for the preparation of VIc without further treatment. For analysis a small sample was recrystallized from benzene. The colorless plates melted at 145–147°.

Anal. Calcd. for $C_9H_9NO_3$: C, 60.32; H, 5.06; N, 7.82. Found: C, 60.25; H, 5.05; N, 7.78.

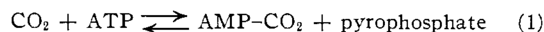
LINCOLN 8, NEBRASKA

COMMUNICATIONS TO THE EDITOR

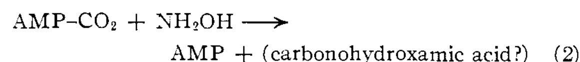
THE ROLE OF ADENOSINE TRIPHOSPHATE IN THE ENZYMATIC ACTIVATION OF CARBON DIOXIDE[1]

Sir:

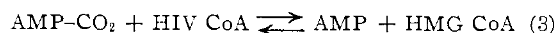
Although many compounds are known to undergo chemical modification or "activation" prior to entrance into biochemical reactions, carbon dioxide has generally been considered to participate simply as such or as bicarbonate ion in a variety of carboxylations in living cells. As recently proposed,[2] however, the reaction of carbon dioxide with ATP[3] may furnish a reactive intermediate capable of carboxylating HIV CoA (an intermediate in leucine metabolism). Further study of this system has led to the discovery of the bicarbonate- and hydroxylamine-dependent cleavage of ATP to AMP and pyrophosphate, catalyzed by extracts of bacteria, yeast and various animal tissues. The reactions shown are proposed to account for this finding and for the mechanism of carbon dioxide activation



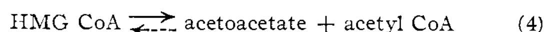
$$CO_2 + ATP \rightleftharpoons AMP\text{–}CO_2 + \text{pyrophosphate} \quad (1)$$



$$AMP\text{–}CO_2 + NH_2OH \longrightarrow AMP + (\text{carbonohydroxamic acid?}) \quad (2)$$



$$AMP\text{–}CO_2 + \text{HIV CoA} \rightleftharpoons AMP + \text{HMG CoA} \quad (3)$$



$$\text{HMG CoA} \rightleftharpoons \text{acetoacetate} + \text{acetyl CoA} \quad (4)$$

The incubation of hydroxylamine and bicarbonate with a heart preparation free of myokinase and pyrophosphatase but containing the carbon dioxide-activating enzyme (H enzyme) results in the degradation of ATP to approximately equimolar amounts of adenylic acid[4] and pyrophosphate (Table I). These products would be expected if this enzyme catalyzes the formation of $AMP\text{-}CO_2$ (the mixed anhydride of adenylic and carbonic acids) according to Reaction 1, and the intermediate decomposes non-enzymatically according to Reaction 2. Attempts to detect carbonohydroxamic acid have so far proved unsuccessful. However, hydroxylamine has been shown to convert synthetically prepared $AMP\text{-}CO_2$[8] to AMP, and synthetically prepared $AMP\text{-}CO_2$ ethyl ester to AMP and a heat-labile hydroxamic acid chromatographically indistinguishable from authentic carbonohydroxamic acid ethyl ester[9] (R_f 0.72 in water-saturated butanol). The H enzyme has been completely separated in this laboratory from fluorokinase (F enzyme), the enzyme in heart extracts which has been shown by Flavin, Castro-Mendoza, and Ochoa[10] to catalyze the fluoride- and bicarbonate-dependent cleavage of ATP to ADP and fluorophosphate.[11] These investigators have proposed[10] that the F enzyme may activate carbon dioxide for propionyl CoA carboxylation.[12] As indicated in Table I, the H enzyme has no signi-

TABLE I

SEPARATION OF H AND F ENZYMES CATALYZING THE BICARBONATE-DEPENDENT CLEAVAGE OF ATP

The test system contained 500 μmoles of potassium bicarbonate, 10 μmoles of ATP, 2 μmoles of Versene, 50 μmoles of magnesium chloride, and, where indicated, crystalline pyrophosphatase[5] (0.3 mg. of protein), 10-fold purified H enzyme (2.3 mg. of protein) or 30-fold purified F enzyme (1.0 mg. of protein), and 200 μmoles of neutralized hydroxylamine or 300 μmoles of potassium fluoride, in a final volume of 3.0 ml. Incubation, 30 minutes at 38°. In control experiments, 500 μmoles of tris-(hydroxymethyl)-aminomethane buffer, *p*H 8.1, was substituted for bicarbonate.

Additions	μMoles orthophosphate formed[6]	μMoles pyrophosphate formed[7]
H enzyme + NH_2OH	0.04	0.76
H enzyme + NH_2OH + pyrophosphatase	1.50	0.02
H enzyme + KF	0	0
F enzyme + NH_2OH	0	0
F enzyme + KF	(1.97 μmoles of fluorophosphate[a])	

[a] Estimated colorimetrically[7] using an authentic sample of fluorophosphate as a standard. The absence of pyrophosphate was demonstrated by paper chromatography.

(1) Supported by grants from the United States Public Health Service (Grant No. A-993C) and the Michigan Memorial-Phoenix Project of the University of Michigan.

(2) M. J. Coon, *Federation Proc.*, **14**, 762 (1955).

(3) Abbreviations: adenosine triphosphate, ATP; adenosine diphosphate, ADP; adenylic acid, AMP; adenosine phosphoryl carbonate, $AMP\text{-}CO_2$; β-hydroxyisovaleryl coenzyme A, HIV CoA; β-hydroxy-β-methylglutaryl coenzyme A, HMG CoA.

(4) Assayed by the action of adenylic deaminase according to H. M. Kalckar, *J. Biol. Chem.*, **167**, 429 (1947).

(5) Kindly furnished by Dr. M. Kunitz.

(6) Estimated according to C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

(7) Estimated according to R. M. Flynn, M. E. Jones and F. Lipmann, *J. Biol. Chem.*, **211**, 791 (1954).

(8) B. K. Bachhawat J. F. Woessner and M. J. Coon, *Federation Proc.*, **15**, 214 (1956).

(9) L. W. Jones, *Am. Chem. J.*, **20**, 1 (1898).

(10) M. Flavin, H. Castro-Mendoza and S. Ochoa, *Biochim. et biophys. acta*, **20**, 591 (1956).

(11) This compound was incorrectly identified as pyrophosphate in an earlier report.[2] Fluorophosphate and pyrophosphate are readily distinguishable from orthophosphate but not from each other by colorimetric test[7] and by paper electrophoresis in citrate buffer, *p*H 9.0.

(12) M. Flavin, P. J. Ortiz and S. Ochoa, *Nature*, **176**, 823 (1955).

ficant activity in the fluoride assay system, and the F enzyme has none in the hydroxylamine assay system.

In the absence of hydroxylamine, Reaction 1 can be coupled with Reaction 3 (catalyzed by HIV CoA carboxylase[13]) to yield HMG CoA. The action of the HMG CoA cleavage enzyme[14] converts this product quantitatively to equimolar amounts of acetyl CoA and acetoacetate (Reaction 4). The data in Table II demonstrate that the H

TABLE II

CARBON DIOXIDE FIXATION BY THE COUPLED ACTION OF H ENZYME AND β-HYDROXYISOVALERYL CoA CARBOXYLASE

The test system contained 500 μmoles of potassium bicarbonate, 5 μmoles of Versene, 50 μmoles of magnesium chloride, 10 μmoles of glutathione, 20 μmoles of ATP, 2 μmoles of HIV CoA, and, where indicated, H enzyme (2.3 mg. of protein), F enzyme (2.0 mg. of protein), and a pig heart fraction containing HIV CoA carboxylase but free of H and F enzymes (5.2 mg. of protein), in a final volume of 3.0 ml. An excess of HMG CoA cleavage enzyme was present in each experiment. Incubation was for 60 minutes at 38°. HIV CoA was omitted in control experiments.

Enzymes added	μMoles acetoacetate formed[15]
H enzyme + carboxylase	0.60
F enzyme + carboxylase	0
H enzyme	0.07
Carboxylase	0.02

enzyme is active in this system whereas the F enzyme is without effect. It is concluded, therefore, that the H enzyme and the carbon dioxide-activating enzyme are probably identical, but that the F enzyme plays no role in carbon dioxide activation for HIV CoA carboxylation. In accord with these findings, the coupling of Reactions 1, 3 and 4 in a heart extract free of pyrophosphatase has been found to furnish approximately equimolar amounts of pyrophosphate and acetoacetate.

Since the results presented establish two enzymatic steps in the carboxylation of HIV CoA, it seemed of particular interest to determine which of these might be lacking in biotin deficiency.[16] Enzyme extracts prepared from the livers of biotin-deficient rats possess no HIV CoA carboxylase activity, but have the same content of H enzyme, F enzyme, and HMG CoA cleavage enzyme as do those prepared from normal livers.[17] The H enzyme has recently been purified 650-fold from pig heart extracts and found to require the presence of Zn^{++} ions for maximal activity.

(13) B. K. Bachhawat, W. G. Robinson and M. J. Coon, *J. Biol. Chem.*, **219**, 539 (1956).

(14) B. K. Bachhawat, W. G. Robinson and M. J. Coon, *ibid.*, **216**, 727 (1955).

(15) Estimated by a modification of the method of S. S. Barkulis and A. L. Lehninger, *J. Biol. Chem.*, **190**, 339 (1951).

(16) G. W. E. Plaut and H. A. Lardy, *J. Biol. Chem.*, **186**, 705 (1950).

(17) Unpublished experiments carried out by the authors in collaboration with Dr. J. F. Woessner and Dr. Henry A. Lardy.

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OPTICAL ROTATORY DISPERSION STUDIES. X.[1] DETERMINATION OF ABSOLUTE CONFIGURATION OF α-HALOCYCLOHEXANONES[2]

Sir:

The determination of the absolute configuration of carbonyl compounds by rotatory dispersion measurements[1,3] involves a comparison of the rotatory dispersion curve of the unknown substance with those of reference ketones (or aldehydes) which have been sterically related to D-glyceraldehyde. In connection with an extensive study[4] of the effect of α-substitution upon the rotatory dispersion curves of ketones, there has been made an observation which appears to offer a means of establishing the absolute configuration of cyclohexanones *without requiring a reference compound of known absolute configuration.*

While introduction of an *equatorial* halogen atom (chlorine, bromine but *not* fluorine)[4] in the α-position of a cyclohexanone does not seem to affect the sign of the rotatory dispersion curve of the halogen-free parent ketone,[5] this is not necessarily the case with *axial* bromine or chlorine substituents. In the latter instance, the stereochemistry of the O=C—C—X ($-\overset{O}{\overset{\|}{C}}-\overset{X}{\overset{|}{C}}-$) chromophore as a whole becomes dominant and *controls the sign of the Cotton effect.* The gross shape of the dispersion curve can be predicted by examining a model of the appropriate ketone in the following manner:

Place the cyclohexanone ring in such a way that the carbonyl carbon atom is the "head" of the chair and look along the O=C bond as shown by the arrow in I. As demonstrated in Table I, if

TABLE I

Axial α-haloketone	Cotton effect: Halogen-free ketone	Cotton effect: α-Haloketone
3α-Bromoandrostan-2-one-17β-ol propionate	Positive	Positive
7α-Bromocholestane-3β,5α-diol-6-one 3-acetate	Negative	Positive
7α-Bromocholestane-3β,5α-diol-6-one 3,5-diacetate	Negative	Positive
6β-Bromocholestan-3β-ol-7-one acetate	Negative	Positive
9α-Bromoergostan-3β-ol-11-one acetate	Positive	Positive
12α-Bromoergostan-3β-ol-11-one acetate	Positive	Negative
12α-Chloro-11-ketotigogenin acetate	Positive	Negative
12α,23-Dibromo-11-ketotigogenin acetate	Positive	Negative
Methyl 11β-bromo-3α-acetoxy-12-ketocholanate	Positive	Negative
2α-Bromofriedelin	Negative	Negative
4α-Bromofriedelin	Negative	Positive

the axial α-chlorine or bromine atom lies to the *right* (*ca.* 105° angle[7]) of the observer (I, X = Cl or Br), *the single Cotton effect will be positive;* if

(1) Paper IX, C. Djerassi and W. Klyne, *Chemistry and Industry*, 988 (1956).

(2) Supported by Grant No. CY-2919 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

(3) C. Djerassi, R. Riniker and B. Riniker, THIS JOURNAL, **78**, 6362 (1956).

(4) C. Djerassi, *et al.*, to be published. The rotatory dispersion curves of the ketones listed in Table I will be reproduced in that article.

(5) For example, cholestan-3-one, 2α-bromocholestanone and 4α-bromocholestanone all exhibit a positive single Cotton effect curve.[6]

(6) For nomenclature in rotatory dispersion work see C. Djerassi and W. Klyne, *Proc. Chem. Soc.*, 55 (1957).

(7) R. C. Cookson, *J. Chem. Soc.*, 181 (1954).