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PHOSPHORIBOSYL-AMINOIMIDAZOLE-SUCCINOCARBOXAMIDE SYNTHETASE FROM *NEUROSPORA CRASSA*

I. PARTIAL PURIFICATION AND PROPERTIES*

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SUMMARY

An assay for phosphoribosyl-aminoimidazole-succinocarboxamide synthetase from *Neurospora crassa* has been developed. This enzyme catalyzes the reaction:

$$\text{ATP} + \text{5-amino-4-imidazole-carboxylic acid ribonucleotide} + \text{aspartate} \overset{Mg^{2+}}{\rightleftharpoons} \text{5-amino-4-imidazole-}N\text{-succinocarboxamide ribonucleotide} + \text{ADP} + P_i$$

The assay utilizes the reversibility of this reaction. The enzyme can be purified 100-fold by ammonium sulfate fractionation, hydroxylapatite chromatography, and DEAE-Sephadex chromatography. Mg^{2+}, ADP, and phosphate or arsenate are required for the reverse reaction. The divalent cations Mn^{2+} and Co^{2+} can partially replace Mg^{2+} as activators. The optimum pH is 6.0, and the reaction rate is greater at 37° than at 25° or 30°. The K_m for the reverse reaction is $1.2 \cdot 10^{-4}$–$1.3 \cdot 10^{-4}$ M. Soluble protein preparations from adenine-3B mutants contain synthetase activity, whereas preparations from adenine-3A mutants do not. It is concluded that the synthesis of phosphoribosyl-aminoimidazole-succinocarboxamide synthetase is controlled by the adenine-3A locus in *N. crassa*.

INTRODUCTION

Extensive genetic data concerning the adenine-3 (*ad-3*) mutants of *Neurospora crassa* have been accumulated by DE SERRES[1] and co-workers. This is one of the most thoroughly studied genetic systems in a eukaryotic organism, but no biochemical or enzymological investigations have been undertaken previously.

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Abbreviations: AIR, aminoimidazole ribonucleotide; CAIR, 5-amino-4-imidazole-carxylic acid ribonucleotide; SAICAR, 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide; AICAR, 5-amino-4-imidazole-carboxamide ribonucleotide; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *n*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

It has been assumed that the *ad-3A* and *ad-3B* loci code for the synthesis of two enzymes that catalyze the conversion of aminoimidazole ribonucleotide (AIR) to 5-amino-4-imidazole-carboxylic acid ribonucleotide (CAIR) and of CAIR to 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide (SAICAR)[1]. French (cited by BUCHANAN[2] and personal communication) performed enzyme assays on cell-free extracts from *ad-3A* and *ad-3B* mutants but was unable to determine the enzymic reaction controlled by each locus. BERNSTEIN[3] assayed for the aminoimidazole compounds accumulated by several adenine-requiring mutants of *N. crassa* but was also unable to determine the reaction controlled by each *ad-3* locus.

With the introduction of a method for synthesizing SAICAR[4], it became feasible to assay for the enzyme phosphoribosyl-amino-imidazole-succinocarboxamide synthetase (5'-phosphoribosyl-4-carboxy-5-aminoimidazole:L-aspartate ligase (ADP), EC 6.3.2.6), which normally catalyzes the conversion of CAIR to SAICAR, by assaying for the reverse reaction, the conversion of SAICAR to CAIR. LUKENS AND BUCHANAN[5] and MILLER AND BUCHANAN[6] demonstrated this reverse reaction in enzyme preparations obtained from pigeon liver. This report will show that (1) a suitable assay for this enzyme from *N. crassa* has been developed; (2) the locus coding for the synthesis of this enzyme has been identified; and (3) several of the properties of the enzyme have been determined.

MATERIALS AND METHODS

Mutant and wild-type strains of *N. crassa* were obtained from the stock collection of Dr. F. J. De Serres of the Biology Division of the Oak Ridge National Laboratory. Wild-type strain 74-OR23-1A was used for all of the enzyme purification and characterization studies.

SAICAR was prepared by the method of HUANG[4]. The author is indebted to Chas. Pfizer and Co., for *Arthrobacter albidus* strain ATCC 15243, and to F. and M. Schaefer Brewing Company for the brewer's yeast used in the production of SAICAR.

Synthetase activity was determined by measuring the rate of conversion of SAICAR to CAIR in a reaction mixture containing potassium phosphate, magnesium chloride or magnesium acetate, ADP, 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer and SAICAR at the concentrations indicated in the text and legends.

The colorimetric method of BRATTON AND MARSHALL[7] as modified by LUKENS AND BUCHANAN[8], and FLAKS AND LUKENS[9] was used for the detection and identification of the aminoimidazole compounds. By using this method and known molar extinction coefficients, each of the aminoimidazole compounds can be identified, and with the exception of CAIR, quantitatively measured.

Since CAIR is unstable and has no usable extinction coefficient, it was measured in the form of AIR. CAIR was converted to AIR by placing the samples at pH 1.5 for 10 min at room temperature. The absorbance values obtained with increasing concentrations of AIR were nonlinear at values above 0.30 *A* unit. All assays were performed, therefore, on samples diluted before the acid treatment to a level that would give an absorbance between 0.05 and 0.10 unit.

SAICAR is the ideal precursor for analyses employing the BRATTON–MARSHALL[7] procedure, because it does not form a colored BRATTON–MARSHALL derivative at 25°, whereas the other aminoimidazole compounds do. If the colorimetric assay is per-

formed at 4°, SAICAR produces a colored derivative that can be used for identification and quantification. In order to observe the conversion of SAICAR to CAIR, the more rapid conversion of SAICAR to 5-amino-4-imidazole-carboxamide ribonucleotide (AICAR) by adenylosuccinase must be prevented. This may be accomplished by utilizing, as the enzyme source, a mutant lacking adenylosuccinase activity or by performing the assay in a reaction mixture buffered at pH 6.0. Adenylosuccinase activity is negligible at this pH, whereas synthetase activity is optimum. The latter method was used in this study.

Soluble protein fractions were prepared by a modification of the method of BARNETT, BROWN AND EPLER[10]. Cultures of *N. crassa* were grown in 125-ml erlenmeyer flasks containing 30 ml of glycerol complete medium[11]. Conidia from two flasks were harvested into 50 ml of sterile water and inoculated into a 12-l flask containing 10 l of VOGEL'S[12] medium with polyglycol antifoam (20 ppm). The flasks were incubated at 30° for 36–40 h with sufficient aeration to provide a constant stirring action. The mycelial growth was harvested by filtering it through cheesecloth and squeezing out as much of the culture medium as possible. The mycelial mats were rinsed by resuspension in ice water and filtering as before. The mycelial mats were weighed and suspended in 4° breaking buffer (250 g of mycelia/750 ml of breaking buffer) composed of 0.01 M EDTA adjusted to pH 7.5 with sodium hydroxide, 0.01 M 2-mercaptoethanol, 15% glycerol, and 20 ppm of polyglycol antifoam. The cells were broken by passing this suspension through a Gaulin Press, first at 3000 lb/inch^2 and then at 10 000 lb/inch^2. Centrifugation of the homogenate at 15 000 $\times$ *g* for 30 min at 4°, followed by centrifugation of the resultant supernatant at 105 000 $\times$ *g* for 1.5 h at 4°, removed the cellular fragments and ribosomes. The supernatant obtained from this procedure is referred to as the soluble protein fraction.

RESULTS

Enzyme purification

All steps in the purification of the enzyme were performed at 4°. 750 ml of a soluble protein fraction containing 4.3 mg of protein/ml was saturated with $(NH_4)_2SO_4$ and stirred slowly for 30 min. The precipitated protein was removed by centrifugation at 20 000 $\times$ *g* for 40 min. The supernatant was saved, and the precipitate was resuspended in approx. 500 ml of 3.0 M $(NH_4)_2SO_4$ in 0.01 M Tris (pH 7.5) containing 0.01 M 2-mercaptoethanol and 15% glycerol. This procedure was repeated using two 500-ml extractions at concentrations of 3.0, 2.5, 2.0, 1.5, 1.0, and 0.0 M $(NH_4)_2SO_4$ in the same buffer. Samples were taken from the saturated $(NH_4)_2SO_4$ precipitate and from each of the supernatant fractions. They were dialyzed 5 times for 1 h (5-h total) against 1-l volumes of 0.001 M Tris (pH 7.5) containing 0.01 M 2-mercaptoethanol and 15% glycerol, and assayed for synthetase activity. Over one-half (55%) of the total enzyme activity was recovered in the 2.0 M $(NH_4)_2SO_4$ supernatant fraction (Table I).

The 2.0 M $(NH_4)_2SO_4$ supernatant fraction was concentrated for column chromatography by placing it in dialysis tubing and covering the tubing with polyethylene glycol (Carbowax 4000) or by precipitating the protein with saturated $(NH_4)_2SO_4$ and redissolving it in a small volume of buffer. The concentrated material was then dialyzed against the desired buffer.

TABLE I

SUMMARY OF ENZYME PURIFICATION

Source of enzyme	*Total protein (mg)*	*Total activity (units)**	*Specific activity (units/mg)*	*Purification (-fold)*
Satd. $(NH_4)_2SO_4$ ppt.	3200	0.74	$2.3 \cdot 10^{-4}$	—
2.0 M $(NH_4)_2SO_4$ supernatant	285	0.40	$1.4 \cdot 10^{-3}$	6
Hydroxylapatite eluate	48	0.28	$5.6 \cdot 10^{-3}$	24
DEAE-Sephadex eluate	6	0.14	$2.3 \cdot 10^{-2}$	100

* The standard unit of enzyme activity of 1 μmole/min is used.

The enzyme was further purified on hydroxylapatite (Hypatite-C) in a 1.5 cm × 90 cm column. The column was equilibrated with 0.005 M potassium phosphate buffer (pH 6.8) containing 0.01 M 2-mercaptoethanol and 5% glycerol. A solution of 200–400 mg of protein in 10 ml of the same buffer was applied to the column and eluted with a linear phosphate gradient using 1 l of 0.005 M potassium phosphate buffer and 1 l of 0.2 M potassium phosphate buffer, both at pH 6.8 and containing 0.01 M 2-mercaptoethanol and 5% glycerol. The fractions containing enzyme activity were concentrated by the use of carbowax. About 68% of the activity applied to the column was recovered.

Additional purification of the enzyme was achieved with DEAE-Sephadex A-25 ion-exchange resin. A 1.5 cm × 90 cm column was equilibrated with 0.01 M Tris (pH 7.5) containing 0.01 M 2-mercaptoethanol, 15% glycerol, and 0.1 M KCl. A solution containing 20–45 mg of protein in 2 ml of the same buffer was applied to the column, and a linear gradient elution was performed using 1 l of 0.1 M KCl and 1 l of 0.5 M KCl, both in 0.01 M Tris (pH 7.5) containing 0.01 M 2-mercaptoethanol and 15% glycerol. The fractions containing enzyme activity were concentrated with carbowax and dialyzed against 0.001 M Tris (pH 7.5) containing 0.01 M 2-mercapto-

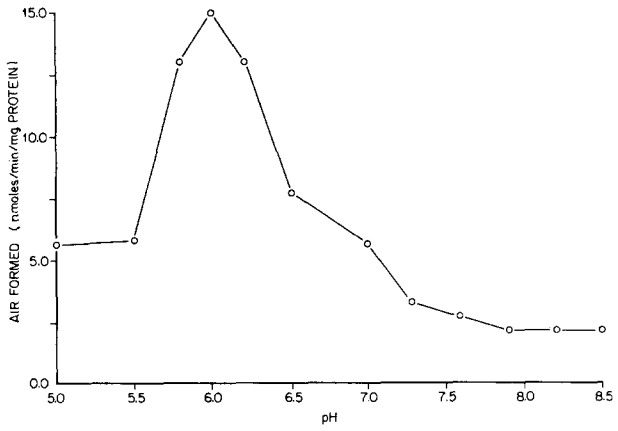
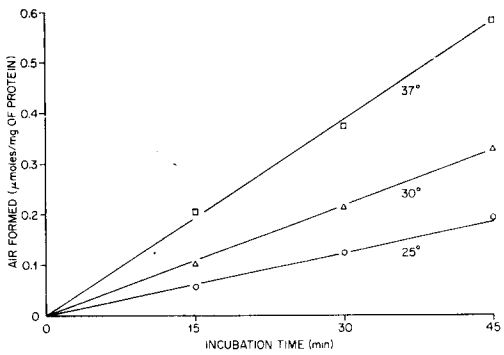


Fig. 1. Effect of pH on synthetase activity. The enzymic reaction mixture contained 0.02 mg of protein and the following expressed in μmoles/ml: $MgCl_2$, 34; ADP, 10; SAICAR, 0.1; and potassium phosphate buffer at the indicated pH, 95. The total volume was 0.525 ml; incubation was at 37° for 60 min.



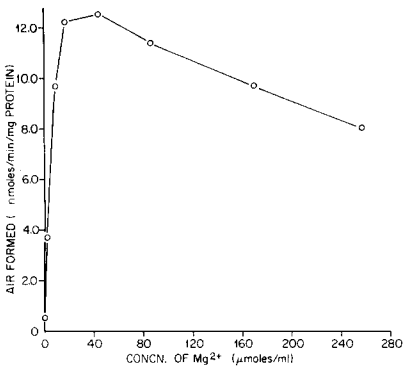


Fig. 2. Effect of temperature on synthetase activity. The enzymic reaction mixtures contained 0.03 mg of protein and the following, expressed in μmoles/ml: ADP, 10; $MgCl_2$, 42; potassium phosphate buffer (pH 6.0), 95; MES (pH 6.0), 38; and SAICAR, 0.1. The total volume was 0.525 ml.

Fig. 3. Effect of Mg^{2+} concentration on synthetase activity. The reaction mixture contained 0.02 mg of protein and the following, expressed in μmoles/ml: ADP, 10; potassium phosphate buffer (pH 6.0), 95; SAICAR, 0.1; and from 0 to 260 μmoles $MgCl_2$/ml. The total volume was 0.525 ml; incubation was at 37° for 60 min.

ethanol and 15% glycerol. About 50% of the activity applied to the column was recovered.

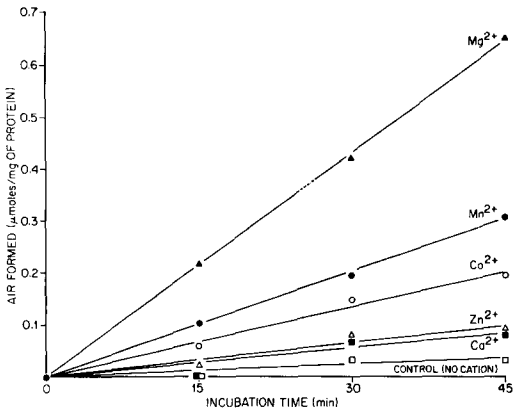
The specific activity of the enzyme preparation obtained by this three-step purification procedure was 100 × greater than that of the original saturated $(NH_4)_2SO_4$ precipitate. This purification procedure provided an enzyme preparation satisfactory for use in characterization studies.

Enzyme characterization

The optimum pH for the conversion of SAICAR to CAIR was determined by using potassium phosphate buffers at pH values between 5.0 and 8.5 as the phosphate component of the enzymic reaction mixture (Fig. 1). The same procedure was used in concurrent experiments, but additional buffers were added to prevent a pH change due to phosphate utilization. MES was used as the buffer for pH 5.0–6.5 and *n*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) for pH 7.0–8.5. In addition to the phosphate buffer, 50 μmoles of buffer at the desired pH were added to each enzymic reaction vessel. The rates obtained in this manner were the same as those obtained with the phosphate buffer alone. A pronounced peak was observed at pH 6.0.

The effect of temperature on the conversion of SAICAR to CAIR is shown in Fig. 2. 37° was chosen as the standard incubation temperature.

The dependency of the reaction on Mg^{2+} was studied by assaying the rate of conversion of SAICAR to CAIR in enzymic reaction mixtures containing from 0 to 260 μmoles of $MgCl_2$/ml (Fig. 3). Low activity at the lower concentrations of Mg^{2+} indicates a Mg^{2+} dependency; the reaction is inhibited above a concentration of about 45 μmoles of Mg^{2+}/ml. Other cations were tested for their ability to substitute for



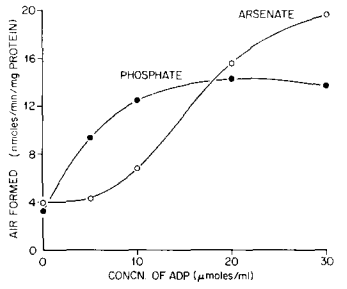


Fig. 4. Effect of cations on synthetase activity. The enzymic reaction mixture contained 0.03 mg of protein and the following, expressed in μmoles/ml: ADP, 10; potassium phosphate buffer (pH 6.0), 95; MES (pH 6.0), 38; SAICAR, 0.1; and the cation as its chloride salt, 5. The total volume was 0.525 ml; incubation was at 37°.

Fig. 5. Effect on synthetase activity of ADP in the presence of arsenate or phosphate. The enzymic reaction mixture contained 0.03 mg of protein and the following ,expressed in μmoles/ml: $MgCl_2$, 42; potassium phosphate buffer (pH 6.0), 95 or sodium arsenate, 38; MES (pH 6.0), 20; SAICAR, 0.1; and from 0 to 30 μmoles of ADP/ml. The total volume was 0.525 ml; incubation was at 37° for 60 min.

Mg^{2+}. At concentrations of 50 μmoles/ml, all of the cations except Mg^{2+} caused a precipitate in the reaction vessel. Therefore, concentrations equivalent to the concentration of Mg^{2+} normally used could not be tested. Mg^{2+} is the most effective activator at the 5 μmoles/ml concentration tested (Fig. 4). The next most effective cations are Mn^{2+}, Co^{2+}, Zn^{2+}, and Ca^{2+}, respectively, with Zn^{2+} and Ca^{2+} being essentially ineffective.

In the presence of phosphate, the rate of the reaction was dependent upon the concentration of ADP up to a concentration of about 15 μmoles/ml (Fig. 5). When arsenate was used in the above experiment at a concentration of 38 μmoles/ml in place of the phosphate, it was anticipated that the arsenate would prove more effective than phosphate in allowing the reaction to proceed at low ADP levels, since arsenate is believed to bind to ADP during ADP-catalyzed reactions to form an ADP–arsenate complex which rapidly dissociates to release the ADP to function again. Phosphate, on the other hand, is more tightly bonded during the reaction to form ATP[5]. The results, however, were not as expected (Fig. 5). Arsenate was not as effective as phosphate at low ADP concentrations, and surpassed phosphate in effectiveness at high ADP concentrations.

The effect of phosphate concentration on synthetase activity was investigated by using potassium phosphate buffer as the phosphate source at concentrations of 0–95 μmoles/ml (Fig. 6). Phosphate was limiting at concentrations less than 10 μmoles/ml and slightly inhibitory at 95 μmoles/ml.

Aspartate and ATP are required for the enzymic conversion of CAIR to SAICAR by pigeon liver extracts[5]. The effect of these compounds on the reverse reaction, SAICAR to CAIR, was tested by using the compounds individually and together in the enzymic reaction mixture (Fig. 7). ATP alone had almost no effect on the rate of

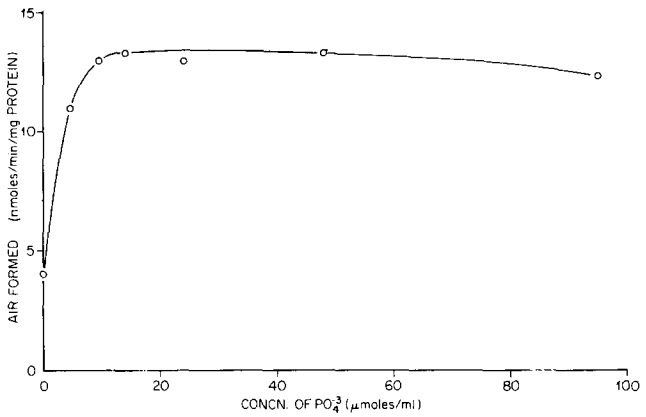


Fig. 6. Effect of PO_4^{3-} concentration on synthetase activity. The enzymic reaction mixture contained 0.03 mg of protein and the following, expressed in μmoles/ml: ADP, 10; $MgCl_2$, 42; MES (pH 6.0), 38; and SAICAR 0.1; and from 0 to 95 μmoles of potassium phosphate (pH 6.0)/ml. The total volume was 0.525 ml; incubation was at 37° for 60 min.

the reaction, and aspartate shortened the linear portion of the reaction slightly; but when both aspartate and ATP were used together, a more pronounced decrease in the linear portion of the reaction was observed.

The K_m for phosphoribosyl-aminoimidazole-succinocarboxamide synthetase was determined by plotting the reaction rates obtained with 8 substrate concentrations varying from $0.2 \cdot 10^{-4}$ to $4 \cdot 10^{-4}$ M by both the LINEWEAVER–BURK[13] method and the EADIE[14] method. The enzymic reaction mixture was composed of 0.02 mg of protein and the following, expressed in μmoles/ml: magnesium acetate, 85; ADP, 10; potassium phosphate buffer (pH 6.2), 95; MES (pH 6.2), 20; and from 0.02 to 0.40 μmole of SAICAR/ml. The total volume was 0.525 ml, and the reaction mixture was incu-

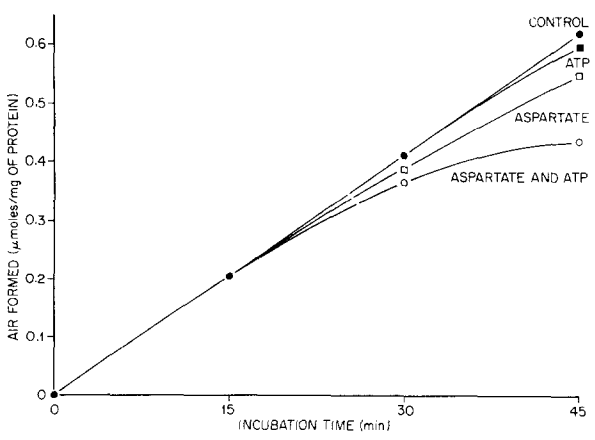


Fig. 7. Effect of ATP and aspartate on synthetase activity. The enzymic reaction mixture contained 0.03 mg of protein and the following, expressed in μmoles/ml: ADP, 10; $MgCl_2$, 42; MES (pH 6.0), 38; potassium phosphate buffer (pH 6.0), 95; and SAICAR, 0.1; and either 10 μmoles of ATP or aspartate/ml, or 10 μmoles of each/ml. The total volume was 0.525 ml; incubation was at 37°.

bated at 37°. At the lower concentrations of substrate, the rate declined rapidly as the substrate was utilized. The initial rates were used in all cases. The Lineweaver–Burk and Eadie methods gave linear plots over the entire concentration range with K_m values of $1.25 \cdot 10^{-4}$ M and $1.27 \cdot 10^{-4}$ M, respectively.

Genetic studies

To further ascertain which of the two *ad-3* loci, *A* or *B*, codes for the synthesis of phosphoribosyl-aminoimidazole-succinocarboxamide synthetase, enzyme preparations were made from two *ad-3A* and two *ad-3B* strains, and tested for synthetase activity in an enzymic reaction mixture containing 0.07–0.12 mg of protein and the following, expressed in μmoles/ml: $MgCl_2$, 85; ADP, 10; potassium phosphate buffer (pH 6.0), 95; MES (pH 6.0), 38; and SAICAR, 0.1. The total volume was 0.525 ml, and the reaction mixture was incubated at 37°. The two *ad-3B* strains had synthetase activity, but the two *ad-3A* strains did not. This finding establishes that phosphoribosyl-aminoimidazole-succinocarboxamide synthetase is coded for by the *ad-3A* locus of *N. crassa*.

DISCUSSION

The partial purification of the enzyme by ammonium sulfate fractionation followed by hydroxylapatite and DEAE-Sephadex chromatography provides a reliable means of increasing the specific activity to a level that is suitable for the enzyme assay system employed. The enzyme is stable during these purification procedures and during storage at 4° in 0.01 M Tris (pH 7.5) containing 0.01 M 2-mercaptoethanol and 15% glycerol. MILLER AND BUCHANAN[6] reported that gradient elution from DEAE-cellulose columns led to a loss of activity. Similar results have been observed occasionally in this investigation, although Sephadex A-25 usually gives satisfactory results.

The discovery that the optimum pH for this enzyme is considerably different from that for the enzyme that catalyzes the conversion of SAICAR to AICAR[15] was beneficial. This finding makes the assay of synthetase activity possible without using mutants unable to carry out the conversion of SAICAR to AICAR. It should be kept in mind that the reverse reaction, SAICAR to CAIR, was studied and that the optimum pH may be different for the forward reaction, CAIR to SAICAR. The low optimum pH may explain the failure of other workers[6] to find appreciable synthetase activity in Neurospora soluble protein preparations. They did not report the pH used in their assay, but it may have been the same as that used in the study of the enzyme from avian liver, *i.e.*, pH 7.3–7.5 (refs. 5, 6). It is also possible that the optimum pH for the avian enzyme lies below the values commonly used.

The observation that Mg^{2+}, phosphate, and ADP are utilized in the reverse reaction is in agreement with the results reported in the avian liver studies[5]. Likewise, the ability of Mn^{2+} and Co^{2+} to partially fulfill the requirement for an activator is in agreement with previous reports[6].

The effect of arsenate reported by LUKENS AND BUCHANAN[5] was not observed with the Neurospora enzyme. They reported that only catalytic amounts of ADP were required for the conversion of SAICAR to CAIR when arsenate was present in the reaction mixture. With the Neurospora enzyme, arsenate is actually inferior to

phosphate in promoting the reaction at low ADP concentrations and only slightly exceeds phosphate in effectiveness at higher ADP concentrations.

The effect of ATP and aspartate on the reverse reaction had not been reported previously. It is not surprising, however, that shortening of the linear portion of the reaction occurs, since these compounds are known to be utilized in the forward reaction CAIR to SAICAR, and an equilibrium is probably being established.

From these observations it appears clear that the basic reaction taking place in *N. crassa* is the same as that proposed for the avian liver system and that the two enzymes are similar. The determination of the enzymic reaction controlled by one of the two *ad-3* loci of *N. crassa* is now possible. The observations that *ad-3B* strains are capable of converting SAICAR to CAIR, whereas *ad-3A* strains are not, indicates that the *ad-3A* locus codes for the synthesis of phosphoribosyl-aminoimidazole-succinocarboxamide synthetase.

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