Imidazole Compounds Accumulated by Purine Mutants of *Neurospora crassa*

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SUMMARY

A procedure is given for the detection of imidazole compounds accumulated in the mycelium of adenine mutants of *Neurospora crassa*. Of five such compounds detected, four have been tentatively identified. The distribution of these imidazoles among the mutants investigated allows a correlation between the adenine loci and the steps of purine biosynthesis.

INTRODUCTION

Extensive enzymic studies with avian liver systems have led to the elucidation of the complete pathway of adenosine-5'-monophosphate biosynthesis (Buchanan, 1958–59). The seven reaction steps following imidazole ring closure are shown in Fig. 1. From studies of the corresponding reaction pathway in *Neurospora crassa*, Giles, Partridge & Nelson (1957) have presented evidence that E mutants (Table 1) are blocked in the conversion of inosinic acid to adenylosuccinic acid ribotide. F mutants

Fig. 1. Steps in purine biosynthesis controlled by adenine loci as judged from imidazole accumulation by mutants of *Neurospora crassa*.

Key: AMP, adenosine-5'-phosphate; AMP-S, adenylosuccinic acid ribotide; IMP, inosinic acid; FAICA-ribotide, 5-formamido-4-imidazolecarboxamide ribotide; AICA-ribotide, 5-amino-4-imidazolecarboxamide ribotide; SAICA-ribotide, 5-amino-4-imidazole-*N*-succinocarboxamide ribotide; CAI-ribotide, 5-amino-4-imidazolecarboxylic acid ribotide; AI-ribotide, 5-amino-imidazole ribotide.

* Giles *et al.* (1957) have shown that mutants at the *ad-4* locus are deficient in a de-acylase which splits both SAICA-ribotide and AMP-S.

† *ad-8* mutants were shown also by Giles *et al.* (1957) to be blocked in the conversion of IMP to AMP-S.

and mutant 44206 were also shown to lack activity for a bifunctional enzyme which catalysed the de-acylation of 5-amino-4-imidazole-*N*-succinocarboxamide ribotide and adenylosuccinic acid ribotide to 5-amino-4-imidazolecarboxamide ribotide

and adenosine-5'-phosphate, respectively. Dr T. French is reported (Buchanan, 1958-59) to have found in wild-type *N. crassa* all the purine biosynthetic enzymes present in the avian liver system. In the present work mutants representing several adenine loci of *N. crassa* were studied for their ability to accumulate imidazole compounds, intermediate in purine biosynthesis. All adenine mutants found to date have been allocated to eight loci (Barratt, Newmeyer, Perkins & Garnjobst, 1954; Giles *et al.* 1957); these are summarized in Table 1.

Table 1. *Summary of mutants of Neurospora crassa assigned to the eight adenine loci*

Locus	Mutants
<i>ad-1</i>	3254*
<i>ad-2</i>	70004, 27663, 20705
<i>ad-3</i>	35203, 38701, 38709, 45601, 68306, A†, B
<i>ad-4</i>	44206, 44415, F
<i>ad-5</i>	71104, J
<i>ad-7</i>	28610
<i>ad-6</i>	44411
<i>ad-8</i>	E

* Mutants with numerical designations came from the early mutant searches of Beadle & Tatum (1945).

† Letter designations were given by Giles *et al.* (1957) to groups of mutants related by heterokaryon complementation tests.

METHODS

Extraction of mycelia

The cultures of *Neurospora crassa* used as stocks, or as a source of conidial inocula, were grown in test tubes containing a complete medium described by Horowitz (1947). To obtain large quantities of mycelium, mutants were grown in carboys containing 10 l. of minimal medium (Beadle & Tatum, 1945) supplemented with limiting amounts of adenine sulphate (usually 30 µg./ml.). All mutants were grown at 25°, except the temperature sensitive strains (44206, 44415, 70004) which were grown at 35°. After 3-4 days of growth under forced aeration the mycelia were harvested by pouring the contents of the carboys through cheesecloth. The mycelial mats were placed in a Waring Blender and boiling water was added (about 1 l./100 g. wet mycelium). After disintegration the extracts were filtered through Whatman no. 1 filter paper on a Buchner funnel. The filtrates were then lyophilized overnight, leaving thick syrupy extracts in the flasks. Five to 10 ml. of water were sufficient to take up the extract from 100 g. mycelium. Residual material were separated by centrifugation and washed several times with 1 ml. portions of water which were added to the soluble fraction. The solutions contained all the imidazoles. The residues were discarded.

Detection of accumulated imidazoles

The redissolved extracts were spotted directly, or with dilution, on Whatman no. 1 paper and resolved by ascending chromatography in a variety of solvents. Isopropanol, water, and conc. aqueous NH₄OH (sp. gr. 0.880) in the volume ratios 70:20:10 and 70:40:10 were the most useful systems. After drying, the chromatograms were sprayed very lightly with diazosulphonic acid reagent (Ames & Mitchell,

1952), and again very lightly with 5% (w/v) aqueous Na_2CO_3 solution. By this procedure five distinct imidazole compounds not present in wild-type mycelium were detected in extracts from the mycelia of the mutant strains.

Isolation of Compounds I and II (Table 3)

To 14 ml. of the water soluble extract of 135 g. wet weight of mutant 44206 mycelium (see Methods) an equal volume of methanol was added. A precipitate was formed which was washed with 50% (w/v) methanol in water. The soluble fractions, containing virtually all of Compounds I and II, were combined to a total volume of 23.5 ml.

A Dowex exchange resin (1-X2, 200–400 mesh), from which the fines had been removed by repeated washings, was equilibrated with 2 M-formic acid. This was placed in a column 2.2 cm. in diam. to a volume of 170 cm.³. Water at 3.5° was circulated through a jacket which surrounded the column. The flow rate of solvent was kept at about one drop every 15 sec. by maintaining the system under controlled positive pressure. A mixing vessel of 125 ml. capacity was included in the system to allow gradient elution according to the procedure of Thompson (1955). The column was first washed with distilled water and then the sample was added. For elution, the series of solutions listed in Table 2 were used in the order given. Successive solutions were added only after the pH value of eluted fractions did not change measurably. By using an automatic fraction collector, 5–10 ml. portions were collected. Compound II began to elute at pH 1.9 (solution 7); it came off the column in a total volume of 273 ml. After an additional 135 ml. of eluent had passed through, compound I began to appear and came through in the next 82 ml.

Table 2. *Solutions used for the serial elution of chromatographic columns*

No.	Solution	Molarity of NH_4^+	Molarity of anion	Measured pH value
1	Distilled water	.	.	.
2	Ammonium acetate buffer	0.4	1.2	4.28
3	Ammonium acetate buffer	0.4	1.0	4.40
4	Ammonium formate buffer	0.4	1.1	3.28
5	Acetic acid	.	1.0	2.53
6	Formic acid	.	1.0	1.97
7	Formic acid	.	6.0	1.17

Separation of Compounds III and IV (Table 3)

An extract of mutant 45601 containing both Compounds III and IV was subjected to a chromatographic procedure similar to that described above. Compound III was eluted by water very close to the front, and Compound IV came off soon after. Though separation was achieved, both fractions were impure. However, they were satisfactory for chromatographic comparisons.

Abbreviations

The following abbreviations, modified from Buchanan (1958–59), are used: AMP, adenosine-5'-phosphate; AMP-S, adenylosuccinic acid ribotide; IMP, inosinic

acid; FAICA-ribotide, 5-formamido-4-imidazolecarboxamide ribotide; AICA-ribotide, 5-amino-4-imidazolecarboxamide ribotide; SAICA-ribotide, 5-amino-4-imidazole-*N*-succinocarboxamide ribotide; CAI-ribotide, 5-amino-4-imidazolecarboxylic acid ribotide; AI-ribotide, 5-amino-imidazole ribotide.

RESULTS

The colour reactions and distribution of the five detected imidazoles among the mutants are detailed in Tables 3 and 4.

Table 3. *Colour reactions of accumulated compounds*

		Compounds				
		I	II	III	IV	V
Colour development	Before Na ₂ CO ₃	Red-orange	Bright orange	Bright yellow	No colour	Yellow
	After Na ₂ CO ₃	Fades to grey	Fades to grey	Grey spots—fades	Red	Blue
Probable identity		SAICA-ribotide	SAICA-riboside	AI-riboside	Unknown	AICA-riboside

Table 4. *Distribution of accumulated imidazoles*

		Compounds				
Locus	Mutants	I	II	III	IV	V
<i>ad-1</i>	3254	—	+	—	—	+
<i>ad-2</i>	70004	—	—	—	—	—
	27663	—	—	—	—	—
	20705	—	—	—	—	—
		—	—	—	—	—
<i>ad-3</i>	35203	—	—	+	+	—
	38701	—	—	+	+	—
	38709	—	—	+	+	—
	45601	—	—	+	+	—
<i>ad-4</i>	44206	+	+	—	±	—
<i>ad-5</i>	71104	—	+	—	±	+
<i>ad-6</i>	28610	—	—	—	—	—
<i>ad-7</i>	44411	—	—	—	—	—

The following designations were used: +, compound present in mycelial extract; ±, compound present, but in low concentration; —, compound not detected.

Characterization

Compound I at pH 7 had an ultraviolet absorption peak at 269 *mμ*. By the method of Mejbaum (1939) it was shown to contain a pentose moiety. A sample of SAICA-ribotide generously provided by Dr T. French proved to be identical with Compound I, both in colour development and chromatographic mobility. When Compound I and the known SAICA-ribotide were hydrolysed by alkaline phosphatase each formed a product identical on chromatograms with Compound II.

Compound II at pH 7 absorbed maximally at 268 *mμ*, and was also shown to contain a pentose moiety. By using the procedure of Allen (1940) it was proved to lack a phosphate group. On hydrolysis at 105° with conc. HCl, for 15 hr. in a sealed

tube, Compound II yielded aspartic acid and glycine. The presence of aspartic acid on hydrolysis, the shape of the ultraviolet absorption curve, and position of the absorption maximum compared well with the properties reported for SAICA-ribotide (Gots & Gollub, 1957; Lukens & Buchanan, 1959*a*). The phosphate determination and the data from hydrolysis with alkaline phosphatase further showed that Compound II was SAICA-riboside.

Compound III formed an orange-red Bratton-Marshall reaction product (Bratton & Marshall, 1939) which absorbed maximally at 500–502 m μ . No absorption maximum in the ultraviolet region was detected. That Compound III failed to bind to an anion exchange resin argues against the presence of carboxyl or phosphate group. The Bratton-Marshall product of AI-ribotide is reported to be salmon-orange and to absorb maximally at 500 m μ (Lukens & Buchanan, 1959*b*). Also this compound is reported to have no ultraviolet absorption maximum above 210 m μ . All the properties observed for Compound III are consistent with its being AI-riboside.

The Bratton-Marshall reaction product of Compound IV was orange-red and absorbed maximally at 534–538 m μ . This corresponds to none of the maxima reported for the imidazoles involved in purine biosynthesis. However, since the concentration of this compound appeared to decrease as more care was taken in preparation of extracts, it seems likely that Compound IV was a reaction product of Compound III.

Ames & Mitchell (1952) reported that, of 16 compounds tested by their diazotization procedure, only 5-amino-4-imidazolecarboxamide (AICA) gave a blue reaction product. Subsequently AICA-ribotide was observed by the present author to give this blue colour. Compound V gave a colour reaction identical with that of the aglycone and AICA-ribotide. Chromatographic evidence suggested that Compound V was either AICA or AICA-riboside.

DISCUSSION

Knowing the distribution and probable identity of the detected imidazole compounds it is possible to correlate the adenine loci and the steps of purine biosynthesis (Fig. 1). Since *ad-2*, *ad-6* and *ad-7* mutants of *Neurospora crassa* can use IMP or hypoxanthine in place of adenine as a growth supplement (Mitchell & Houlahan, 1946) and yet accumulate no imidazole compounds, they can be assigned to steps preceding imidazole ring closure. This conclusion is in accord with an observation of Mitchell & Houlahan (1946). All *ad-3* mutants accumulate a distinctive purple pigment in their growth media. The purple pigment, which seems to have associated with it a 305 m μ ultraviolet absorption maximum, is probably a reaction product of Compound III. It was also shown by these authors that the double mutants of 35203 (*ad-3*) in conjunction with *ad-2*, *ad-6* and *ad-7* mutants, accumulate no purple pigment. The double mutant 35203, 44206 (*ad-4*) does accumulate the pigment. This again places the *ad-2*, *ad-6* and *ad-7* block before, and the *ad-4* block after, the reaction controlled by *ad-3*.

Colourless solutions of SAICA-riboside (Compound II) will turn red in time, especially at low pH values or on exposure to air. The reddish appearance of mutant 44206 (*ad-4*) mycelia is probably due to this effect. The percentage yield of SAICA-riboside and SAICA-ribotide in the dried mycelia of mutant 44026 can be calculated

from the observed optical densities at the ultraviolet absorption maxima and the extinction coefficient given by Lukens & Buchanan (1959*a*). The yields of the riboside and ribotide were found to be 2.1 and 0.023 %, respectively, of the dry weight of its mycelia.

The two intermediates CAI-ribotide and FAICA-ribotide and the corresponding ribosides, known to be the least stable of the imidazoles, were not detected in any extracts.

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