groups rather than the thioether linkage. Olah and White (1968) have observed protonation of the thioether linkage and the ureido carbonyl oxygen atom of biotin in FSO_3H-SbF_5.

Results of studies of nonenzymatic unimolecular decarboxylation of carbamates, and acyl transfer from esters of *N*-carboxyimidazolidone (Caplow, 1965, 1968) suggest that the principal determinants underlying the reactivity of *N*-carboxybiotin in enzymatic carbon dioxide transfer are the basicity of the cofactor, electrophilic activation of the carboxylate group, and the catalysis by the enzyme directed to activation of the nucleophilic acceptor. The absence of any measurable interaction between the ureido system and the thioether linkage in neutral or protonated biotin suggests that the sulfur atom does not play a significant role in enzymatic transcarboxylation, as suggested previously (Mildvan *et al.*, 1966).

References

Arnett, E. M. (1963), *Progr. Phys. Org. Chem. 1*, 223.
Bowen, C. E., Rauscher, E., and Ingraham, L. L. (1968), *Arch. Biochem. Biophys. 125*, 865.
Caplow, M. (1965), *J. Am. Chem. Soc. 87*, 5774.
Caplow, M. (1968), *J. Am. Chem. Soc. 90*, 6795.
Clark, W. M. (1960), Oxidation–Reduction Potentials of Organic Systems, Baltimore, Md., Williams & Wilkins Co., p 149.
Glasel, J. A. (1966), *Biochemistry 5*, 1851.
Hofmann, K., Melville, D. B., and DuVigneaud, V. (1941), *J. Biol. Chem. 141*, 207.
Melville, D. B., Dittmer, K., Brown, G. B., and DuVigneaud, V. (1943), *Science 98*, 497.
Melville, D. B., Pierce, J. G., and Partridge, C. W. H. (1949), *J. Biol. Chem. 180*, 299.
Mildvan, A. S., Scrutton, M. C., and Utter, M. F. (1966), *J. Biol. Chem. 241*, 3488.
Olah, G. A., and White, A. M. (1968), *J. Am. Chem. Soc. 90*, 6087.
Paul, M. A., and Long, F. A. (1957), *Chem. Rev. 57*, 1.
Stewart, R., and Muenster, L. J. (1961), *Can. J. Chem. 39*, 401.

On the Reported Presence of Biotin in Carbamyl Phosphate Synthetase*

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ABSTRACT: Purified carbamyl phosphate synthetase from frog and beef liver mitochondria and from *Escherichia coli* was tested for the presence of biotin as a coenzyme. Avidin preparations which were proved effective in inhibiting pyruvate carboxylase had no effect on either the beef liver or the *E. coli* carbamyl phosphate synthetase. Also, a direct assay for biotin using *Neurospora crassa* as a test organism showed no significant amount of biotin in any of the three enzymes Thus biotin is not a coenzyme in the carbamyl phosphate synthetase reaction.

The results on the *E. coli* enzyme are in direct conflict with the conclusion of the report (Wellner, V. P., Santos, J. I., and Meister, A. (1968), *Biochemistry 7*, 2848) that biotin was present and functional.

The formation of carbamyl phosphate from bicarbonate, ammonia, and 2 moles of ATP by carbamyl phosphate synthetase includes the ATP-dependent activation of CO_2 as a postulated partial reaction (Anderson and Meister, 1965; Metzenberg *et al.*, 1958). This has led in the past to a number of negative, and therefore unpublished, experiments concerning the possible inhibition by avidin, and possible presence of biotin, in the frog liver mitochondrial enzyme (M. Marshall, R. L. Metzenberg, and P. P. Cohen, unpublished results).

The recent report of the presence of biotin in the glutamine-utilizing *Escherichia coli* carbamyl phosphate synthetase (Wellner *et al.*, 1968) and the accompanying suggestion that both the ammonia- and glutamine-utilizing animal enzymes might also contain biotin has led to a reinvestigation of this question in the frog liver mitochondrial carbamyl phosphate synthetase and the extension of the study to include the mitochondrial enzyme from beef liver. As a control, *E. coli* carbamyl phosphate synthetase was also prepared and tested.

Two recent papers have stated the absence of biotin in the rat liver mitochondrial carbamyl phosphate synthetase (Guthöhrlein and Knappe, 1968) and the lack of inhibition by avidin of the frog liver mitochondrial enzyme and the pigeon liver glutamine-utilizing enzyme (Peng and Jones, 1969).

Experimental Section

Materials. Two samples of avidin were used, both products

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of Nutritional Biochemicals Corp. Pronase and TES[1] were purchased from Calbiochem and *d*-biotin and dithiothreitol from Sigma Chemical Co.

Purified frog liver mitochondrial carbamyl phosphate synthetase was prepared as reported previously (Marshall *et al.*, 1958). Beef liver mitochondrial carbamyl phosphate synthetase was purified to a specific activity of 25 (compared with 35 for the frog enzyme) by DEAE-cellulose chromatography and gel filtration on Sephadex G-200, in buffers containing glycerol and dithiothreitol (R. B. Huston and P. P. Cohen, unpublished method). Carbamyl phosphate synthetase from *E. coli* was prepared according to Anderson and Meister (1965) as modified by Anderson and Marvin (1968). We are indebted to Dr. Paul M. Anderson for making available the details of this modification.

Purified pyruvate carboxylase from rat liver (McClure, 1969) was provided by William R. McClure and Prof. H. A. Lardy of the Enzyme Institute.

Ornithine transcarbamylase from beef liver was partially purified to a specific activity of 2000 (μmoles of citrulline/mg of protein per 10 min) using some of the steps of the method of Marshall (M. Marshall, unpublished method).

Methods. The activity of carbamyl phosphate synthetase from frog and beef liver was determined in an incubation system containing: ATP (5 μmoles), $Mg(CH_3CO^-{}_2)_2$ (15 μmoles), NH_4Cl (50 μmoles), $KHCO_3$ (10 μmoles), ornithine (5 μmoles), acetylglutamate (10 μmoles), TES (50 μmoles), ornithine transcarbamylase (1 unit), and water to give a final volume of 1 ml. All substrates were adjusted to pH 7.4. After 15 min at 37°, citrulline was determined colorimetrically (Hunninghake and Grisolia, 1966). As previously defined (Marshall *et al.*, 1958), 1 unit of enzyme results in the synthesis of 1 μmole of citrulline under these conditions.

The activity of carbamyl phosphate synthetase from *E. coli* was determined in the incubation system of Table I of Wellner *et al.* (1968), with the modification that ornithine and ornithine transcarbamylase were added and citrulline was determined, as in the assay for the beef and frog enzymes. For the *E. coli* enzyme, one unit is defined (Anderson and Meister, 1965) as that amount of enzyme which will form 1 μmole of carbamyl phosphate per hr in the assay system.

Protein concentration was determined by a turbidimetric method (Luck *et al.*, 1958).

Biotin was determined using an Oak Ridge wild-type strain, 74-OR8-1a, of *Neurospora crassa* as the test organism. This was provided by R. L. Metzenberg and originally obtained from the Fungal Genetics Stock Center, Dartmouth College. Biotin standards or enzyme hydrolysates and 20 ml of Fries salts medium (cited by Beadle and Tatum, 1945) without biotin and containing 1% (w/v) sucrose as the carbon source were added to 125-ml flasks and autoclaved. The flasks were then inoculated with about 1000 conidiospores and incubated 4 days at room temperature. The mycelia were collected, blotted to remove excess medium, and the dry weight of the pads was determined. The optimum range of the assay is from 0.5 to 8 μμmoles of biotin (5–40 mg dry wt), with a detection limit of about 0.1 μμmole.

Hydrolysis of the enzyme prior to biotin assay was done in a final volume of 0.2 ml with 6 N H_2SO_4, or in a final volume of 0.35 ml with 2 mg of Pronase. For H_2SO_4 hydrolysis, covered or sealed tubes were autoclaved 1 hr at 120°, neutralized, diluted, and assayed. For Pronase digestion, the enzymes were first denatured by heating 5 min in boiling water; Pronase was then added and the stoppered tubes were incubated at 37°. In the experiment shown in Table II, the water bath was inadvertently turned off between 30 and 45 hr of the 48-hr incubation. The reaction was stopped by autoclaving for 15 min, the mixtures were diluted and an aliquot was assayed for biotin.

TABLE I: Effect of Avidin on Enzyme Activity.[a]

Expt	Enzyme	Time (hr)	Temp (°C)	mg of Avidin/mg of Carbamyl Phosphate Synthetase	Units/mg
Ia	Beef	0		0	20.8
		1.5	37	0	21.4
		1.5		0.41	19.3
		1.5		2.06	19.8
Ib		20	25	0	20.4
		20		0.41	21.0
		20		2.06	21.4
II	*E. coli*	0	25	0	297
		0		1.41	292
		1		0	261
		1		1.41	264
III	*E. coli*	0	25	0	304
		1		0	295
		1		1.37	285

[a] The incubations for expt I contained 86 μg of carbamyl phosphate synthetase and, as indicated, 35 and 175 μg of avidin in 0.1 ml of a solution containing 35 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, and 20% (w/v) glycerol (pH 7.5). For expt II and III the buffer was 70 mM KPO_4–0.06 mM EDTA (pH 7.6). Experiment II contained 85 μg of carbamyl phosphate synthetase and, as indicated, 120 μg of avidin in a volume of 0.4 ml. Experiment III contained 17 μg of carbamyl phosphate synthetase and 23 μg of avidin in a volume of 0.08 ml. After incubations as indicated, aliquots were assayed as described in the text.

Results

As shown by the experiments of Table I, the beef liver carbamyl phosphate synthetase is not significantly inhibited by avidin. In contrast to the results of Wellner *et al.* (1968), the *E. coli* carbamyl phosphate synthetase also was not inhibited. The data are representative of a number of experiments on each enzyme, using two separate preparations of avidin (from the same supplier). Both avidin preparations were tested with rat liver pyruvate carboxylase, a known biotin enzyme, and

[1] Abbreviation used: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

TABLE II: Assays for Biotin Content.[a]

Expt	Hydrolysis Method	Enzyme	mg of Protein Hydrolyzed	μg of Potein Assayed	μμmoles of Biotin	g of Protein/mole of Biotin
1	H_2SO_4	Beef carbamyl phosphate synthetase	1.00	54	<0.5	$>1.08 \times 10^8$
		Frog carbamyl phosphate synthetase	1.32	66	0.65	1.02×10^8
		Pyruvate carboxylase	0.033	1.5	15.2	9.9×10^4
2	H_2SO_4	Frog carbamyl phosphate synthetase	0.220	44	0.15	2.94×10^8
		E. coli carbamyl phosphate synthetase	0.115	2.30	<0.1	$>2.3 \times 10^7$
		Pyruvate carboxylase	0.030	0.60	6.1	10.0×10^4
3	Pronase	*E. coli* carbamyl phosphate synthetase	0.17	3.4	0	∞
				17.0	<0.1	$>1.7 \times 10^8$
		Pyruvate carboxylase	0.030	0.60	6.3	9.5×10^4

[a] The indicated samples were hydrolyzed and assayed for biotin by the methods described in the text.

found to inhibit completely at a weight ratio of approximately 1.

Representative biotin assays are shown in Table II. Neither frog nor beef carbamyl phosphate synthetase shows evidence of being a biotin-containing enzyme. Also, no biotin could be found in the *E. coli* carbamyl phosphate synthetase. The validity of the biotin assay procedure is shown by its ability to detect biotin in hydrolysates of pyruvate carboxylase, a known biotin enzyme. Thus, the conclusion must be that neither the frog, beef, nor *E. coli* enzymes contain biotin.

Discussion

The experiments shown here fail to support the suggestion of Wellner *et al.* (1968) that all forms of carbamyl phosphate synthetase, animal as well as bacterial, acetylglutamate dependent and ammonia utilizing, as well as acetylglutamate independent and glutamine utilizing, may contain biotin as a coenzyme. Further, the data are in direct conflict with the data of Wellner *et al.* (1968) and their conclusion that the carbamyl phosphate synthetase from *E. coli* is a biotin enzyme. Avidin preparations which effectively inhibited pyruvate carboxylase had no significant effect on carbamyl phosphate synthetase from beef liver mitochondria or from *E. coli*. An assay capable of detecting biotin in H_2SO_4 or Pronase digests of pyruvate carboxylase showed no significant amount of biotin to be present in carbamyl phosphate synthetase from any of the three sources. The loss of activity in the ammonium sulfate step of the *E. coli* carbamyl phosphate synthetase purification as reported by Wellner *et al.* (1968) was not seen, and activation of this fraction by biotin did not occur.

No definite reason for this conflict of data on the *E. coli* enzyme can be given, but some possibilities can be discussed. The preparations in the two studies were similar, but not identical. The preparation used in this study did, however, have a specific activity as great as that reported by Anderson and Meister (1965) and as great as that presumably used in the study by Wellner *et al.* (1968).

If the data of both studies are considered to be unequivocal with respect to the biotin assays, and the preparation used by Wellner *et al.* (1968) did contain biotin while the equally active enzyme used in this study did not, the only possible conclusion would be that biotin is not required for the activity of carbamyl phosphate synthetase. Alternatively, either the assay used in the present study did not detect biotin which was present, or the assays used by Wellner *et al.* (1968) gave a response which was not due to biotin.

While the use of *N. crassa* as a test organism is not common, it is a useful assay system and has been reported to respond to biotin, oxybiotin, biocytin, desthiobiotin, and biotin sulfoxide (Snell, 1950). Thus this assay system allows the use of a Pronase digestion, which does not destroy biotin, and allows the detection of the released biocytin.

Loss of biotin during hydrolysis as a reason for lack of detection can be ruled out, since two different methods of hydrolysis (H_2SO_4 and Pronase) were used, each of which was able to detect not only the biotin in pyruvate carboxylase, but also free biotin added to the various samples of carbamyl phosphate synthetase before hydrolysis. Thus, no methodological reason is apparent for the lack of detection of biotin in the enzyme preparations used in the present study.

The major assay used by Wellner *et al.* (1968) is based on the decrease in absorbance when a dye, 4′-hydroxyazobenzene-2-carboxylic acid, is displaced from avidin by biotin, which is bound much more tightly ($K_{dissn} = 5.8 \times 10^{-6}$ for the dye, $\sim 10^{-15}$ for biotin) (Green, 1965). However, any compound which will compete with the dye would be expected to give a response. Since the dye shows no obvious structural relationship to biotin, it is obvious that a competing compound would not need to be related to biotin. Thus, the dye binding assay would appear to be useful for quantitation of a sample known to be free of interfering compounds, but may

not be sufficiently specific to be used as proof of the presence of biotin.

A second assay for biotin, using yeast, was also reported by Wellner *et al.* (1968). While in this case also a response which is not due to biotin may be possible, we have no explanation for the findings reported.

The results of the experiments of Wellner *et al.* (1968) with avidin also suggest the possibility that the enzyme preparation used by Wellner *et al.* (1968) may have contained biotin or some interfering compound which gave a response in the assays used, while the enzyme preparation used in the present study was free of any such compound. In the absence of steric restrictions imposed by the enzyme, a compound which was bound to avidin in the dye displacement assay could also be expected, when attached to the enzyme, to provide a site for the binding of avidin to the enzyme. If this site were properly located, the binding of avidin could conceivably cause inhibition as a result of either a change in conformation or a direct masking of the active site, even if the group providing the binding site had no function in the action of the enzyme. (It should be noted that both the dye displacement assay for biotin and the inhibition experiments depend on the binding of avidin to some group or compound, not necessarily biotin, and should therefore not be considered as completely independent experiments.)

It would appear, then, that carbamyl phosphate synthetase, regardless of its type or source, does not contain biotin as a required coenzyme.

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We wish to acknowledge our thanks to Dr. Robert L. Metzenberg for providing us with *Neurospora* cultures and advice on the biotin assays.

Added in Proof

Guthöhrlein and Knappe (1969) recently reported that assay of purified rat liver carbamyl phosphate synthetase failed to reveal the presence of biotin in two microbiological assay systems, *L. arabinosus* and *S. cerevisiae*. The inhibition of rat liver carbamyl phosphate synthetase by a highly purified avidin preparation was observed. However, the authors demonstrated the same degree of inhibition by other basic proteins (protamine and lysozyme). This effect was not reversed by biotin but was reversed by serum albumin. The authors concluded that the inhibition was due to the basic nature of the proteins used. Since some preparations of avidin are known to be complexed with acidic components (Fraenkel-Conrat *et al.*, 1952), the degree of basicity of different avidin preparations thus could vary independently of biotin-binding capacity. The different inhibitory effects of avidin observed by ourselves and by Wellner *et al.* (1968) and those reported by Guthöhrlein and Knappe (1969) could thus reflect differences in basicity of the avidin preparations used in these studies.

References

Anderson, P. M., and Marvin, S. V. (1968), *Biochem. Biophys. Res. Commun. 32*, 928.

Anderson, P. M., and Meister, A. (1965), *Biochemistry 4*, 2803.

Beadle, G. W., and Tatum, E. L. (1945), *Am. J. Botany 32*, 678.

Fraenkel-Conrat, H., Snell, N. S., and Ducay, E. D. (1952), *Arch. Biochem. Biophys. 39*, 80.

Green, N. M. (1965), *Biochem. J. 94*, 23C.

Guthöhrlein, G., and Knappe, J. (1968), *European J. Biochem. 7*, 119.

Guthöhrlein, G., and Knappe, J. (1969), *European J. Biochem. 8*, 207.

Hunninghake, D., and Grisolia, S. (1966), *Anal. Biochem. 16*, 200.

Luck, J. M., Rasmussen, P. S., Satake, K., and Tsvetikov, A. N. (1958), *J. Biol. Chem. 233*, 1407.

Marshall, M., Metzenberg, R. L., and Cohen, P. P. (1958), *J. Biol. Chem. 233*, 102.

McClure, W. R. (1969), *Federation Proc.* (in press).

Metzenberg, R. L., Marshall, M., and Cohen, P. P. (1958), *J. Biol. Chem. 233*, 1560.

Peng, L., and Jones, M. E. (1969), *Biochem. Biophys. Res. Commun. 34*, 335.

Snell, E. E. (1950), *in* Vitamin Methods, Vol. 1, György, P., Ed., New York, N. Y., Academic, p 327.

Wellner, V. P., Santos, J. I., and Meister, A. (1968), *Biochemistry 7*, 2848.