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19. Teruhisa Noguchi : Studies on the Biosynthesis of Nucleic Acids. I. On the Rôle of 4-Aminoimidazole-5-carboxamide as a Precursor of Polynucleotide Purines.

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A new diazotizable arylamine was found to accumulate in the culture medium of *Escherichia coli* in the presence of a bacteriostatic amount of sulfanilamides.[1] The arylamine was isolated but its structure was not elucidated. Subsequently, Shive *et al.*[2] identified this amine as 4-aminoimidazole-5-carboxamide (AICA) which seemed to be an important intermediate precursor in the biosynthesis of nucleic acid purines. Recently, Buchanan, Greenberg, and their co-workers[3~5] concluded that AICA riboside or ribotide is one of the important intermediates in the *de novo* synthesis of inosinic acid from glycine, ribose-5'-phosphate, and other small-molecule precursors. Each enzymatic step has now been almost elucidated.[6]

Meanwhile, the enzyme systems which are involved in the course of polynucleotide biosynthesis from AICA still remain unknown.

The purpose of this study was to find out whether 4-aminoimidazole[4-^{14}C]-5-carboxamide(AICA-^{14}C) would be incorporated into the polynucleotide purines in cell-free enzyme systems. If the incorporation could be successfully observed, this will make it possible to isolate enzymes involved in polynucleotide biosynthesis.

Present study will thereby contribute to the elucidation of the enzymes as well as the metabolic intermediates which are related to the mode of action of antimetabolites, such as 8-azaguanine, 6-mercaptopurine, and 2,6-diaminopurine.[7]

Materials and Methods

As enzyme systems, rat or pigeon liver slices and homogenate were used. The animals were sacrificed by decapitation. The livers were removed quickly, chilled on cracked ice, excised with scissors, washed once briefly with incubation medium, and sliced with a tissue slicer. To make a 20% homogenate, 10 g. of the liver was homogenized in 50 cc. of 0.34*M* sucrose for 2 mins. with Teflon pestle. About 2 g. of the slices or 10 cc. of the homogenate was transferred to an incubation vessel (50 cc. Erlenmyer flask), containing the labeled compound and the substrate shown in Table I. The incubations were carried out at 37° for 100 mins.

The synthesis of the AICA-^{14}C used will be described in the following paper.[8] The specific activity of the AICA-^{14}C was 14,500 cpm./micromole.**

The extraction of nucleic acids was carried out by the procedure shown in Chart 1, except the ^{32}P experiments. The detail of this procedure will be described in the next paper.[7]

To separate deoxyribose nucleic acid (DNA) from ribose nucleic acid (RNA), sodium nucleate was digested at 37° for 18 hrs. with 2 cc. of 1*N* NaOH. The deoxypolynucleotides were isolated from ribomononucleotides by acidification of the solution with 6*N* HCl, (0.2 vol.) and 4% $HClO_4$ (1 vol.), followed by the addition of 2 vols. of EtOH. DNA was precipitated by centrifugation,

* Hongo, Tokyo (野口照久).

** Counts per minute per micromole.

1) M. R. Stetten, C. L. Fox : J. Biol. Chem., **161**, 333(1945).
2) W. Shive, W. W. Ackermann, M. Gordon, M. E. Getzendaner, R. E. Eakin : *Ibid.*, **169**, 725 (1947). 3) M. P. Schulman, J. M. Buchanan : *Ibid.*, **196**, 513(1952); **202**, 241(1953).
4) B. Levenberg, S. C. Hartman, J. M. Buchanan : Federation Proc., **14**, 243(1955).
5) G. R. Greenberg : *Ibid.*, **12**, 211, 651(1953).
6) Personal communication from Dr. J. M. Buchanan to Dr. Y. Miura(1955).
7) T. Noguchi : This Bulletin, **4**, 97(1956). 8) T. Noguchi : *Ibid.*, **4**, 130(1956).

Table I. Composition of the Incubation Media

a) Incubation medium for the slices :

Krebs-Ringer phosphate (pH 7.4) 15 cc. with	K-pyruvate	0.02 *M*
	Glucose	0.02 *M*

When AICA-^{14}C was used as the labeled compound, the solution F was added.

Solution F :	AICA-^{14}C	0.06 *mM*
	Ribose-5′-phosphate	0.06 *mM*
	Na Formate	0.06 *mM*
	Ca leucovorin	50 γ

When formate-^{14}C was used, the following solution was added.

	Formate-^{14}C 10 μc. in	0.06 *mM*
	Ribose-5′-phosphate	0.06 *mM*
	Ca leucovorin	50 γ

In the case ^{32}P was used as the tracer element, 50 μc. of $H_3{}^{32}PO_4$ was added to the medium.

b) Incubation medium for the homogenate :

	KCl	0.07 *M*
	$MgCl_2$	0.0033 *M*
	Cytochrome c	0.00002 *M*
	K-Phosphate buffer (pH 7.4)	0.007 *M*
	K-ATP	0.001 *M*
	α-Ketoglutarate	0.003 *M*
	Solution F	Total 15 cc.

the supernatant liquid containing RNA was adjusted to pH 8.2 with NaOH and 2 vols. of EtOH was added to precipitate RNA. This procedure was repeated twice.

After hydrolysis with 1*N* HCl at 100° for 1 hr., adenine and guanine were obtained by the descending paper chromatography of Wyatt.[9] The spots were detected by ultraviolet lamp and adenine was eluted with 0.1*N* HCl, guanine with 1*N* HCl. The quantities of adenine and guanine were determined by Beckman spectrophotometer at 262.5 mμ for adenine and at 249 mμ for guanine.

Chart 1. Procedure of Nucleic Acid Extraction

15 cc. of culture media + cold conc. $HClO_4$ with final concn. of 4%
↓ centrifugation
Residue + 25 cc. of cold 4% $HClO_4$, washed with carrier
↓ centrifugation
Residue + 10 cc. of cold 3% $HClO_4$, washed twice
↓ centrifugation
Residue + 10 cc. of cold 2% $HClO_4$, washed twice
↓ centrifugation
Residue + 10 cc. of cold 1% $HClO_4$, washed once
↓ centrifugation
Residue + 10 cc. of cold 1% $HClO_4$ + 95% EtOH (1:1)
↓ centrifugation
Residue + 10 cc. of cold 95% EtOH
↓ centrifugation
Residue + 10 cc. of cold 95% EtOH + ether (3:1) extd. 3 times in hot water
↓ centrifugation
Residue + 30 cc. of 10% NaCl, extd. 3 times with boiling in for 15 mins.
↓ centrifugation
Supernatatant + 4 vols. of 95% EtOH, over night at −5°
↓ centrifugation
Sodium nucleate

9) G. R. Wyatt : Biochem. J. (London), **48**, 584(1951).

The radioactivities were determined at the infinite thinness level in a Q-gas flow counter. The contaminated radioactivity by adsorption was checked by "Zero-time" incubation experiments in which no radioactivity was observed in polynucleotide purines.

Results

As shown in Table II, AICA-^{14}C was incorporated into adenine and guanine of RNA by rat liver slices. The specific activites were about 2 times higher in adenine than in guanine. In the case of regenerating liver, generally higher specific activities were observed than in the case of normal liver.

Table II. Incorporation of AICA-^{14}C into RNA-Purines by Rat Liver Slice

	Specific activity[a]		Rel. spec. activity[b]		
	Adenine	Guanine	Adenine	Guanine	Ad./Gu.
Normal liver	102	54	0.71	0.32	2.2
Regenerating liver	176	69	1.21	0.47	2.6

a) Specific activity : Counts per min./μM of isolated purines.

b) Relative specific activity (%)

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

Encouraged by these data, attempts were made on the same experiments with rat liver homogenate, with the fortified medium as presented in Table I (b). The results are shown in Table III.

Table III. Incorporation of the AICA-^{14}C into RNA-Purines by Rat Liver Homogenate

	Specific activity		Rel. spec. activity		
	Adenine	Guanine	Adenine	Guanine	Ad./Gu.
Normal liver	70	14	0.48	0.10	4.8
Regenerating liver	145	26	1.00	0.18	5.6

AICA-^{14}C was incorporated into RNA-adenine and -guanine by the cell-free system. The specific activity of adenine in the homogenate was about 70~80% of those in the slices, whereas that of guanine was generally lower (20~30%) in the homogenate than in the slices. The ratio of the relative specific activities of adenine to guanine were about 2.2 (normal liver) and 2.6 (regenerating liver) in the slices and about 4.8 (normal liver) and 5.6 (regenerating liver) in the homogenates. This fact will indicate that, in the cell-free system, the interconversion of AICA into guanine is more difficult than in the slices.

The evidence that the enzyme system even in homogenates are very active in the regenerating liver will indicate that this will be a good source for isolating the polynucleotide-forming enzymes.

Discussion

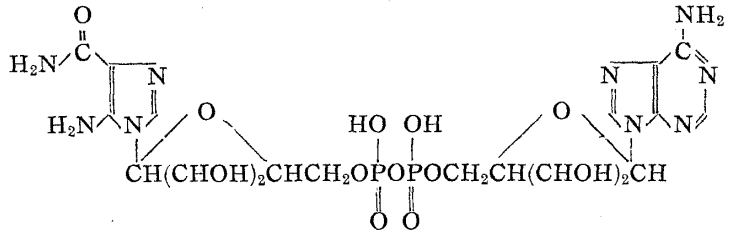
The most significant recent development in the field of polynucleotide biosynthesis may be the isolation of various ribonucleoside-5'-phosphates. These compounds may occupy a key position in the present concepts of polynucleotide synthesis. The occurrence of AICA riboside and ribotide has been confirmed by Gots[10] and Greenberg.[11] Involvement of thymidine in the utilization of this base is suggested.[12] The mechanism of AICA ribotide formation from AICA has been clarified by Kornberg and his co-workers.[13] With a purified enzyme from pigeon liver, it was shown that 5'-phosphoribosylpyrophosphate (PRPP) is formed from ribose-5'-phosphate (R-5'-P) and ATP. AICA condenses with PRPP to form AICA ribotide.

$$\text{ATP} + \text{R-5'-P} \rightleftharpoons \text{PRPP} + \text{AMP}$$
$$\text{PRPP} + \text{AICA} \rightleftharpoons \text{AICA ribotide} + \text{PP}$$

10) J. S. Gots : Nature, **172**, 256(1953).
11) G. R. Greenberg : Federation Proc., **13**, 745(1954).
12) J. M. Wever, W. Shive : J. Am. Chem. Soc., **75**, 4628(1953).
13) A. Kornberg, I. Lieberman, E. S. Simms : *Ibid.*, **76**, 2027, 2844(1954).

In addition, Woolley[14] reported the formation of a new AICA dinucleotide compound from cozymase (DPN) and AICA :

AICA + DPN ⟶ Nicotinamide + H^+ +



Although the exact rôle of this compound still remains unknown, the new compound may also be some "active" intermediate of polynuleotide synthesis. Guanosine-5'-triphosphate and uridine-5'-triphoshate were obtained from rabbit muscles.[15] All of the ribonucleoside triphoshates were found in tumor and other tissues.[16,17]

The occurrence of deoxyadenosine diphosphate and triphosphate in muscles and kidneys has been suggested.[18] The studies of Kalckar and his co-workers[19] have revealed important information about the function and biogenesis of ribonucleotide triphosphates.[20] The following enzymic reactions were observed :

Uridine triphosphate + ADP ⇌ Uridine diphosphate + ATP

Such a phosphate shift may be of general importance as indicated by the discovery of the reaction:[21]

ATP + inosine diphosphate ⇌ ADP + inosine triphosphate

More recently, Roll *et al.*[22] studied the incorporation of the phosphorus of ^{32}P-labeled purine nucleotide into RNA of rat *in vivo*; Heidelberger *et al.*[23] carried out similar experiment with rat liver slices; they claimed that no significant incorporation of the intact nucleotides occurred and an assumption was made that the nucleotides were dephosphorylated by these systems and that the observed uptake of ^{32}P into nucleic acids was due to the incorporation of the inorganic or other organic phosphate resulting from the nucleotide breakdown.

These data concerning the biosynthesis of polynucleotides will serve for the interpretation of the author's present results as well as other experimental data collected in the preliminary experiment.

In the preliminary experiments, the author observed some stimulatory effect of non-labeled AICA on the incorporation of formate-^{14}C or ^{32}P into the polynucleotides as shown in Tables IV and V.

By the coexistence of 10^{-3} *M* of AICA, the relative incorporation of formate-^{14}C was greater into adenine than into guanine of the polynucleotides, while the reverse was true if the AICA was absent in the medium (*de novo* synthesis : Control). When AICA was present, ^{32}P was incorporated more than 250% of the control. It would seem that AICA might play some rôle as a part of coenzyme-like substances. However, the new data obtained in this experiment demonstrated clearly the rôle

14) D. W. Woolley : *Ibid.*, **77**, 1065(1955).
15) R. Bergkvist, A. Deutsch : Acta Chem. Scand., **7**, 1307(1953).
16) R. B. Hurlbert, H. Schmitz, H. F. Brumm, V. P. Potter : J. Biol. Chem., **209**, 23(1954).
17) L. Hecht, V. R. Potter, E. Herbert : Biochim. et Biophys. Acta **13**, 132(1954).
18) H. Z. Salle, P. B. Wilber, A. E. Cohen, M. R. Kane : *Ibid.*, **13**, 156(1954).
19) H. M. Kalckar : Science, **119**, 479(1954).
20) I. Lieberman, A. Kornberg, E. S. Simms : J. Am. Chem. Soc., **76**, 3608(1954).
21) P. Berg, W. K. Joklik : J. Biol. Chem., **210**, 657(1954).
22) P. M. Roll, H. Weinfeld, G. B. Brown : Biochim. et Biophys. Acta, **13**, 141(1954).
23) K. C. Leibman, C. Heidelberger : J. Biol. Chem., **216**, 823(1955).

Table IV. Effect of AICA on Formate-^{14}C[a] Incorporation into Polynucleotide Purines by Pigeon Liver Slices

Substrate		Specific activity[b]		Rel. spec. activity[c]		
		Adenine	Guanine	Adenine	Guanine	Ad./Gu.
None(Control)	RNA	468	526	0.334	0.376	0.9
	DNA	189	240	0.135	0.172	0.8
AICA $10^{-3}M$	RNA	701	452	0.500	0.322	1.6
	DNA	226	132	0.161	0.094	1.7

a) Specific activity (S. A.) of formate-^{14}C in medium : 140,000 cpm./μM.
b) Specific activity : cpm./μM of isolate purines.
c) Relative specific activity (%)

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

Table V. Effect of AICA on ^{32}P-Incorporation into RNA by Rat Liver Slices

Substrate	Mol. concn.	S. A.[a]	R. S. A.[b]	I. R.(%)[c]
None (Control)	—	23.8	1.90	100
AICA	10^{-3}	60.7	4.85	255
	10^{-4}	55.8	4.35	229
	10^{-5}	23.9	1.90	100

a) Specific activity (S. A.) : cpm./γ P.
b) Relative specific activity (R. S. A.)(%)

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

c) Incorporation ratio (I. R.)(%) $= \frac{\text{R. S. A. of test}}{\text{R. S. A. of control}} \times 100$

of AICA as the true intermediate of polynucleotide biosynthesis and as the precursor of polynucleotide adenine moiety rather than the precursor of polynucleotide guanine moiety.

The author conjectured, therefore, that AICA may first compose a compound such as AICA-ribose-P-P-adenosine or "active" AICA riboside, then the purine ring will be formed, and change into "active" purine ribosides; finally they will be incorporated into polynucleotides.

The existence of non-labeled AICA in the medium would stimulate the formation of the "active" AICA riboside, thus it would contribute in stimulating the incorporation of ^{32}P or formate-^{14}C into polynucleotide.

The question of whether or not the AICA *per se* is an intermediate in polynucleotide synthesis has long been unsolved. The author believes that the present experiment makes an important contribution towards solving this problem.

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Summary

By employing 4-aminoimidazole[4-^{14}C]-5-carboxamide, formate-^{14}C, and ^{32}P, the rôle of the carboxamide as a precursor of polynucleotide purines was examined.

The *in vitro* incorporation of the labeled carboxamide into polynucleotide adenine and guanine was found by rat liver slices and by cell-free systems. The specific activity was about 2~5 times higher in adenine than in guanine. In the case of regenerating liver, generally higher specific activity was observed than in the case of normal liver.

The stimulatory effect of the non-labeled carboxamide in the course of the incorporation of formate-^{14}C and ^{32}P into polynucleotides was demonstrated.

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