BBA 8241

INHIBITION OF PROTEIN SYNTHESIS IN CELL-FREE SYSTEMS BY HOMOCITRULLYLAMINO ADENOSINE

ARMAND J. GUARINO, MARY LOU IBERSHOF AND RICHARD SWAIN

The Department of Biological Chemistry, The University of Michigan, Ann Arbor, Mich. (U.S.A.)

(Received October 5th, 1962)

SUMMARY

Homocitrullylamino adenosine inhibits the incorporation of [^{14}C]amino acids into a hot acid-insoluble form in cell-free systems derived from *Escherichia coli* and rat liver. The concentration necessary for one-half maximal inhibition in the *E. coli* system is $1.1 \cdot 10^{-4}$ M. Addition of the intact molecule is necessary for the maximum inhibition. The inhibition does not appear to involve the activation of the amino acids or their transfer to s-RNA, but rather exerts its effect, like puromycin, at the level involving the s-RNA ribosomal complex.

INTRODUCTION

We recently reported on the isolation and characterization of HCAA, a nucleoside obtained from mycelia of *Cordyceps militaris*[1]. The structural similarity of this compound to puromycin is shown in Fig. 1. Since puromycin has been shown to inhibit the incorporation of [^{14}C]amino acids into protein in cell-free extracts of liver[2], bacteria[3], thymus[4], ascites tumor[5], and reticulocytes[6,7], we decided to test HCAA to see if it might exert a similar effect. In the studies reported here we have found that HCAA, like puromycin, inhibits the incorporation of amino acids into protein in cell-free extracts of both *Escherichia coli* and rat liver.

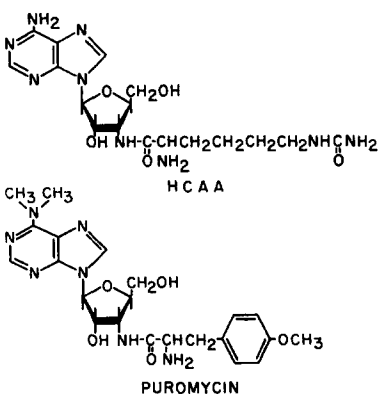

Fig. 1. Structure of homocitrullylamino adenosine and puromycin.

Abbreviation: HCAA, homocitrullylamino adenosine.

MATERIALS AND METHODS

E. coli R2 was grown in shaking culture at 37° in a medium containing 1 % glucose, 1 % yeast extract, and 0.25 M potassium phosphate buffer (pH 6.5). The cells were harvested early in the log phase of growth in a Sharples centrifuge at 10° and the paste obtained was stored at −20°. The various cell-free fractions used as either sources of enzyme, s-RNA, or ribosomes were obtained as follows: The cells were ground with 2.5 g alumina (Alcoa A305)/g of frozen cells, followed by extraction with 2–3 volumes of buffer containing 0.01 M Tris hydrochloride (pH 7.8), 0.01 M magnesium acetate, 0.006 M mercaptoethanol and 0.06 M potassium chloride (standard buffer of MATTHAEI AND NIRENBERG[8]). The mixture was centrifuged at 0° for 20 min at 30 000 × *g*, and this centrifugation repeated two more times, the final one being for 60 min. The supernatant fluid (crude 30 S) was dialyzed against 60 volumes of the standard buffer (dialyzed 30 S) followed by centrifugation at 105 000 × *g* for 2 h. The supernatant fluid from this centrifugation (dialyzed 100 S) was carefully separated from the precipitate (ribosomes), which were resuspended in standard buffer. All fractions were stored at −20°.

s-RNA was obtained by extraction of the dialyzed 100 S fraction at 24° with an equal volume of 88 % phenol (Mallinckrodt, "Gilt Label"). The turbid solution was centrifuged at 1200 × *g* for 15 min and the phenol layer was re-extracted with another volume of standard buffer minus the mercaptoethanol. The aequeous layers were combined and extracted with ether 3 times to remove phenol. Residual ether was removed by aeration with nitrogen. Solid potassium acetate was added with stirring to give a final concentration of 2 %, followed by 2.5 volumes of 95 % ethanol. The heavy white precipitate resulting from this treatment was collected by centrifugation, resuspended in water, and treated with deoxyribonuclease at 24° for 15 min, which resulted in a noticeable lowering in the viscosity of the solution. The s-RNA was exhaustively dialyzed against large volumes of water and dried by lyophilization.

Deacylation of the s-RNA was achieved by incubation in 0.5 M Tris hydrochloride (pH 8.8) for 45 min at 35° according to the procedure of VON EHRENSTEIN AND LIPMANN[10]. The deacylated s-RNA was dialyzed overnight against water and dried by lyophilization.

Reacylation of the s-RNA with amino acids was carried out by incubating 100 mg s-RNA in a final volume of 10 ml with 1 μmole each of 21 amino acids including [^{14}C]leucine (containing 12 μC), 0.4 ml (dialyzed 100 S), 1 mmole Tris hydrochloride (pH7.2), 50 μmoles magnesium acetate, 30 μmoles ATP, 200 μmoles phosphoenolpyruvate, and 0.4 mg pyruvate kinase for 10 min at 37° according to the procedure of VON EHRENSTEIN AND LIPMANN[10]. The reaction was stopped by adding phenol and the s-RNA reisolated by the procedure of VON EHRENSTEIN AND LIPMANN[10]. The final lyophilized product had a specific activity of 3000 counts/min/mg s-RNA.

In experiments in which HCAA was tested as an inhibitor of protein synthesis in rat-liver homogenates, the livers of 125 g rats were minced with scissors, and homogenized in a Potter–Elvehjem homogenizer in 2.5 volumes of medium X of ZAMECNIK AND KELLER[9]. The homogenate was centrifuged at 4000 × *g* for 20 min and the supernatant fluid (crude liver extract) was used in the studies to be reported below.

GTP, sodium phosphoenolpyruvate, ATP, and creatine phosphate were purchased from Sigma Chemical Co. Pyruvate kinase (Boehringer) was purchased from Calbiochem. DL [1-^{14}C]Leucine (specific activity 8–12 mC/mmole) was also purchased

from Calbiochem. Uniformly labeled amino acids from Chlorella hydrolysate was a product of Volk. Homocitrullylamino adenosine was isolated from the mycelia of *Cordyceps militaris*[1]. Puromycin was purchased from Nutritional Biochemicals. Chloromycetin was obtained from Parke Davis Co. Protein was determined by the method of ROBINSON AND HOGDEN[11]. Homocitrulline was synthesized according to the procedure of STEVENS AND ELLMAN[12]. 3'-Amino 3'-deoxyadenosine was obtained as a product of alkaline methanolysis of HCAA[1].

RESULTS

Initial experiments showed HCAA inhibited protein synthesis, therefore the level necessary to produce one-half maximal inhibition was determined and compared with that for puromycin and chloromycetin. The incubation period for this experiment was 10 min at which time the incorporation of label into protein was still proceeding at a linear rate in the uninhibited system. The levels of inhibitor necessary for half-maximal inhibition as derived from the graph in Fig. 2 were $1.1 \cdot 10^{-4}$ M for HCAA, $1.8 \cdot 10^{-5}$ M for chloromycetin and $2.2 \cdot 10^{-6}$ M for puromycin.

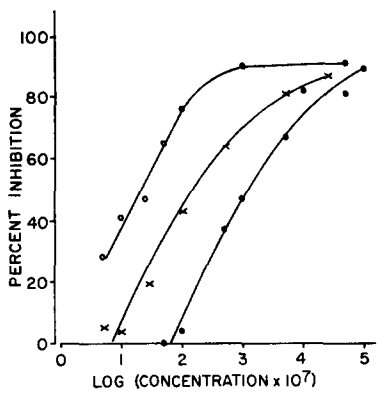

Fig. 2. Inhibition of protein synthesis by different concentrations of puromycin, chloromycetin, and HCAA. The incubations were carried out essentially as described by MATTHAEI AND NIRENBERG[8]. Each reaction vessel contained in a final volume of 1 ml: 100 μmoles Tris buffer (pH 7.8), 10 μmoles magnesium acetate, 6 μmoles mercaptoethanol, 50 μmoles potassium chloride, 1 μmole ATP, 5 μmoles phosphoenolpyruvate, 0.03 μmole GTP, 20 μg pyruvate kinase, 0.25 μmole tryptophan, 70 000 counts/min ^{14}C-labeled Chlorella hydrolysate (specific activity 0.17 mC/mg), and 3.4 mg protein (dialyzed 30 S). The concentrations of inhibitors present are indicated above. Reaction vessels were incubated for 10 min at 35°, and stopped by the addition of 1 ml 10 % trichloroacetic acid. Vessels were placed in a 90° water bath for 15 min. The precipitated protein, collected by centrifugation, was washed 4 times with 5 % trichloroacetic acid, 2 times with 95 % ethanol, and 2 times with ether. The precipitates, still wet with ether, were dissolved in 2 ml concentrated formic acid, and 0.5-ml aliquots plated and dried for counting on glass planchets. The incorporation of radioactivity into protein is corrected by subtracting the radioactivity incorporated in a control tube in which trichloroacetic acid was added prior to the addition of enzyme. O–O, puromycin; ×–×, chloromycetin; ●–●, HCAA.

That the inhibitory nature of HCAA was not due to its hydrolytic products is shown in Table I. Homocitrulline and 3'-amino 3'-deoxyadenosine as well as the two substances together were tested at a concentration which for the added intact molecule gave approximately half-maximal inhibition. Although some inhibition was obtained with these compounds it is obvious from the data that the addition of intact HCAA produced a better inhibition than its hydrolytic products.

TABLE I

INHIBITION OF PROTEIN SYNTHESIS IN CELL-FREE EXTRACTS OF *E. coli* BY HCAA AND ITS HYDROLYTIC PRODUCTS

These experiments were performed as described in the legend of Fig. 2 with the following exceptions: Incubation time was 15 min instead of 10 min. The concentrations of inhibitors added are indicated below.

Additions	*Counts/min/mg protein*
Complete	459
Complete + homocitrulline (10^{-4} M)	408
Complete + 3'-amino 3'-deoxyadenosine (10^{-4} M)	418
Complete + 3'-amino 3'-deoxyadenosine (10^{-4} M) + homocitrulline (10^{-4} M)	394
Complete + HCAA (10^{-4} M)	221

The synthesis of protein from the amino acids may be thought of as taking place in two major steps, the activation and transfer of the amino acids to s-RNA, and secondly, the transfer of the activated amino acid from s-RNA to the ribosomal protein, with subsequent release from the ribosome. In cell-free preparations, puromycin appears to exert its inhibitory effect on this second sequence of events[5,13,14]. In an effort to determine whether HCAA might be inhibiting at a similar place the experiments enumerated below were carried out.

The first experiment involved comparison of the radioactivity incorporated into the hot and cold trichloroacetic acid-precipitable protein. If the inhibition occurs at a site similar to that reported for puromycin in cell-free systems, then one might expect that the decrease in counts observed in the cold trichloroacetic acid-precipitable fraction would be accounted for chiefly by the decrease in counts in the hot trichloroacetic acid-precipitable fraction (which measures total protein). Results of an experiment designed to prove this point are given in Table II. As may be noted in this experiment the decrease in radioactivity in the cold trichloroacetic acid-precipitable fraction in the presence of the inhibitors can be accounted for chiefly by the decrease in the radioactivity in the hot trichloroacetic acid-precipitable fraction which measures protein. This implies that no inhibition occurred in the activation and transfer of the amino acids to s-RNA.

Another approach taken to prove this point involved a series of experiments in

TABLE II

INCORPORATION OF [^{14}C]AMINO ACIDS INTO THE COLD AND HOT TRICHLOROACETIC ACID-PRECIPITABLE FRACTIONS OF *E. coli* EXTRACTS IN THE PRESENCE OF HCAA OR PUROMYCIN

These experiments were performed as described in the legend of Fig. 2 with the following exceptions: 2.9 mg dialyzed 30 S protein was used as the enzyme source. Incubations were carried out for 15 min. For the cold trichloroacetic acid-precipitable protein, washings were carried out as described in Fig. 2 except all washings and centrifugations were done at 4°. The concentration of HCAA was $5 \cdot 10^{-4}$ M while that for puromycin was $1 \cdot 10^{-4}$ M. The values given are in counts/min/mg protein.

Additions	*Cold trichloroacetic acid*	*Hot trichloroacetic acid*
Complete	1668	484
Complete + HCAA	1504	125
Complete + puromycin	1386	30

which the precipitated protein was washed exhaustively and centrifuged in the cold, then heated for 15 min at 90°, and the radioactivity liberated in the supernatant fraction counted in the presence and absence of HCAA or puromycin. The results of such an experiment are seen in Table III. In experiments in which significant inhibition of label into protein was observed (Lower part of Table III), the incorporation of label into s-RNA was not appreciably altered.

TABLE III

INCORPORATION OF [^{14}C]AMINO ACIDS INTO S-RNA IN THE PRESENCE AND ABSENCE OF HCAA OR PUROMYCIN

These experiments were performed as described in the legend of Fig. 2 with the following exceptions: Incubations were carried out for 15 min. Supernatant fractions were obtained by stopping the reaction with cold trichloroacetic acid, washing and centrifuging the precipitated protein at 4°, then heating the precipitated washed protein with trichloroacetic acid at 90° for 15 min. Aliquots of the supernatant fraction obtained by this procedure were then plated directly for counting after three successive treatments with ether to remove the trichloroacetic acid. The hot trichloroacetic acid-precipitable protein was then washed and counted as described in Fig. 2. The concentration of HCAA and puromycin was $1 \cdot 10^{-4}$ M.

Additions	*Counts/min/mg*
Supernatant fraction (s-RNA)	RNA*
Complete	3600
Complete + HCAA	3510
Complete + puromycin	3730
Hot trichloroacetic acid-precipitable protein	Protein
Complete	346
Complete + HCAA	165
Complete + puromycin	25

* RNA was estimated by assuming 24 absorbancy units were equivalent to 1 mg of RNA.

The third experiment demonstrating that the inhibition by HCAA involved the s-RNA ribosomal system is shown in Table IV. Here, s-RNA charged with labeled leucine was used as the source of amino acids for protein synthesis thereby bypassing the systems responsible for the activation and transfer of the amino acids to s-RNA. To control the possibility that the s-RNA may be releasing free amino acids, and the inhibition observed was actually on the activating or transfer system to s-RNA, a

TABLE IV

INHIBITION OF AMINO ACID INCORPORATION FROM S-RNA INTO PROTEIN

These experiments were performed as described in the legend of Fig. 2 with the following exceptions: 1 mg s-RNA charged with [^{14}C]leucine, prepared as described in the text, was used instead of the ^{14}C-labeled Chlorella hydrolysate and tryptophan. 4 mg unwashed ribosomes prepared as described in the text were used instead of 3.4 mg dialyzed 30 S fraction. Incubation time was 15 min. In Expt. 2, 0.05 μmole each of 21 amino acids including leucine were added. The concentration of HCAA present in Expt. 3 was $5 \cdot 10^{-4}$ M. The concentration of puromycin present in Expt. 4 was $5 \cdot 10^{-4}$ M.

Expt.	*Additions*	*Total counts incorporated*
1	Complete	296
2	Complete + amino acids	240
3	Complete + HCAA	92
4	Complete + puromycin	12

vessel was used containing non-labeled leucine and the 21 other amino acids. If a significant deacylation occurred during the incubation, then the specific activity or the total counts incorporated into the protein should be greatly lowered. From the results of this experiment, it can be seen that HCAA does inhibit the overall conversion from s-RNA to ribosomal protein, and the overall deacylation during the course of the experiment is not significant.

Puromycin has been observed to have an inhibitory effect on protein synthesis in mammalian as well as bacterial systems. Chloromycetin, on the other hand, inhibits the bacterial but not the mammalian system. To see whether or not HCAA resembled puromycin in this regard several experiments were carried out with a crude rat-liver homogenate. The results of a typical experiment are shown in Table V. As noted in the bacterial system, the inhibitory effect of HCAA is not as pronounced as that observed with puromycin at the same molar concentration. Although it is not indicated in this series of experiments, chloromycetin at a concentration of $2 \cdot 10^{-4}$ M did not inhibit in this system. Therefore HCAA appears in this respect to resemble puromycin.

TABLE V

INHIBITION OF PROTEIN SYNTHESIS IN CRUDE RAT-LIVER HOMOGENATES BY HCAA AND PUROMYCIN

Incubations were carried out in a final volume of 1.0 ml. The complete system contained 0.7 ml of a crude rat-liver homogenate prepared as described in the text, 1 μmole ATP, 7.3 mg creatine phosphate, and 0.04 μC [^{14}C]leucine (specific activity 12 mC/mmole). Inhibitor additions were made as indicated below. Reaction vessels were incubated in an atmosphere of nitrogen–carbon dioxide (95 : 5) for 1 h at 37°. At the end of this period of time, the reaction was stopped by the addition of an equal volume of 10 % trichloroacetic acid. The vessels were then heated at 90° for 15 min. The subsequent treatment of the precipitated protein was the same as that indicated in the legend of Fig. 2. A control vessel was also run in which the addition of the acid preceeded that of the enzyme. All subsequent treatment of the control was the same as that for the experimental vessels. The values given below have been corrected by subtracting the counts obtained in the control vessel from the experimental vessels.

Additions	*Total counts incorporated*
Complete	1660
Complete + HCAA ($2 \cdot 10^{-3}$ M)	360
Complete + HCAA ($5 \cdot 10^{-4}$ M)	880
Complete + puromycin ($5 \cdot 10^{-4}$ M)	64

DISCUSSION

The structural similarity of HCAA to puromycin is shown in Fig. 1. Both molecules may be thought of as being derivatives of 3′-amino 3′-deoxyadenosine but differing in two major respects. Puromycin has a dimethyl substitution on the amino group of adenine while HCAA does not, and secondly, the amino acid substitutions are different on the 3′-amino position of the nucleoside. The results of this work have shown that 3′-amino 3′-deoxyadenosine is not an effective inhibitor of protein synthesis, while the work of RABINOVITZ AND FISHER[5] have demonstrated that the dimethyl substituted 3′-amino 3′-deoxyadenosine also is not an effective inhibitor of protein synthesis. Therefore it appears that a substitution on the free nucleoside is necessary for the maximal inhibitory effect. Substitution of homocitrulline on the 3′-amino group yields a compound that is about one-fiftieth as effective as puromycin. The leucine analog of puromycin which contains leucine in place of *O*-methyl tyrosine has also

been shown to be a less effective inhibitor than puromycin[5]. Therefore it would seem that the maximal inhibitory action might be due to the *O*-methyl tyrosine substitution since the leucine and homocitrulline derivatives are much less effective and the free aminonucleoside and its dimethyl substituted product do not inhibit at all.

The fact that HCAA does inhibit protein synthesis even though less effective than puromycin is still consistent with the view that compounds of this type may be acting as analogs of amino-acyl-s-RNA[2].

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the United States Public Health Service (CY-5221). One of us (R.S.) is a National Science Foundation Predoctoral Fellow 1961-1962.

REFERENCES

1 N. M. KREDICH AND A. J. GUARINO, *J. Biol. Chem.*, 236 (1961) 3300
2 M. B. YARMOLINSKY AND G. L. DE LA HABA, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 1721.
3 D. NATHANS AND F. LIPMANN, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 497.
4 V. G. ALLFREY, J. W. HOPKINS, J. H. FRENSTER AND A. E. MIRSKY, *Ann. N.Y. Acad. Sci.*, 88 (1960) 722.
5 M. RABINOVITZ AND J. M. FISHER, *J. Biol. Chem.*, 237 (1962) 477.
6 R. S. SCHWEET, J. BISHOP AND A. MORRIS, *Lab. Invest.*, 10 (1961) 992.
7 E. H. ALLEN AND R. S. SCHWEET, *J. Biol. Chem.*, 237 (1961) 760.
8 J. H. MATTHAEI AND M. W. NIRENBERG, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1580.
9 P. C. ZAMECNIK AND E. B. KELLER, *J. Biol. Chem.*, 209 (1954) 337.
10 G. VON EHRENSTEIN AND F. LIPMANN, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 941.
11 H. W. ROBINSON AND C. G. HOGDEN, *J. Biol. Chem.*, 135 (1940) 707.
12 C. M. STEVENS AND P. B. ELLMAN, *J. Biol. Chem.*, 182 (1950) 75.
13 A. J. MORRIS AND R. S. SCHWEET, *Biochim. Biophys. Acta*, 47 (1961) 415.
14 D. W. ALLEN AND P. C. ZAMECNIK, *Biochim Biophys. Acta*, 55 (1962) 865.