [^{32}P]orthophosphate was obtained from Oak Ridge National Laboratory. Inorganic [^{32}P]pyrophosphate was synthesized by the pyrolysis of $K_2H^{32}PO_4$.

Enzyme Preparation.—The enzyme system was prepared by a combination of methods very similar to those used in studies of hippuric acid synthesis (Cohen and McGilvery, 1947a,b; Chantrenne, 1951; Schachter and Taggart, 1953). All operations were carried out at 0–5°. Kidneys were removed from chickens which were 4–9 weeks old. The kidneys were homogenized in a glass homogenizer fitted with a standard-clearance (0.10–0.15 mm) Teflon pestle in enough 0.154 M (isotonic) potassium chloride to give a 15% homogenate. The homogenate was centrifuged for 15 minutes at 2000 *g*. The supernatant was discarded, and the residue was washed twice with a volume of 0.154 M potassium chloride equal to that used in homogenization and again centrifuged at 2000 *g*. To this washed residue was added 10 volumes of cold (−35°) acetone with constant stirring. The acetone precipitate was collected on a funnel, washed several times with cold acetone, allowed to dry on the funnel for about 15 minutes, and stored in a vacuum desiccator over phosphorus pentoxide at −20°. The enzyme activity of such a powder was stable for at least several months.

Prior to study, the acetone powder was extracted with 12 volumes of 0.02 M potassium phosphate buffer at *p*H 7.6. The resulting suspension was centrifuged at 10,000 *g* for 10 minutes. The supernatant was dialyzed against the same buffer for 4–18 hours. This type of dialyzed acetone-powder extract was used as the enzyme source for most experiments reported here; however whole kidney homogenates and other systems have also been studied.

Assay of Enzymatic Activity.—Reaction mixtures containing enzyme and substrates (as shown later) were incubated at 37° for 30 or 60 minutes with shaking. The reactions were stopped by the addition of 0.5 volume of ethanol followed by heating in a boiling-water bath for 10 minutes and then by centrifugation. In most experiments, a zero-incubation-time control was included in which the enzyme was added after the 0.5 volume of ethanol. Carrier amounts of 2-*N*-, 5-*N*-, and dibenzoylornithine and benzoic acid were added as needed to the supernatants from the reaction mixtures. Aliquots of each were applied to strips of Whatman No. 1 paper 56 cm in length and subjected to descending chromatography using 1-butanol saturated with 5 N NH_4OH or methyl ethyl ketone–2 N NH_4OH (1:1) (Baldwin *et al.*, 1960). Benzoic acid, monobenzoylornithines, and ornithuric acid were located by viewing the papers under ultraviolet light. Ornithine was identified with ninhydrin. The approximate R_F values using 1-butanol saturated with 5 N NH_4OH were 0.22 for monobenzoylornithine, 0.34 for benzoic acid, and 0.52 for ornithuric acid. Ornithine remained at the origin. 2-*N*-Benzoyl- and 5-*N*-benzoylornithine were not satisfactorily separated by this system. Each of the zones was cut out and counted in a gas-flow counter without elution from the paper. Using the counts it was possible to calculate the per cent of the ^{14}C substrate which was incorporated into a benzoyl derivative. The results are expressed as mμmoles incorporated per mg of enzyme protein during the incubation period. Protein concentration was determined by the method of Lowry *et al.* (1951), using crystalline bovine serum albumin as a standard. The methyl ethyl ketone–2 N NH_4OH (1:1) solvent was also used in experiments in which it was necessary to distinguish 2-*N*- from 5-*N*-benzoylornithine. To accomplish this separation it was necessary to allow the solvent to run off the bottom of the paper for 10 hours or more.

In some cases when benzoyl-CoA was used as a substrate, the reaction was followed by measuring the decrease in 280 mμ absorbance resulting from the cleavage of the thioester bond of the benzoyl-CoA (Schachter and Taggart, 1954). The exchange of ^{32}P between inorganic pyrophosphate and ATP was studied by the method of DeMoss and Novelli (1956).

Results

In the first series of experiments carried out with the enzyme preparation, a monobenzoylornithine was used as the substrate in order to limit the number of benzoylations being studied. The results in Table I show that the enzyme system was able to catalyze the benzoylation of 5-*N*-benzoyl-L-ornithine by benzoic acid to form ornithuric acid. The reaction required the presence of ATP and CoA. Omission of Mg^{2+} had little effect; however EDTA caused almost complete inhibition. The apparent formation of about 10 mμmoles of ornithuric acid in the absence of enzyme or other cofactors was shown to be owing to contamination of the ornithuric acid zone by about 1% of the [^{14}C]benzoic acid during the chromatography. Results similar to those in Table I were obtained when the chromatography was carried out using methyl ethyl

Table I

Incorporation of Benzoic Acid into Ornithuric Acid in the Presence of 5-*N*-Benzoyl-L-ornithine[a]

Reaction Mixture	Benzoic Acid Incorporated into Ornithuric Acid (mμmoles/mg protein/30 min)
Complete	135
Zero incubation time	10
Enzyme omitted	10
5-*N*-Benzoyl-L-ornithine omitted	11
ATP omitted	13
CoA omitted	11
$MgCl_2$ omitted	112
$MgCl_2$ omitted, 10 μmoles EDTA added	17

[a] Complete reaction mixture contained 5.0 μmoles Tris-HCl buffer at *p*H 8.0, 3.0 μmoles $MgCl_2$, 3.0 μmoles ATP, 0.1 μmole CoA, 3.0 μmoles 5-*N*-benzoyl-L-ornithine, 1.0 μmole [1-^{14}C]benzoic acid (2.5 mc/mmole), and about 1 mg acetone-powder-extract protein in 1.0 ml. Incubation was for 30 minutes. Chromatography was with 1-butanol saturated with 5 N NH_4OH.

ketone–2 N NH_4OH (1:1) in place of 1-butanol saturated with 5 N NH_4OH. In this case the apparent blank values were only about 2 mμmoles.

The results in Table II indicate that the enzyme preparation could catalyze the benzoylation of L-ornithine to form monobenzoylornithine and ornithuric

TABLE II

INCORPORATION OF BENZOIC ACID INTO BENZOYLORNITHINES IN THE PRESENCE OF L-ORNITHINE[a]

Reaction Mixture	Benzoic Acid Incorporated into: (mμmoles/mg protein/30 min) Monobenzoyl-ornithine	Ornithuric Acid
Complete	71	46
Zero incubation time	3	9

[a] Conditions were the same as those described for Table I with the following exceptions: 3.0 μmoles of L-ornithine in place of 5-*N*-benzoyl-L-ornithine and 3.0 rather than 1.0 μmole of [1-^{14}C]benzoic acid.

acid. The 46 mμmoles of [1-^{14}C]benzoic acid incorporated into ornithuric acid represents the synthesis of only 23 mμmoles of that compound, or about one-third as much as of the monobenzoyl derivative. Similar results were obtained when DL-[2-^{14}C]ornithine was used in the presence of unlabeled benzoic acid. Again there was about three times as much monobenzoyl- as dibenzoylornithine formed.

The next experiments tested the ability of the enzyme preparation to catalyze ornithine benzoylation in the presence of benzoyl-CoA. The results (Table III)

TABLE III

INCORPORATION OF ORNITHINE INTO BENZOYLORNITHINES IN THE PRESENCE OF BENZOYL-CoA[a]

Reaction Mixture	Ornithine Incorporated into: (mμmoles/mg protein/30 min) Monobenzoyl-ornithine	Ornithuric Acid
Complete	87	31
Zero incubation time	4	1

[a] Complete reaction mixture contained 5.0 μmoles Tris-HCl buffer at *p*H 8.0, 0.46 μmole benzoyl-CoA, 1.0 μmole DL-[2-^{14}C]ornithine (1.0 mg/mmole), and about 1.0 mg acetone-powder-extract protein in 1.0 ml. Incubation was for 30 minutes. Chromatography was with 1-butanol saturated with 5 N NH_4OH.

show that mono- and dibenzoylornithine were formed in about a 3:1 ratio and that benzoyl-CoA replaced the requirement for benzoic acid, ATP, and CoA.

Synthetic benzoyl-AMP was tested as an intermediate in the reaction. The compound was active in the enzymatic formation of mono- and dibenzoylornithine from ornithine, replacing the requirement for ATP and benzoic acid. This benzoylation was almost entirely dependent upon the presence of CoA. However, in the absence of enzyme there was considerable monobenzoylornithine but little dibenzoylornithine formation, both of which were independent of the presence of CoA. The nonenzymatic formation of monobenzoylornithine exceeded that which occurred in the presence of enzyme but absence of CoA. Interpretation of these findings must await kinetic studies of the enzymatic and nonenzymatic benzoylations as well as any other reactions resulting in benzoyl-AMP breakdown.

TABLE IV

COMPARISON OF 2-*N*-BENZOYL-L-ORNITHINE AND 5-*N*-BENZOYL-L-ORNITHINE AS SUBSTRATES FOR ORNITHURIC ACID SYNTHESIS[a]

Reaction Mixture	Buffer	Benzoic Acid Incorporated into Ornithuric Acid Using: (mμmoles/mg protein/hr) 2-*N*-Benzoyl-L-ornithine	5-*N*-Benzoyl-L-ornithine
(1) Acetone-powder extract as enzyme source			
Complete	Tris-HCl	15	185
Zero incubation time		<1	1
CoA omitted		1	1
Complete	Phosphate	21	244
Zero incubation time		1	1
(2) Whole-kidney homogenate as enzyme source			
Complete	Tris-HCl	26	50
Zero incubation time		<1	1
Complete	Phosphate	36	63
Zero incubation time		1	2

[a] Complete reaction mixture contained 5.0 μmoles Tris-HCl or potassium phosphate buffer at *p*H 8.0, 3.0 μmoles $MgCl_2$, 3.0 μmoles ATP, 0.1 μmole CoA, 1.0 μmole [1-^{14}C]-benzoic acid (2.5 mc/mmole), 3.0 μmoles 2-*N*- or 5-*N*-benzoyl-L-ornithine, and about 3.0 mg of enzyme protein in 1.0 ml. Incubation was for 1 hour. Chromatography was with methyl ethyl ketone–2 N NH_4OH (1:1).

Results in Table IV show the relative effectiveness of 2-*N*- and 5-*N*-benzoylornithine as substrates for ornithuric acid formation. It can be seen that, in contrast to results with 5-*N*-benzoyl-L-ornithine, there was very little ornithuric acid synthesis from 2-*N*-benzoyl-L-ornithine catalyzed by the acetone-powder extract. Because these results were in apparent disagreement with those of McGilvery and Cohen (1950) the reactions were studied with whole kidney homogenate as the enzyme source using both Tris and phosphate buffers. Under these conditions (Table IV) ornithuric acid formation from 2-*N*-benzoyl-L-ornithine was more than 50% of that from 5-*N*-benzoyl-L-ornithine. Phosphate buffer increased the formation from both but did not markedly alter the ratio. The absolute values in the presence of phosphate buffer are in fairly good agreement with the earlier results measuring *p*-aminoornithuric acid synthesis (McGilvery and Cohen, 1950). The zero-time values are lower in Table IV than in previous tables, because more enzyme protein was used, but the results are still expressed on a per-mg basis.

In order to determine which monobenzoylornithine was synthesized enzymatically, experiments were carried out as described in Table V. Very little, if any, 2-*N*-benzoylornithine formation was catalyzed by acetone powder extract or whole homogenate in the presence of either Tris or phosphate buffer. If present, the formation of 2-*N*-benzoylornithine was less than about 3% of that of 5-*N*-benzoylornithine.

The nature of the monobenzoyl intermediate was further investigated by study of the enzymatic acylation with benzoyl-CoA. The reaction was followed by measuring the decrease in 280 mμ absorbance resulting from benzoyl-CoA breakdown dependent upon the presence of acyl acceptor. It was possible to measure L-orni-

TABLE V
INCORPORATION OF ORNITHINE INTO MONOBENZOYLORNITHINES[a]

Reaction Mixture	Buffer	Ornithine Incorporated into: (mμmoles/mg protein/hr) 2-*N*-Benzoylornithine	5-*N*-Benzoylornithine
(1) Acetone-powder extract as enzyme source			
Complete	Tris-HCl	2	102
Zero incubation time		<1	<1
CoA omitted		1	<1
Complete	Phosphate	3	176
Zero incubation time		<1	<1
(2) Whole-kidney homogenate as enzyme source			
Complete	Tris-HCl	<1	15
Zero incubation time		<1	<1
Complete	Phosphate	1	33
Zero incubation time		1	<1

[a] Conditions were the same as those described in Table IV with the exception of 3.0 μmoles of L-ornithine being used in place of 2-*N*- or 5-*N*-benzoyl-L-ornithine.

thine-dependent (14 mμmoles), 5-*N*-benzoyl-L-ornithine-dependent (15 mμmoles), and 2-*N*-benzoyl-L-ornithine-dependent (7 mμmoles) breakdown of benzoyl-CoA catalyzed by the 2000 *g* supernatant of whole-kidney homogenate. However, acetone-powder extract catalyzed L-ornithine-dependent (56 mμmoles) and 5-*N*-benzoyl-L-ornithine-dependent (64 mμmoles), but almost no 2-*N*-benzoyl-L-ornithine-dependent (4 mμmoles), benzoyl-CoA cleavage. The figures in parenthesis represent the amount of substrate-dependent benzoyl-CoA breakdown per mg enzyme protein in 30 minutes. Though the 2000 *g* residue was used for the preparation of the acetone powder, the supernatant contained significant amounts of the benzoyl-transferase activities indicated above.

Preliminary experiments have been carried out attempting to determine the number of enzymes involved in the benzoylation reactions being studied. Benzoic acid–activating enzyme activity, as measured by benzoic acid–dependent exchange of ^{32}P between inorganic pyrophosphate and ATP, has been detected in dialyzed whole-kidney homogenates and acetone-powder extracts. Acetone-powder extracts have been fractionated using ammonium sulfate precipitation and DEAE-cellulose-column chromatography. The fractionations have been followed by measurements of benzoic acid activation and L-ornithine-, 2-*N*-benzoyl-L-ornithine-, and 5-*N*-benzoyl-L-ornithine-dependent benzoyl-CoA breakdown. Only one of these activities has been obtained free of the other three. When the fraction of acetone-powder extract which precipitates between 35 and 50% saturation with ammonium sulfate was placed over a DEAE-cellulose column in 0.005 M phosphate buffer at *p*H 8.0, the protein fraction which came through prior to the start of a 0.0–0.2 M NaCl gradient catalyzed only 5-*N*-benzoyl-L-ornithine-dependent breakdown of benzoyl-CoA. Both ammonium sulfate fractionation and DEAE-cellulose chromatography have resulted in fractions which showed marked enrichment of benzoic acid activation or L-ornithine-dependent benzoyl-CoA breakdown activity relative to the other reactions being measured, but were not free of the other activities. Because acetone-powder extract is a poor source of 2-*N*-benzoyl-L-ornithine-dependent benzoyl-CoA–breakdown activity, attempts have been made to purify this activity from 2000 *g* supernatant of whole kidney homogenate. To date these attempts have not accomplished the purification of this activity relative to the others being studied.

DISCUSSION

The results presented here are consistent with a mechanism for ornithuric acid synthesis similar to that proposed for hippuric acid (Chantrenne, 1951; Schachter and Taggart, 1953), phenylacetylglutamine (Moldave and Meister, 1957), and others. The reaction apparently involves the ATP-dependent synthesis of benzoyl-CoA followed by benzoyl-transfer reactions, first primarily to the 5- and then to the 2-amino group of ornithine. Chicken kidney preparations can catalyze the benzoylation of 2-*N*-benzoyl-L-ornithine, but little if any of this compound is formed in the system. The results suggest that separate enzymes are responsible for the activation of benzoic acid, benzoyl transfer from CoA to L-ornithine, and benzoyl transfer to 5-*N*-benzoyl-L-ornithine. Whether or not benzoyl transfer to 2-*N*-benzoyl-L-ornithine is catalyzed by an enzyme other than one of the foregoing has not been established.

REFERENCES

Baldwin, B. C., Robinson, D., and Williams, R. T. (1960), *Biochem. J. 76*, 595.

Chantrenne, H. (1951), *J. Biol. Chem. 189*, 227.

Cohen, P. P., and McGilvery, R. W. (1947a), *J. Biol. Chem. 169*, 119.

Cohen, P. P., and McGilvery, R. W. (1947b), *J. Biol. Chem. 171*, 121.

DeMoss, J. A., and Novelli, G. D. (1956), *Biochim. Biophys. Acta 22*, 49.

Jaffe, M. (1877), *Ber. deut. chem. Ges* (now *Chem. Ber.*) *10*, 1925.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

McGilvery, R. W., and Cohen, P. P. (1950), *J. Biol. Chem. 183*, 179.

Moldave, K., and Meister, A. (1957), *J. Biol. Chem. 229*, 463.

Schachter, D., Manis, J. G., and Taggart, J. V. (1955), *Am. J. Physiol. 182*, 537.

Schachter, D., and Taggart, J. V. (1953), *J. Biol. Chem. 203*, 925.

Schachter, D., and Taggart, J. V. (1954), *J. Biol. Chem. 208*, 263.

Schulze, E., and Winterstein, E. (1898), *Z. Physiol. Chem. 26*, 1.

Sörensen, S. P. L. (1905), *Chem. Zentr. 1905 II*, 460.