INCORPORATION OF THE AMINO ACID MOIETIES OF AMINO ACID-ADENYLIC ACID ANHYDRIDES INTO PROTEINS[1]

Sir:

Recent studies have led to the suggestion that AA-AMP[2] anhydrides are intermediates in incorporation of AA into proteins in cell-free systems.[3,4] In an effort to elucidate the nature of such incorporation, we have prepared a number of AA–AMP anhydrides by treating N-carbobenzoxy-amino acids with AMP in the presence of N,N′-dicyclohexylcarbodiimide; the blocking group was removed from the purified carbobenzoxy-AA-AMP anhydride by catalytic hydrogenation.[5] The products exhibited consistent ionophoretic mobility,[6] gave the corresponding AA-hydroxamates, yielded equivalent quantities of AA and AMP on hydrolysis, and formed ATP enzymatically.[6]

When C^{14}-AA-AMP anhydrides were incubated with enzyme preparations, the protein subsequently isolated contained significant radioactivity (Table I). No incorporation occurred when the anhydride was hydrolyzed prior to study. When the enzyme preparations were heat-denatured, incorporation was greater than with unheated enzyme. The C^{14}-AA of the proteins (heated and unheated) were released over a period of 16 hours by hydrolysis with 6 *N* HCl at 105°; treatment of these proteins with 1-fluoro-2,4-dinitrobenzene, and then acid hydrolysis[7] gave DNP-amino acid preparations containing 70 to 80% of the C^{14} originally incorporated.

TABLE I: TRANSFER OF C^{14} FROM C^{14}-AMINO ACID-AMP ANHYDRIDES TO PROTEINS

Reaction mixtures[a]	Incorporation into protein (c.p.m./mg.) Glycine	Tryptophan
E + AA-AMP	30	16
E + AA + AMP	0.3	0
E (heated; 100°; 10 min.) + AA-AMP	286	166
E (heated; 100°; 10 min.) + AA + AMP	0.3	0

[a] Contained enzyme preparation (E; 2 ml. of supernatant solution (containing microsomes) obtained by centrifuging a 25% rat liver homogenate at 12,000 × *g*[8]), gly-1-C^{14}-AMP (2.5 μmoles; 3.6×10^5 c.p.m.) or DL-try-3-C^{14}-AMP (1 μmole; 1.9×10^5 c.p.m.) in a volume of 2.5 ml.; incubated at 38° for 30 minutes. E catalyzed incorporation of gly-C^{14} and try-C^{14} in the presence of an ATP-generating system, as described by Zamecnik and Keller.[9]

Transfer of C^{14}-AA from C^{14}-AA-AMP to heated proteins may be regarded as non-enzymatic acylation of protein amino groups, perhaps mainly those of lysine residues. That heated proteins incorporated more C^{14} than did unheated proteins suggests that denaturation exposes more reactive groups. To what extent labelling of unheated proteins is enzymatic remains to be determined. Non-enzymatic acylation of proteins may also occur when C^{14}-AA are incubated with enzyme and ATP-generating systems; thus, the high reactivity[8] of AA-AMP anhydrides may explain some of the incorporation previously reported.[9,10,11] However, it is not excluded that under physiological conditions, selective mechanisms (*e.g.*, binding to enzymes) may direct transfer of the AA-moieties of AA-AMP anhydrides to specific acceptors and ultimately to specific positions in the peptide chains of proteins.[12]

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(2) Abbreviations: amino acid, AA; adenylic acid, AMP; amino acid-adenylic acid anhydride, AA-AMP; adenosine triphosphate, ATP; glycine, gly; tryptophan, try; ribonucleic acid, RNA.

(3) M. B. Hoagland, *et al.*, *J. Biol. Chem.*, **218**, 345 (1956).

(4) P. Berg, *Federatiau Proc.*, **16**, 152 (1957).

(5) Ribose acylated derivatives appear excluded by paper ionophoresis in borate and other buffers and by periodate titration. Acylation of the 6-amino group was excluded by treating carbobenzoxy-try-AMP with nitrous acid to give carbobenzoxy-try-inosinate.

(6) M. Karasek, *et al.*, accompanying paper.

(7) H. Frankel-Conrat, *et al.*, *Methods of Biochemical Analysis*, **2**, 359–425 (1955).

(8) Purified liver RNA preparations (A. Gierer and G. Schramm, *Nature*, **177**, 702 (1956) also were labelled readily when incubated with C^{14}-AA-AMP; after incubating such C^{14}-RNA with heated and unheated proteins, significant radioactivity was found in the proteins subsequently isolated. The nature of this incorporation is being investigated.

(9) P. C. Zamecnik and E. B. Keller, *J. Biol. Chem.*, **209**, 337 (1954).

(10) M. B. Hoagland, *et al.*, *Biochim. et Biophys. Acta*, **24**, 215 (1957).

(11) The concentrations of AA-AMP anhydrides presumably formed in incorporating systems consisting of AA and ATP-generating systems would be lower than in the present studies; it is therefore difficult to make a meaningful comparison of the labelling in the two systems.

(12) While this work was in progress, we learned that C. Zioudrou, S. Fujii and J. S. Fruton have independently synthesized C^{14}-tyrosine-AMP and C^{14}-glycyltyrosine-AMP by a similar procedure, and observed labelling of heated and unheated rat liver mitochondria by these compounds and their N-carbobenzoxy derivatives. They also conclude that non-enzymatic acylation is responsible for the labelling. (We thank Dr. Fruton for communicating these findings to us prior to publication.)

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ENZYMATIC SYNTHESIS AND REACTIONS OF TRYPTOPHAN-ADENYLIC ACID ANHYDRIDE[1]

Sir:

Several enzyme preparations catalyze AA[2] activation reactions which yield AA-hydroxamate when enzyme is incubated with AA, ATP, Mg^{++}, and hydroxylamine.[3,4,5,6] Although available evidence[7,8,9] is consistent with intermediate formation of AA-AMP anhydrides similar to those postulated in acetate[10] and fatty acid[11,12] activation and in phenylacetylglutamine and hippurate synthesis,[13]

(1) Supported in part by grants from the National Science Foundation and National Institutes of Health.

(2) Abbreviations: amino acid, AA; amino acid-adenylic acid anhydride, AA-AMP; adenosine triphosphate, ATP; pyrophosphate, P-P; tryptophan, try.

(3) M. B. Hoagland *et al.*, *J. Biol. Chem.*, **218**, 345 (1956).

(4) P. Berg, *ibid.*, **222**, 1025 (1956).

(5) E. W. Davie *et al.*, *Arch. Biochem. Biophys.*, **65**, 21 (1956).

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

(7) J. A. Demoss *et al*, *Proc. U. S. Nat. Acad. Sci.*, **42**, 325 (1956).

(8) P. Berg, *J. Biol. Chem.*, **222**, 1015 (1957).

(9) M. B. Hoagland *et al.*, *Biochim. Biophys. Acta*, **26**, 215 (1957).

(10) P. Berg, *J. Biol. Chem.*, **222**, 1015 (1956).

(11) W. P. Jencks and F. Lipmann, *ibid.*, **225**, 207 (1957).

(12) H. S. Moyed and F. Lipmann, *J. Bact.*, **73**, 117 (1957).

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

enzymatic formation of such anhydrides has not been observed. This communication presents evidence for the enzymatic synthesis of try-AMP.

The total yield (15 mg.) of try-activating enzyme from 5 kg. of beef pancreas[5] was incubated with try-3-C^{14}, Mg^{++}, and ATP (Table I). The reaction mixture was lyophilized and extracted with glacial acetic acid; on addition of ether to the extract a precipitate formed, leaving try-3-C^{14} in solution. Paper ionophoresis of the precipitate gave a radioactive band (A) corresponding to authentic try-AMP.[14] Elution of A yielded an alkali-labile compound that reacted with hydroxylamine to form a C^{14}-hydroxamate, which on paper chromatography in five solvents gave R_F values identical with try-hydroxamate. The negatively charged band (B) has not yet been identified. In a similar experiment with added try-AMP (1 μmole), about 10 times more C^{14} was found in band A, suggesting exchange between try and try-AMP. This was demonstrated by incubating try-3-C^{14} (0.08 μmole; 5×10^5 c.p.m.) and try-AMP (1 μmole) with enzyme followed by addition of hydroxylamine; C^{14}-try-hydroxamate (3420 c.p.m.) was identified as described above.[15] We have found that this enzyme also catalyzes ATP synthesis from P-P and AA-AMP anhydrides (*e.g.*, AMP anhydrides of D-try,[16] L-try, D-phenylalanine,[16] L-phenylalanine, L-isoleucine, L-leucine, L-glutamine, glycine, L-alanine, L-proline, L-tyrosine, L-valine). Thus, incubation of AA-AMP (1 μmole), P-P (2 μmoles), $MgCl_2$ (10 μmoles), and enzyme (0.4 mg.) in 1 ml. of 0.1 M tris-(hydroxymethyl)-aminomethane buffer (*p*H 7.2) gave 0.1 to 0.2 μmole of ATP in 5 minutes at 37°. Benzoyl-AMP, carbobenzoxy-try-AMP, and β-alanine-AMP were inactive.[17] The low specificity with respect to ATP synthesis contrasts markedly with the strict specificity for L-try in hydroxamate formation.[5] The present data indicate net synthesis of a compound with the properties of try-AMP in the absence of hydroxylamine as trapping agent, and thus support the concept that try-AMP is formed in the activation reaction.[18] It would appear that any mechanism proposed for the activation phenomenon should account for the observations that the activating enzyme preparation catalyzes exchange between try and try-AMP, and synthesis[19] of ATP from P-P and various AA-AMP anhydrides.

TABLE I: PAPER IONOPHORESIS[a]

Compound	Position of band (mm./2 hours)	C.p.m.
Tryptophan standard	− 5.0	
Try-AMP standard	−30.2	
Band A	−30.2	3100
Band B	+12.1	450

[a] Reaction mixture consisted of enzyme[5] (15 mg.), ATP (10 μmoles), $MgCl_2$ (10 μmoles), DL-try-3-C^{14} (0.08 μmole; 5×10^5 c.p.m.), crystalline pyrophosphatase (10 γ) in 2 ml. of 0.05 *M* tris-(hydroxymethyl)-aminomethane buffer (*p*H 8.0); incubated at 37° for 30 minutes and processed as described in text. Precipitate was analyzed ionophoretically (R. Markham and J. B. Smith, *Biochem. J.*, **52**, 552 (1952)) in 0.05 *M* ammonium formate buffer (*p*H 4.5) at 20 volts per cm. for 2 hours.

(14) Prepared as described by Castelfranco, *et al.*, accompanying paper.

(15) A value of 101 c.p.m. was obtained non-enzymatically.

(16) The optical purity of the AA moiety was greater than 99.5%; A. Meister, *et al.*, *J. Biol. Chem.*, **192**, 535 (1951).

(17) L-Try-inosinate did not form inosine triphosphate.

(18) W. C. Rhodes and W. D. McElroy recently have shown enzymatic synthesis of adenyloxyluciferin by firefly luciferase (personal communication).

(19) Such synthesis recently has been reported by Novelli (*Proc. Natl. Acad. Sci.*, **44**, 86 (1958)) and with yeast methionine-activating enzyme by Berg (*Fed. Proc.*, **16**, 152 (1957); personal communication).

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STERIC CONSIDERATIONS IN THE ENZYMATIC COURSE OF THE HYDROXYLATION OF STEROIDS*

Sir:

Experiments to determine the mode of attack of the steroid hydroxylases on their substrates have been carried out. The two major possibilities considered in hydroxylation reactions involving $>C<^{H}_{H}$ groupings were: (1) a direct replacement of the H from the position to be hydroxylated, or (2) a Walden type inversion with the removal of the H from the alternate position to the one hydroxylated. For this purpose pregnane-3,20-dione-11α,12α-H^3[1] was incubated with *Rhizopus nigricans*[2] and the 11α-hydroxylated analog isolated; progesterone-11α,12α-H^3 prepared by the method of Holysz[3] from pregnanedione-11α,12α-H^3 was perfused through surviving bovine adrenal glands[4]

TABLE I

INCUBATION OF PREGNANEDIONE-11α,12α-H^3 WITH RHIZOPUS NIGRICANS

Compounds isolated	Counts/min./μM[a]
Pregnanedione, m.p. 121–122°[b]	3.25×10^7[c]
11α-Hydroxy-pregnanedione, m.p. 123–125°	0.97×10^7

PERFUSION OF PROGESTERONE-11α,12α-H^3 THROUGH BOVINE ADRENALS

Compounds isolated	Counts/min./μM
Progesterone, m.p. 124–125°	6.90×10^6
17α-Hydroxy-progesterone, m.p. 219–223°	6.93×10^6
Corticosterone, m.p. 179–181°	6.70×10^6
Hydrocortisone, m.p. 213–215°	6.73×10^6

[a] All counts were determined in a Packard Tri-Carb liquid scintillation counter Model 314. [b] All melting points were observed on a Fisher–Johns hot stage and are corrected. [c] Accuracy ±3%.

and the 11β-hydroxylated products, corticosterone and hydrocortisone, isolated. Resolution of incubated material in both experiments was effected by silica gel column chromatography using mixtures of benzene and ethyl acetate for elution. All steroids were purified to a constant count by additional column or paper chromatography, when necessary, and by repeated crystallizations. Iden-

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(1) Synthesis and discussion of H^3 distribution to be published.

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(3) R. P. Holysz, *ibid.*, **75**, 4432 (1953).

(4) O. Hechter, A. Zaffaroni, R. P. Jacobsen, H. Levy, R. W. Jeanloz, V. Schenker, and G. Pincus, "Recent Progress in Hormone Research," Vol. VI, Academic Press, New York, 1951, p. 215.