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20. Teruhisa Noguchi : Studies on the Biosynthesis of Nucleic Acids. II.[1] On the Rôle of Antimetabolites as an Inhibitor of Polynucleotide Biosynthesis.

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4-Aminoimidazole-5-carboxamide (AICA) has been shown by isotopic tracer techniques to be a purine precursor in a number of biological systems, such as pigeons,[2] rats,[3] mice,[4] yeast,[5] and bacteria.[6] However, these experiments were demonstrated mostly *in vivo*.

In the preceding paper,[1] the author described the incorporation of 4-aminoimidazole[4-^{14}C]-5-carboxamide (AICA-^{14}C) into polynucleotide purines *in vitro* and assumed the rôle of AICA as an important intermediate of polynucleotide biosynthesis.

The present paper will demonstrate the effect of AICA analog, 4-aminotriazole-5-carboxamide (Aza-AICA) and certain purine antimetabolites as well as folic acid antimetabolites on the incorporation of ^{32}P- and ^{14}C-labeled compounds into polynucleotides of rat or pigeon liver slices. Thus, certain aspects of their mode of action will be elucidated in detail.

The purpose of the whole study is primarily aimed at gaining some basic knowledge of the metabolic transformation involved in the biosynthesis of the nucleic acids. Secondly, little is known about the rôle of antimetabolites for these studies and it might offer some useful suggestions for studies on the antitumor agents.[7,8,9]

Chart 1. Extraction of Nucleic Acids

20 cc. of culture media + 20 cc. of cold 8% $HClO_4$(final concn. 4%)
↓ centrifugation (acid-soluble fraction was removed)
Residue + 10 cc. of cold 4% $HClO_4$ with carrier
↓ centrifugation
Residue + 10 cc. of cold 50% EtOH
↓ centrifugation
Residue + 10 cc. of cold 95% EtOH
↓ centrifugation
Residue + 10 cc. of 95% EtOH + ether (3:1), extd. 3 times in hot water
↓ centrifugation
Residue + 10 cc. of 10% NaCl, extd. 3 times at 100° for 15 mins.
↓ centrifugation
Supernatant (total vol. 30 cc.) + 120 cc. of 95% EtOH, over night at −5°, pH 8.2
↓ centrifugation
Na Nucleate

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Materials and Methods

The materials and experimental methods were identical with those used in the preceding paper,[1] except the procedure of the nucleic acid extraction used in ^{32}P-experiments.

As the adsorption rate of labeled inorganic phosphate on the surface of aliquots was not negligible in this case, the method shown in Chart 1 was adopted.

After acidification of the solution of Na nucleate with 6*N* HCl and 4% $HClO_4$, 2 vols. of 95% EtOH was added and the mixture kept over night in a refrigerator at −5°. The DNA fraction was centrifuged off and Na-RNA was obtained by precipitation on adding 2 vols. of 95% EtOH to the supernatant at pH 8.2.

The extraction of inorganic phosphate with *iso*-BuOH was carried out according to Yoshizawa's report.[10] Owing to this extraction, ca. 95% of the adsorpted inorganic-^{32}P was removed.

The radioactivity incorporated into polynucleotides was calculated from the difference of the radioactivities observed in the parallel "Zero-time" experiments.

Results

The effects of Aza-AICA on the incorporation of ^{32}P, AICA-^{14}C, and formate-^{14}C are shown in Tables I～III.

Table I. Effect of Aza-AICA[a] upon ^{32}P Incorporation into Ribonucleotide by Rat Liver Slices

Substrate	Mol. concn.	S. A.[b]	R. S. A.[c]	I. R. (%)[d]
Aza-AICA	10^{-3}	5.05	0.405	21.4
	10^{-4}	13.3	1.06	55.8
	10^{-5}	23.8	1.89	100.0
None (Control)	—	23.8	1.90	100.0

a) As Aza-AICA hydrochloride

b) Specific activity (S. A.) : cpm/γ P

c) Relative specific activity (R. S. A.)(%)

$$= \frac{\text{S. A. of isolated ribonucleic acid phosphorus}}{\text{S. A. of inorganic phosphorus in medium}} \times 100$$

d) Incorporation Ratio (I. R.)(%) $= \frac{\text{R. S. A. of Tests}}{\text{R. S. A. of Control}} \times 100$

Table II. Effect of AICA and Aza-AICA upon Formate-^{14}C[a] Incorporation into Ribonucleotide Purines by Pigeon Liver Slices

Substrate	Specific activity[b]		Rel. spec. activity[c]		
	Adenine	Guanine	Adenine	Guanine	Ad./Gu.
Aza-AICA 10^{-3}	354	236	0.253	0.168	1.5
AICA 10^{-3} + Aza-AICA 10^{-3}	652	390	0.465	0.278	1.7
None (Control)	468	526	0.334	0.376	0.9

a) S. A. of formate-^{14}C in medium : 140,000 cpm/μM

b) Specific activity : cpm/μM of isolated purines.

c) Relative specific activity (%)

$$= \frac{\text{Specific activity of purine isolated from polynucleotide}}{\text{Specific activity of formate in medium}} \times 100$$

Table III. Effect of Aza-AICA upon AICA-^{14}C Incorporation into Ribonucleotide Purines by Rat Liver Slices

Substrate	Mol. concn. (mM/15 cc.)	Specific activity		Rel. Spec. activity		
		Adenine	Guanine	Adenine	Guanine	Ad./Gu.
Aza-AICA	0.06	73	14	0.50	0.10	5.0
None (Control)	—	176	69	1.21	0.47	2.6

The incorporation of ^{32}P into RNA by rat liver slices was inhibited by the effect of Aza-AICA (Table I). This inhibitory effect was also observed in the case of the incorporation of AICA-^{14}C into polynucleotide purines (Table III) as well as in the case of the incorporation of formate-^{14}C (Table II).

These data indicate that Aza-AICA is a competitive antimetabolite of AICA which is important intermediate of polynucleotide purine biosynthesis.

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The inhibitory effect of 6-mercaptopurine, 2,6-diaminopurine, or 8-azaguanine on the incorporation of ^{32}P into RNA by rat liver slices is demonstrated in Table IV. The effect was generally lower than that of Aza-AICA.

Incorporation of AICA-^{14}C or formate-^{14}C into polynucleotide purine was inhibited by folic acid atimetabolites (aminopterin) in a concentration of 100 γ/15 cc. of the incubation medium. The results are shown in Tables V and VI.

Table IV. Effect of Antipurines upon ^{32}P-Incorporation into Ribonucleotide of Rat Liver Slices

Substrate	Mol. concn.	S. A.	R. S. A.	I. R. (%)
6-Mercaptopurine	10^{-3}	60.0	1.69	80
	10^{-4}	61.0	1.72	82
2,6-Diaminopurine	10^{-3}	26.8	0.75	36
	10^{-4}	49.2	1.38	66
8-Azaguanine Na	10^{-3}	37.5	1.05	50
	10^{-4}	64.5	1.80	86
None (Control)	—	75.0	2.10	100

Table V. Effect of Aminopterin upon Formate-^{14}C Incorporation into Ribonucleotide Purines by Pigeon Liver Slices

Substrate	Concn. (γ/cc.)	Specific Activity		Rel. spec. activity		
		Adenine	Guanine	Adenine	Guanine	Ad./Gu.
Aminopterin	100	301	273	0.215	0.195	1.1
None (Control)	—	468	526	0.334	0.376	0.9

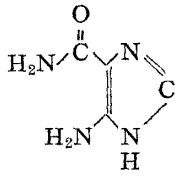
Table VI. Effect of Aminopterin upon AICA-^{14}C Incorporation into Ribonucleotide Purines by Rat Liver Slices

Substrate	Concn. (γ/cc.)	Specific activity		Rel. spec. activity		
		Adenine	Guanine	Adenine	Guanine	Ad./Gu.
Aminopterin	100	91	17	0.62	0.11	5.6
None (Control)	—	176	69	1.21	0.47	2.6

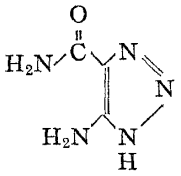
Discussion

In the preceding paper,[1] the author assumed that AICA is incorporated into polynucleotides as an "active" AICA riboside and that the phosphate portion will be supplied from other sources of phosphates.

The antagonistic effect of Aza-AICA for the incorporation of AICA-^{14}C (Table II) will serve to explain the above hypothesis.



4-Aminoimidazole-5-carboxamide (AICA)



4-Aminotriazole-5-carboxamide (Aza-AICA)

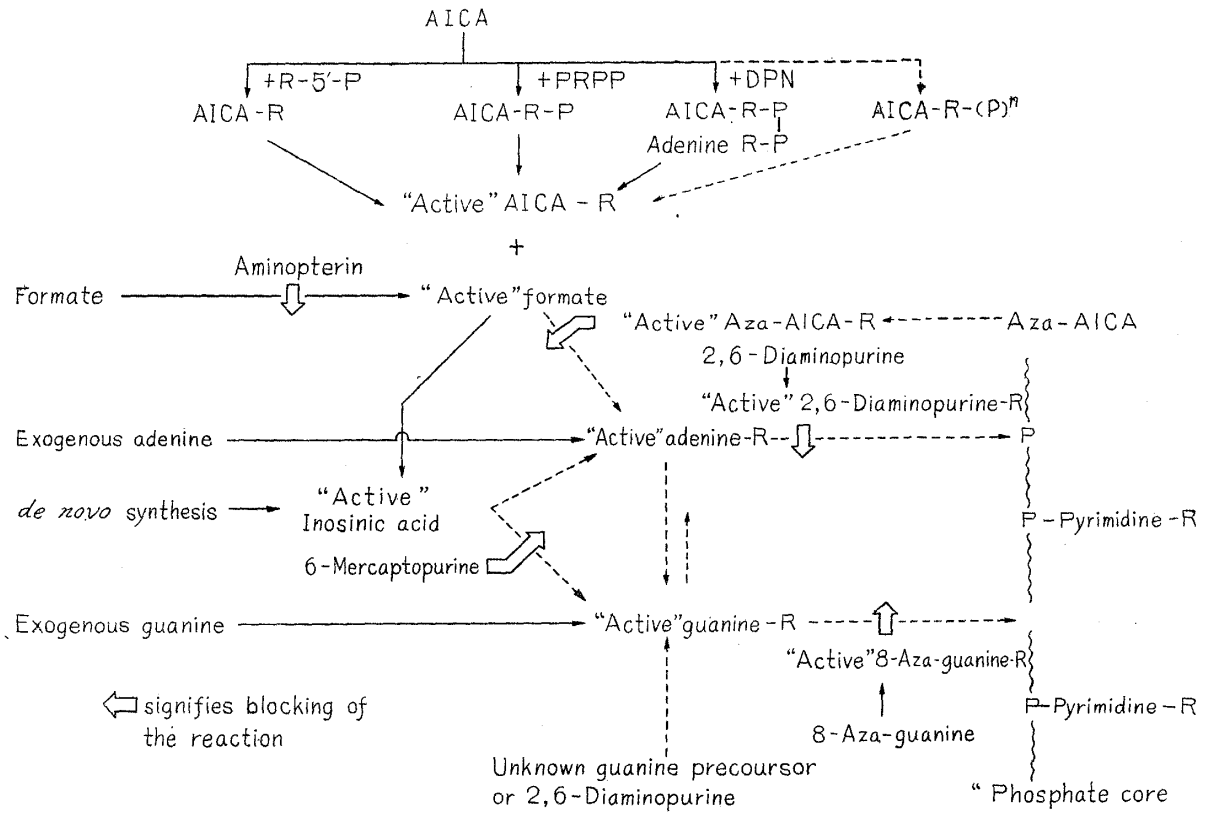
Aza-AICA might compose an "active" Aza-AICA riboside which, in turn, will antagonize "active" AICA riboside. This abnormal riboside would not be incorporated as "active" AICA riboside would be. Subsequently, the incorporation of ^{32}P into RNA was inhibited by Aza-AICA at the concentration of $10^{-3}\sim10^{-4}$ *M* (Table I).

The antagonism between AICA and Aza-AICA is clearly observed in Table II. If AICA exists in the medium at the same concentration as Aza-AICA, "active" AICA riboside would be incorporated into polynucleotide purines to some extent (cf. Table III) after fixation of the labeled formate at the C_2-position, whereas, if Aza-AICA alone exists in the medium, the incorporation of AICA was inhibited and accordingly the incorporation of formate-^{14}C would also be suppressed.

The data concerning purine antimetabolites will also support this view. In acid-

soluble fraction of the medium, the abnormal nucleosides (e.g. 8-azaguanine riboside[11] and 2,6-diaminopurine riboside[12]) were detected by paper chromatography. These abnormal nucleosides would be incorporated to some extent into polynucleotides. However, the normal formation of "phosphate core" in the course of RNA biosynthesis would be impaired by these abnormal nucleosides[8] (cf. Chart 2).

Chart 2. Postulated Scheme of the Mode of Action of Antimetabolites on the Biosynthesis of Polynucleotide Purines



The inhibitory effect of aminopterin on the incorporation of AICA-^{14}C as well as formate-^{14}C will indicate evidently that the purine ring will be formed before the carboxamide enters into the polynucleotide fraction. Thus, it could be assumed that "active" AICA riboside is an intermediate of polynucleotide biosynthesis.

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Summary

The effect of 4-aminotriazole-5-carboxamide and certain purine antimetabolites (e.g. 8-azaguanine, 6-mercaptopurine, and 2,6-diaminopurine) as well as folic acid antimetabolites (e.g. aminopterin) on the incorporation of labeled 4-aminoimidazole[4-^{14}C]-5-carboxamide, formate-^{14}C, and ^{32}P into polynucleotides of rat or pigeon liver slices was demonstrated and certain aspects of their mode of action were discussed.

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