Preparation of 5-Amino-1-β-D-ribosyl-4-imidazolecarboxamide-5′-phosphate and *N*-(5-Amino-1-β-D-ribosyl-4-imidazolecarbonyl)-L-aspartic Acid 5′-Phosphate*

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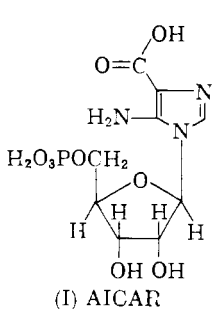
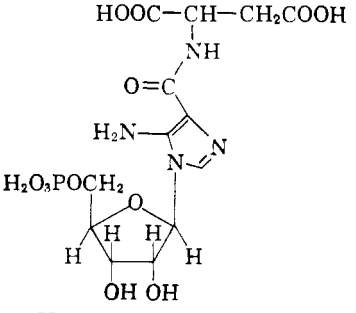
ABSTRACT: 5-Amino-1-β-D-ribosyl-4-imidazolecarboxamide-5′-phosphate (AICAR) and *N*-(5-amino-1-β-D-ribosyl-4-imidazolecarbonyl)-L-aspartic acid 5′-phosphate (succino-AICAR) have been prepared by the appropriate incubation of 5-amino-4-imidazolecarboxamide riboside (AICA riboside) with actively fermenting yeast.

Analytically pure samples of AICAR and succino-AICAR were isolated, for the first time, in the form of their respective crystalline free acids.

It is now well established that 5-amino-1-β-D-ribosyl-4-imidazolecarboxamide-5′-phosphate (AICAR: I)[1] and *N*-(5-amino-1-β-D-ribosyl-4-imidazolecarbonyl)-L-aspartic acid 5′-phosphate (succino-AICAR: II) are intermediates in the biosynthesis of purine nucleotides (Hartman and Buchanan, 1959). AICAR has been prepared enzymatically from 5-amino-4-β-D-imidazolecarboxamide riboside (AICA-riboside) and ATP (Greenberg, 1956) and from 5-amino-4-imidazolecarboxamide (AICA) and 5-phosphoribosylpyrophosphate (Flaks



(I) AICAR



(II) Succino-AICAR

et al., 1957; Lukens and Flaks, 1963). It has also been obtained by chemical degradation of a derivative of inosinic acid (Shaw, 1961).

Succino-AICAR has been prepared enzymatically from 5-aminoimidazole ribotide, aspartic acid, ATP, and carbon dioxide (Lukens and Buchanan, 1959). It was more conveniently prepared by incubation of AICAR with excess fumarate in the presence of adenylosuccinase (Miller *et al.*, 1959). Succino-AICAR was isolated from the culture broth of the adenine-requiring mutant of *Escherichia coli*, strain B-97 (Gots and Gollub, 1957). The total synthesis of succino-AICAR has recently been reported by Shaw and Wilson (1963).

Because of extensive technical difficulties, none of the procedures described above has afforded crystalline or analytically pure samples of AICAR or succino-AICAR. We became interested in this problem when we found that a hypoxanthine-requiring mutant of *Arthrobacter albides*, developed and isolated by John R. DeZeeuw of these laboratories, accumulated substantial quantities (about 0.5 g/liter) of AICA riboside when grown in submerged culture. The product was easily recovered from broth by absorption on IR-120 (H^+), elution with dilute ammonium hydroxide, and removal of amphoteric substances by treatment with Dowex 1 ($formate^-$). It crystallized readily from aqueous methanol and possessed all the properties expected of AICA riboside as described in the literature (Greenberg and Spilman, 1956).

This level of accumulation of AICA riboside is much higher than those previously observed with *E. coli* (Gots, 1953; Greenberg and Spilman, 1956) and approaches that reported by Shiro *et al.* (1962) for a mutant of *Bacillus subtilis*.

Ostern and Terszakowec (1937) and Ostern *et al.* (1938a,b) have reported that when adenosine and inorganic phosphate were incubated in the presence of actively fermenting, mildly autolyzed brewers' yeast, adenosine-5′-monophosphate (AMP) and adenosine-5′-triphosphate (ATP) were formed in good yield. Guanosine and D-ribose were not phosphorylated. The biochemical basis for this process is readily understood from a consideration of the known facts of the enzymology of yeast. In the fermentation of glucose to ethanol, as summarized in equation (1), it is clear that

$$C_6H_{12}O_6 + 2\,H_3PO_4 + 2\,ADP \rightarrow 2\,C_2H_5OH + 2\,CO_2 + 2\,ATP + 2\,H_2O \quad (1)$$

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[1] Abbreviations used in this work: AICAR, 5-amino-β-D-ribosyl-4-imidazolecarboxamide-5′-phosphate; AICA, 5-amino-4-imidazolecarboxamide.

for each mole of glucose metabolized 2 moles of adenosine diphosphate (ADP) are phosphorylated to ATP. Actively fermenting yeast thus provided a convenient and efficient system for the regeneration of ATP. This source of ATP was utilized to phosphorylate adenosine (equation 2), a reaction presumably catalyzed by the adenosine phosphokinase known to be present (Caputto,

$$\text{adenosine} + \text{ATP} \rightarrow \text{AMP} + \text{ADP} \qquad (2)$$

1951). Mild autolysis of yeast cells was required to alter the permeability of the cell membrane, so that the adenosine phosphates formed intracellularly could be excreted into the medium.

By analogy, it appeared possible that the ATP so produced could also be utilized to phosphorylate AICA riboside, as shown in equation (3). The occurrence in yeast of the appropriate enzyme, AICA-riboside phos-

$$\text{AICA riboside} + \text{ATP} \rightarrow \text{AICAR} + \text{ADP} \qquad (3)$$

phokinase, had already been suggested by the work of Greenberg (1956). We found, indeed, that when AICA riboside was treated in a similar manner it was almost completely converted to a more polar substance whose behavior upon paper chromatography was consistent with that reported for AICAR. With the AICA riboside now available in gram quantities it became a simple matter to carry out this reaction on a preparative scale. AICAR was recovered by absorption on Dowex 1 ($formate^-$) and elution with 2 N formic acid. Clusters of fine needles crystallized out of the solution when the most concentrated fractions were cooled in the refrigerator. Elemental analysis indicated that the crystalline product is the monohydrate of AICAR, free acid. Its identity was confirmed (1) by comparison of the ultraviolet spectra at *p*H 1 and *p*H 7 with literature data; (2) by a positive orcinol reaction and a positive periodate reaction, indicating that the 2′- and 3′-hydroxy groups on the ribose are free; and (3) by its conversion, when it was incubated with excess fumarate in the presence of autolyzed yeast, to a more polar, diazotizable amine with the properties of succino-AICAR. The product is, therefore, active as a substrate for the enzyme adenylosuccinase (Miller *et al.*, 1959) which catalyzes the reversible cleavage of succino-AICAR to AICAR (reaction 4).

$$\text{succino-AICAR} \rightleftharpoons \text{AICAR} + \text{fumarate} \qquad (4)$$

The last observation immediately suggested a simple procedure for the preparation of succino-AICAR from AICA-riboside. AICA-riboside was phosphorylated with yeast in the usual manner. When the conversion of AICA-riboside to AICAR was almost complete (in about 5 hours) excess fumarate was added to the reaction mixture and the *p*H was adjusted to 7.0. Upon further incubation the AICAR produced was converted in excellent yield to succino-AICAR. The product was isolated by adsorption on activated carbon, elution with dilute ammonia, precipitation of the material as the barium salt, liberation of the free acid, and finally crystallization of the free acid from aqueous acetone. The crystalline free acid so prepared was found to contain 0.5 mole of acetone of crystallization. When a sample was treated with 2,4-dinitrophenylhydrazine, the expected amount of acetone 2,4-dinitrophenylhydrazone was formed.

This material was identified as succino-AICAR by elemental analysis, paper chromatographic properties, ultraviolet spectra, and by the instability of its diazonium salt in the Bratton-Marshall test for aromatic amines.

The ultraviolet spectra of pure AICAR and succino-AICAR obtained in this work are compared with those reported in the literature in Table I. It is seen that for AICAR our results are in good agreement with those reported earlier. On the other hand, our values of molar extinction coefficients for succino-AICAR are significantly (5–10%) higher than the literature data.

Recently, Kuninaka (1960) reported that a number of purine-5′-ribonucleotides, namely, inosinic, xanthylic, and guanylic acids, are extremely active flavor enhancers or seasoning agents. Accordingly, appropriate taste tests were carried out with AICAR and succino-AICAR. Both compounds were found to possess interesting flavor-enhancing activity. Detailed results of this work will be reported elsewhere.

Experimental

Methods. Paper chromatography was carried out on Whatman No. 4 paper at room temperature. The systems which were most useful in this work and R_F values of relevant compounds are summarized in Table II. When following the course of phosphorylation experiments, a 1-μl aliquot of the reaction mixture was applied on a sheet of paper 13 × 20 cm and the paper was developed on system B for 35 minutes. Compounds were detected by ultraviolet absorption (Katz, 1962) or by spraying with Bratton-Marshall reagents (Greenberg, 1956).

Thin-layer chromatography on silica gel was run on 15 × 20–cm plates prepared by the procedure of Lees and DeMuria (1962). The R_F values for the 2,4-dinitrophenylhydrazone of acetone were: 0.6 in benzene–ether (3:1 by volume); 0.6 in ethyl acetate–hexane (85:15 by volume); and 0.5 in benzene–ethyl acetate (9:1 by volume).

Diazotizable amines were determined by a modified Bratton and Marshall (1939) procedure. Ribose was estimated by the orcinol method (Mejbaum, 1939), and inorganic phosphate by the method of Fiske and Subbarow (1925).

Samples of AICA and AICA-riboside were purchased from the California Corp. for Biochemical Research.

Solvents for measurement of ultraviolet spectra were as follows: *p*H 1.0, 0.1 N HCl; *p*H 7.0, 0.01 M potassium phosphate buffer; *p*H 8.0, 0.025 M Tris-HCl buffer.

Production of AICA-Riboside. Inoculum for the fermentor stage was grown in a Fernbach flask containing

TABLE I: Absorption Spectra of AICAR and Succino-AICAR.

Compound	*p*H	$\lambda_{max(1)}$	E_{max}	$\lambda_{max(2)}$	E_{max}	OD Ratio 250/260
AICAR[a]	1.0	267	11,000	245	9400	0.90
	7.0	268	12,900			0.75
[b]	7.0	268	12,800			0.76
[c]	1.0	268	9,400			0.87
	7.0	269	12,600			0.78
Succino-AICAR[a]	1.0	270	12,350	245	9610	0.87
	7.0	268	14,800			0.81
	8.0	269	14,500			0.74
[d]	1.0	269	11,300	244		0.99
	7.8	269	13,800			0.7
[e]	1.0	269–270	11,000	243		0.91
	8.0	269–270	13,300			0.75

[a] Samples described in this paper. E_{max} calculated on the basis of AICAR·H_2O, mw 356.24, and succino-AICAR·$^1/_2(C_3H_6O)$, mw 483.33. [b] Greenberg (1956). [c] Flaks *et al.* (1957). [d] Shaw and Wilson (1963). [e] Miller *et al.* (1959).

1 liter of medium with the following composition: Bacto peptone, 30.0 g; Bacto meat extract, 3.0 g; Bacto yeast extract, 5.0 g; glucose, 5.0 g; hypoxanthine, 50.0 mg in 1 liter of tap water sterilized at 120° for 15 minutes. After inoculation from a slant of *Arthrobacter albides* mutant 19729, the flask was incubated on a rotary shaker at 28° for 18 hours.

Large-scale growth of culture was carried out in 4-liter glass fermentors (Shull and Kita, 1955) with 2 liters of medium containing, per liter: ammonium chloride, 5.0 g; dipotassium hydrogen phosphate, 1.0 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $FeCl_3 \cdot 6\ H_2O$, 0.01 g; $MnCl_2 \cdot H_2O$, 0.01 g; biotin, 30.0 μg; urea, 8.0 g; hypoxanthine, 80.0 mg; glucose, 50.0 g; autoclaved at 120° for 30 minutes. A drop of Dow-Corning Antifoam was added to each fermentor before sterilization. After sterilization 20 g of calcium carbonate, suspended in water (200 ml) and autoclaved separately, was added to each fermentor. After inoculation with 100 ml inoculum the contents of each fermentor was stirred at 1750 rpm, aerated at a rate of 1 volume air per volume liquid per minute, and incubated at 28° for 72 hours. The broth was estimated by paper chromatography to contain approximately 0.5 g/liter of AICA-riboside plus a trace of AICA.

TABLE II: Paper Chromatography of AICA Derivatives.

Compound	R_F in System[a] A	B	C
AICA	0.95	0.65	0.50
AICA-riboside	0.85	0.65	0.40
AICAR	0.50	0.15	0.0
Succino-AICAR	0.25	0.15	0.15
Adenosine	0.89	0.65	0.45
AMP	0.62	0.15	0.15

[a] Descending systems: A, isobutyric acid–concd NH_4OH–H_2O, 66:1:33; B, methyl ethyl ketone–acetic acid–H_2O, 9:2.5:3; C, methyl ethyl ketone–1-butanol–concd NH_4OH–H_2O, 4:4:1:1.

Isolation of AICA-Riboside. The combined broth from ten fermentors was filtered, brought to *p*H 2 with concd HCl, and passed through a column (8 × 32 cm) containing 1600 ml IR-120 (H^+) at a rate of 1 liter every 20 minutes. The column was washed with 2 liters of deionized water and eluted with 2 N ammonium hydroxide. Fractions of 200 ml each were taken and analyzed by paper chromatography for the presence of Bratton-Marshall positive amine. Fractions 6–44 were combined and evaporated *in vacuo* to a volume of about 100 ml.

The *p*H of the concentrate was raised from 7.5 to 9.5 by the addition of concentrated ammonium hydroxide. The concentrate was passed through a column, 4 × 45 cm, containing about 560 ml of Dowex 1-X8 ($formate^-$). The column was eluted with water at a rate of 6 ml/min, and fractions of 100 ml were taken. Fractions 1, 2, and 3 primarily contained colored bodies and were discarded. Fractions 4–10 contained traces of AICA-riboside and were not worked up further. Fractions 11–39, containing most of the material, were concentrated to a convenient volume. Upon addition of methanol, AICA-riboside crystallized. The product was collected and dried (4.5 g). From the mother liquor another 1.9 g of material was obtained. The total yield from three separate runs was about 20 g. This product assayed colorimetrically at about 93–98% purity and was satisfactory for use in experiments for converting AICA-riboside to AICAR and succino-AICAR.

An analytical sample was obtained by recrystallization from water: mp 210–211°; $[\alpha]_D^{26}$ −63.0 in water (*c*, 1).

Anal. Calcd for $C_9H_{14}N_4O_5$: C, 41.86; H, 5.46; N, 21.70%. Found: C, 41.75; H, 5.76; N, 21.92%.

Phosphorylation Experiments in the Yeast System. Preliminary experiments for the phosphorylation of AICA-riboside in the yeast system were carried out in the same way as for the large-scale preparation of AICAR, except that all quantities were reduced in scale by a factor of 100 or 200 to give final volumes of 10 or 5 ml. The reaction mixtures were incubated in 25 ml Erlenmeyer flasks in a Dubnoff shaker at 30°.

Earlier experiments were carried out with fresh brewers' yeast grown in the following medium: malt extract, 10 g; yeast extract, 4 g, made up to 1 liter with tap water and adjusted to *p*H 7.3 with sodium hydroxide. One liter of medium in a Fernbach flask was sterilized at 120° for 30 minutes. After inoculation from a slant of *Saccharomyces carlsbergensis*, the flask was incubated stationary at 28° for 21 hours. It was shaken vigorously by hand once every 4 hours. The yeast cells were collected by centrifugation. For optimal results, each 10 ml of the phosphorylation mixture was found to require about 3 g of wet yeast cake (equivalent to 1 g dry wt of yeast cells).

Later it was observed that brewers' yeast, freshly received from the brewery as a pressed cake, was as active as the home-grown variety in the phosphorylation of AICA-riboside. Furthermore, when brewers' yeast was grated through a 10-mesh screen and dried in air overnight, very little phosphorylating activity was lost. The dried yeast, in the form of granules, retained almost full activity when stored in the refrigerator for several months. The large-scale experiments described subsequently were all carried out with a dried yeast preparation obtained in this manner from a batch of brewers' yeast from Shaeffer Brewing Co., Brooklyn, N.Y.

Preparation of AICAR. To a 1-liter Erlenmeyer flask fitted with a magnetic stirrer was added 10.0 g glucose, 6.4 g disodium hydrogen phosphate, 4.14 g sodium dihydrogen phosphate monohydrate, 50 g dried brewers' yeast, and water to give a total volume of 500 ml. The mixture was gently stirred at 30° in a constant-temperature water bath.

After 1 hour, 5.0 g glucose, 5.0 g AICA-riboside, 25 mg adenosine, and 5 ml toluene, in that order, were added to the actively fermenting mixture. After 2.5 hours another 2.5 g glucose was added. Immediately following addition of substrate, and every hour subsequently, 1-μl aliquots were spotted on paper chromatograms and developed in system B (Table II). Production of AICAR was detected by the appearance of a new Bratton-Marshall positive spot at R_F 0.15. The reaction was virtually complete in about 6 hours. The incubation mixture was then centrifuged, the supernatant was decanted, and the cells were washed twice with 100 ml water. The combined supernatant and wash (*p*H 6.4) was passed through a column (4 × 42 cm) of Dowex 1 ($formate^-$) containing about 500 ml resin. The column was washed with 2 liters water and then eluted with 2 N formic acid at a rate of one 100-ml fraction every 15 minutes. The fractions were analyzed for the presence of AICAR by paper chromatography. AICAR began to be detectable at fraction 26 and was strongest at fractions 29–31. Upon cooling of the solution in the refrigerator, clusters of fine needles crystallized in these fractions and were collected. The filtrate from these fractions and other fractions containing AICAR (26–28, 32–90) were freeze-dried. The freeze-dried solid, weighing 6.4 g, was suspended in 60 ml water and brought to *p*H 6.5 with sodium hydroxide. The slightly colored solution was treated with 1.4 g Darco KB for 15 minutes and filtered. The filtrate was adjusted to *p*H 3.0 with concd phosphoric acid. Upon standing, masses of crystals of AICAR appeared. Under the microscope they are seen to consist of clusters of fine needles. These were collected, washed with methanol, and dried in air. The combined crystalline material weighed 5.45 g. It gave a positive orcinol test, and reacted readily with periodate. It contained no detectable inorganic phosphate.

For analysis a sample was dissolved in water by neutralization, and the free acid reprecipitated at low *p*H, $[\alpha]_D^{26}$ −67.6 in 0.1 M $NaHCO_3$ (*c*, 1).

Anal. Calcd for $C_9H_{15}N_4O_8P \cdot H_2O$: C, 30.34; H, 4.81; N, 15.73; P, 8.70; H_2O, 5.06%. Found: C, 30.45; H, 4.90; N, 15.62; P, 8.35; H_2O, 5.08%.

The effect of excess fumarate on AICAR in the presence of autolyzed yeast was tested in the following experiment. A mixture of 150 mg glucose, 64 mg Na_2HPO_4, 1 g dry yeast, and 10 ml water was incubated on the Dubnoff shaker at 30°. After 1 hour 20 mg AICAR·H_2O (56 μM), 80 mg disodium fumarate (500 μM), and 0.1 ml toluene were added, and the mixture was shaken for another 4 hours. Paper chromatography of the reaction mixture in system A (Table II) indicated that most of the AICAR (R_F 0.50) had disappeared. There was present a new major spot at R_F 0.25 which showed, after spraying with nitrous acid, a lack of stability characteristic of succino-AICAR.

Preparation of Succino-AICAR. In a 2-liter Erlenmeyer flask fitted with a magnetic stirrer were placed 15.0 g glucose, 12.8 g disodium hydrogen phosphate, 8.3 g sodium dihydrogen phosphate monohydrate, 100 g dried, pressed yeast, and water to give a volume of 1 liter. The flask was immersed in a constant-temperature water bath and stirred at 30°. After 1 hour, 10.0 g AICA-riboside, 10.0 g glucose, 50 mg adenosine, and 10 ml toluene were added, in that order. Stirring was continued at 30°. After 1.5 hours 5.0 g glucose was again added. The stirring was continued for a total of 6 hours. At this time most of the AICA-riboside added had been converted to AICAR.

To the reaction mixture was then added 32 g disodium fumarate. The *p*H of the mixture (about 6.3) was raised to 7.0 with 50% sodium hydroxide. Stirring was continued at 30° for an additional 7 hours. At the end of this period paper chromatographic analysis indicated that practically all the AICAR had been converted to succino-AICAR.

The reaction mixture was centrifuged and the residual cells were washed three times with 350 ml water. The supernatant and wash were combined to give a volume of about 2 liters. The *p*H of this solution was adjusted from 6.9 to 2.5 by the addition of IR-120 (H^+). To the acidified solution was added 175 g Darco G60 and the mixture was stirred vigorously for 45 minutes, whereupon practically all the succino-AICAR present was adsorbed on the carbon. The carbon was collected by filtration and washed well with water. It was then mixed with 1 liter eluting solution consisting of 2 parts ethanol, 1 part ammonium hydroxide, and 7 parts water. After being stirred for 30 minutes, the mixture was filtered and the cake was washed twice with 400 ml of eluting solution. The combined filtrate and wash were concentrated to 500 ml *in vacuo* and freeze-dried to give 25.6 g of a brown solid.

The freeze-dried solid was dissolved in 150 ml water and the *p*H was adjusted from 6.3 to 7.8 with ammonium hydroxide. The solution was decolorized by stirring with 1.5 g Darco G60 for 20 minutes. After filtration, 68 ml of a solution containing 385 mg/ml of barium chloride was added to the clear filtrate with stirring. A voluminous precipitate appeared. Forty ml methanol was added and the mixture was cooled in an ice bath. The crude barium salt was filtered, washed with 200 ml methanol, and dried in air to give 31.1 g product.

The crude barium salt of succino-AICAR was stirred in 1250 ml water at 50°. Most of the material went into solution. After filtration to remove some undissolved material the filtrate was treated with 7 g Darco G60 and filtered. The clear filtrate was concentrated *in vacuo*. The barium salt began to precipitate out of the solution when the volume was about 500 ml. Concentration was continued until the volume was about 120 ml. The product was filtered, washed with methanol, and dried (19.2 g).

The purified barium salt was dissolved in 750 ml water and passed through a column containing about 600 ml IR-120 (H^+). The column was washed with water until no more succino-AICAR appeared. Fractions of 200 ml were taken at a rate of one fraction every 10 minutes. Fractions 2–13, containing the active material, were freeze-dried to give 10.2 g of an almost white powder. This amorphous free acid was dissolved in a minimum amount of water, and acetone was added to the point of cloudiness. Upon standing, the solution began to crystallize in clusters of thin rods. Crystallization was allowed to continue overnight. The first crop weighed 7.3 g and another 1.5 g was collected from the mother liquor upon further treatment with acetone. For analysis a sample was recrystallized from water-acetone. The product analyzed for succino-AICAR free acid containing 0.5 mole of acetone of crystallization; $[\alpha]_D^{26} = -26.3$ (*c*, 1 in 0.1 M $NaHCO_3$).

Anal. Calcd for $C_{13}H_{19}N_4O_{12}P \cdot 1/2(C_3H_6O)$: C, 36.03; H, 4.95; N, 11.59; P, 6.41. Found: C, 35.76; H, 4.87; N, 11.19; P, 6.19.

The fact that the crystals contain acetone of solvation was shown by reacting 48 mg of material in 1 ml solution with 2,4-dinitrophenylhydrazine hydrochloride. Immediate precipitation of a 2,4-dinitrophenylhydrazone occurred. The product was filtered to give 12.3 mg of yellow needles (theoretical yield, 11.9 mg). The material was shown to be a dinitrophenylhydrazone of acetone by chromatographic comparison with an authentic sample and by mixture melting point.

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