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The Metabolism of Exogenous Adenine and Purine Analogues by Fungi

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Cordycepin, a metabolic product of the fungus *Cordyceps militaris* (Linn.) Link, has been shown to be 9-cordyceposidoadenine, the sugar cordycepose being a 3-deoxy branch-chain pentose (Cunningham, Hutchinson, Manson & Spring, 1951; Bentley, Cunningham & Spring, 1951). *C. militaris* has been grown on media containing [8-¹⁴C]adenine, and the cordycepin isolated found to be radioactive (Kredich & Guarino, 1961). We have done similar experiments with two concentrations of [8-¹⁴C]adenine. Increasing the concentration of added adenine increased both the yield and the radioactivity/mole of the cordycepin produced. With 500 mg. of adenine in 1 l. of the medium, the counts/mole in the cordycepin iso-

lated were 73 % of those in the adenine. It thus seems probable that the adenine supplied was incorporated as such into the cordycepin.

It was consequently thought that analogues of purines might be similarly incorporated, so *C. militaris* was grown on media containing such compounds and new derivatives were sought in the culture filtrates by paper chromatography. No new cordyceposides have been found, but from an isomer of adenine two ribosides have been obtained, and three aromatic amines have been acetylated.

The isomer of adenine, 4-amino-1*H*-pyrazolo-[3,4-*d*]pyrimidine [4-amino-(I)], has been converted into a riboside and into 4-hydroxypyrazolo-

(I) 1H-Pyrazolo[3,4-d]pyrimidine

[3,4-*d*]pyrimidine riboside. The latter compound was not formed from 4-hydroxypyrazolo[3,4-*d*]pyrimidine supplied in the medium as a precursor.

On hydrolysis with hydrochloric acid, both ribosides give ribose and each gives its 4-substituted pyrazolo[3,4-*d*]pyrimidine. The position of attachment of ribose has not been established. These results are analogous to those of Matthews & Smith (1956), who found that azaguanine was converted into azaguanosine and azaxanthosine by *Bacillus cereus*.

From each of the aromatic amines (II–IV) a monoacetyl derivative was formed. This was established by comparing the fungus products with synthetic acetyl derivatives, using paper chromatography and infrared spectra. Similar acetylations of aromatic amines commonly occur in animals and are said to be detoxication mechanisms (Baldwin, 1947).

2,6-Diaminobenzothiazole
(II) 5-Amino-1,2-dimethylbenzimidazole
(III) 5-Amino-2-oxobenzimidazoline
(IV)

The capacity to produce ribosides from 4-aminopyrazolo[3,4-*d*]pyrimidine supplied as a precursor appears to be fairly widespread among fungi. *Aspergillus caespitosus* Raper & Thom, *Gibberella fujikuroi* (Sacc.) Wr., *Mucor racemosus* Fries, *Mucor ramannianus* Dierckx, *Penicillium griseofulvum* Dierckx, *Scopulariopsis brevicaulis* (Sacc.) Bainier, and *Trichoderma viride* Pers. ex Fries were also grown on media containing 4-aminopyrazolo[3,4-*d*]pyrimidine. Both ribosides were obtained from all these except *Scopulariopsis brevicaulis*, which gave 4-aminopyrazolo[3,4-*d*]pyrimidine riboside only.

EXPERIMENTAL

Melting points are uncorrected.

Preparation of precursors

4-Aminopyrazolo[3,4-*d*]pyrimidine. This was prepared by the method of Robins (1956) and purified on a column of Amberlite IR-120 (H⁺ form). After developing the column

with 2N-hydrochloric acid, the eluates were neutralized with ammonia and the precipitate which separated was recrystallized from water (Found: C, 44.3; H, 4.1; N, 52.1. Calc. for C₆H₅N₅: C, 44.4; H, 3.7; N, 51.9%).

4-Hydroxypyrazolo[3,4-*d*]pyrimidine. This was prepared by the method of Robins (1956) (Found: C, 44.5; H, 3.2; N, 41.0. Calc. for C₆H₄N₄O: C, 44.1; H, 3.0; N, 41.2%).

Detection of analogues and their derivatives by paper chromatography

Portions of extracts were chromatographed on Whatman no. 1 paper with the butan-1-ol-aq. ammonia (sp.gr. 0.88)–water (4:1:5, by vol.) solvent system, and new compounds detected by the methods shown below. The *R_F* values shown in Table 2 often varied considerably on repeating a chromatogram. For the purposes of identification, however, agreement to within 0.02 was obtained by running the samples simultaneously on the same sheet.

4-Aminopyrazolo[3,4-*d*]pyrimidine, 4-hydroxypyrazolo[3,4-*d*]pyrimidine, 2,6-diaminobenzothiazole, 2-amino-6-acetamidobenzothiazole, 5-amino-1,2-dimethylbenzimidazole and 5-acetamido-1,2-dimethylbenzimidazole. The papers were dipped in a mixture of equal volumes of 0.4% bromophenol blue in acetone, and aq. 2% silver nitrate; blue spots remained after washing the papers with water (Wood, 1955).

Ribosides. The papers were sprayed with 10% trichloroacetic acid and heated at 105° for 15 min., thus hydrolysing off the ribosyl group. After neutralizing in an atmosphere

of ammonia, the compounds were detected as described above.

5-Acetamido-2-oxobenzimidazoline. The paper was sprayed with 0.5N-nitric acid and heated to 105°, thus giving a yellow spot.

5-Amino-2-oxobenzimidazoline. This was detected by the mercuric nitrate spray method of Vischer & Chargaff (1947).

Pentose. Papers were sprayed with aniline phthalate solution and heated at 105° for 10 min. (Partridge, 1949).

Fungi

The sources of the cultures used in this work were as follows: *Aspergillus caespitosus* (Raper & Thom) A.C.C. 1216; *Cordyceps militaris* (Linn.) Link I.M.I. 58448; *Gibberella fujikuroi* Sawada strain from C.B.S. Baarn; *Mucor racemosus* (Fries) A.C.C. 546; *Mucor ramannianus* Möller A.C.C. 644; *Penicillium griseofulvum* Dierckx A.C.C. 375; *Scopulariopsis brevicaulis* (Sacc.) Bainier N.C.T.C. 580, *Trichoderma viride* Pers. ex Fries A.C.C. 211. I.M.I., N.C.T.C. and A.C.C. refer to organisms maintained by the Commonwealth Mycological Institute, the National Collection of Type Cultures and the Akers Culture Collection respectively.

Media and incubation

The medium described by Cunningham *et al.* (1951) containing 0.5% peptone (Evans), 0.3% Lab Lemco (Supplied by Oxo Ltd.) and 0.1% potassium dihydrogen phosphate with 1% glucose was used except where otherwise stated. Two other media are mentioned below; these contained inorganic salts with either potassium nitrate (medium N) or ammonium tartrate (medium A) as nitrogen source, and have previously been described in detail (Brian, Curtis & Hemming, 1947). Dextrolact, a proprietary brand of glucose, was used, except in later experiments with medium N in which it was replaced by the purer Dextrosol; both were supplied by Corn Products Ltd., 125 Strand, London, W.C. 2. Media were adjusted to pH 5.5 before autoclaving.

After inoculating with the appropriate mycelial or spore suspension, cultures were incubated at 25°.

Production of cordycepin on media containing adenine

Adenine hydrochloride and [8-¹⁴C]adenine sulphate were added to the medium to give 100 and 500 mg. of adenine/l. The medium was dispensed in 250 ml. portions in Glaxo culture vessels (Clayton, Hems, Robinson, Andrews & Hunwicke, 1944), inoculated with a mycelial suspension of *Cordyceps militaris* and incubated for 28 days.

The method of extraction of Cunningham *et al.* (1951) was used, and yields and radioactivity are shown in Table 1.

Growth of fungi on media containing purine analogues and the extraction of derivatives

Media containing 100 mg. of analogue/l. were dispensed in 1 l. portions in Glaxo culture vessels, and incubated for about 40 days. An exception to this was made with media containing 5-amino-2-oxobenzimidazole and 4-hydroxypyrazolo[3,4-*d*]pyrimidine: in these cases 100 ml. portions of medium were dispensed in 300 ml. conical flasks. Culture filtrates were stirred for at least 10 min. with 11 g.

of charcoal (British Drug Houses Ltd., activated for decolorizing purposes)/l. After the charcoal had been filtered off and dried in air at room temperature, it was eluted with acetone in column extractors (Curtis, 1956). The elution of 4-aminopyrazolo[3,4-*d*]pyrimidine riboside was facilitated by adding 20% (v/v) of water to the acetone in the flasks. Except where otherwise stated the concentrated acetone eluates were streaked on sheets of 3MM Whatman chromatography paper and developed with butan-1-ol-aq. ammonia (sp.gr. 0.88)-water (4:1:5, by vol.) solvent system (butanol-rich phase). The compounds were located in a longitudinal strip cut from the sheet, by the methods described above. The appropriate transverse strips were then eluted with acetone in a Soxhlet extractor, and the acetone eluates evaporated to dryness.

Isolation and yields of 4-aminopyrazolo[3,4-*d*]pyrimidine riboside and 4-hydroxypyrazolo[3,4-*d*]pyrimidine riboside

Eight fungi were grown on media containing 100 mg. of 4-aminopyrazolo[3,4-*d*]pyrimidine/l. Seven of these produced both ribosides; the 4-amino compound crystallized from butan-1-ol in yields up to 20 mg./l., and the 4-hydroxy compound from ethanol in yields up to 70 mg./l. With 3% Dextrolact, medium N mostly gave better yields than the peptone-Lemco medium, and medium A gave no yield of either riboside. Possibly this is associated with the fall of pH to 3-4 on medium A, in contrast with the rise to pH 7-8 on the other two media.

The remaining fungus, *S. brevicaulis*, yielded the riboside of the 4-amino compound only. In particular, when grown for 60 days on medium N with 1% Dextrosol it gave 120 mg. of riboside/l.; paper chromatography was unnecessary and the riboside crystallized on concentrating the aqueous acetone eluates from the charcoal.

The crystalline solids from different fungi were all identified by paper chromatography on three solvent systems (Table 2). The identities were confirmed by infrared spectra, except for 4-hydroxypyrazolo[3,4-*d*]pyrimidine

Table 1. Conversion of radioactive adenine into cordycepin by *Cordyceps militaris*

Adenine in medium	10 ⁻⁴ Counts/min./m-mole mg./l.	—	16.05	16.05
		0	100	500
Cordycepin isolated	10 ⁻⁴ Counts/min./m-mole mg./l.	—	4.01	11.8
		55	106	282

Table 2. *R_F* values of analogues, derivatives and hydrolysis products

	Solvent system					
	A	B	C	D	E	F
4-Aminopyrazolo[3,4- <i>d</i>]pyrimidine riboside	0.31	0.42	0.53	—	—	—
4-Hydroxypyrazolo[3,4- <i>d</i>]pyrimidine riboside	0.10	0.39	0.57	—	—	—
4-Aminopyrazolo[3,4- <i>d</i>]pyrimidine	0.63	0.55	0.55	—	—	—
4-Hydroxypyrazolo[3,4- <i>d</i>]pyrimidine	0.23	0.58	0.55	—	—	—
Ribose	0.18	0.31	0.45	0.59	—	—
2-Amino-6-acetamidobenzothiazole	0.73	0.78	—	—	0.90	—
5-Acetamido-1,2-dimethylbenzimidazole	0.79	0.78	—	—	0.7	0.2-0.5
5-Acetamido-2-oxobenzimidazole	0.64	0.67	—	—	—	0.35
2,6-Diaminobenzothiazole	0.52	—	—	—	—	—
5-Amino-1,2-dimethylbenzimidazole	0.58	—	—	—	—	—
5-Amino-2-oxobenzimidazole	0.44	—	—	—	—	—

A: Butan-1-ol-aq. ammonia (sp.gr. 0.88)-water (4:1:5, by vol.). B: Butan-1-ol-acetic acid-water (4:1:5, by vol.). C: Acetone-water (9:1, v/v). D: Phenol saturated with water. E: Ethanol. F: Water.

riboside from *M. racemosus* and *A. caespitosus*, where spectra showed that the solids probably contained this compound.

Exogenous metabolism of 4-hydroxypyrazolo[3,4-d]pyrimidine by Cordyceps militaris

On incorporating 4-hydroxypyrazolo[3,4-d]pyrimidine in the medium, riboside was not detected in the extracts. Paper chromatography showed that the precursor was present up to 17 days' incubation, but was not detected at 24 days.

Characterization of 4-aminopyrazolo[3,4-d]pyrimidine riboside and 4-hydroxypyrazolo[3,4-d]pyrimidine riboside

Neither riboside contained P, S or halogen. 10 mg. of each compound was hydrolysed by heating with 2N-hydrochloric acid (0.2 ml.) for 5 min. at 100°. After removal of Cl⁻ ions by passing through a column of Amberlite IR-45 resin, both hydrolysates were found by paper chromatography on at least three solvent systems to contain ribose, and each its 4-substituted pyrazolo[3,4-d]pyrimidine (Table 2). Pentose was estimated in the ribosides by the method of Borrow & Jefferys (1956), by using ribose standards. It was assumed that the pentose in the ribosides would be completely liberated at the commencement of the 2.5 hr. treatment with 3.7N-hydrochloric acid at 100°.

4-Aminopyrazolo[3,4-d]pyrimidine riboside. Recrystallization from water and from aqueous ethanol gave needles, m.p. 255° (Found: C, 44.8, 44.6; H, 5.1; N, 26.2; pentose 53, 55. C₁₀H₁₃N₅O₄ requires C, 44.9, H, 4.9; N, 26.2; pentose 56%).

4-Hydroxypyrazolo[3,4-d]pyrimidine riboside. Recrystallization from methanol gave fine needles, m.p. 211° (Found: C, 44.6; H, 4.6; N, 20.9; pentose, 53, 57. C₁₀H₁₂N₄O₅ requires C, 44.8; H, 4.5; N, 20.9; pentose 56%).

Isolation of derivatives produced by fungi from aromatic amines

The derivative of 2,6-diaminobenzothiazole crystallized from ethyl acetate in yields of 19 mg./l. of medium had m.p. 212–214°. That from 5-amino-1,2-dimethylbenzimidazole crystallized from ethyl acetate in yields of 49 mg./l. and had m.p. 242° after recrystallizing from chloroform and ethanol.

The derivative of 5-amino-2-oxobenzimidazole was exceptional in that paper chromatography was unnecessary; the derivative crystallized on concentrating the eluate from the charcoal, giving yields of 36 mg. of solid/l., decomposing at 330–360°.

Synthesis of acetyl derivatives of aromatic amines

The three aromatic amines were acetylated with pyridine and acetic anhydride at room temperature, giving the following compounds: (a) 2-amino-6-acetamidobenzothiazole, m.p. 238° [Found: N, 20.4; acetyl 20.0. Calc. for C₉H₈N₂OS: N, 20.3; acetyl 20.8% (for 1)]; Kaufman & Shulz (1935) recorded m.p. 239°; (b) 5-acetamido-1,2-dimethylbenzimidazole, m.p. 242°, which was not depressed on admixture with the derivative from the fungus. Pinnow & Pistor (1894) recorded m.p. 238.5; (c) 5-acetamido-2-oxobenzimidazole; this compound was recrystallized from

water; it decomposed at 362° approx. [Found: C, 56.6; H, 4.8; N, 22.0; acetyl, 26.6. C₉H₈N₂O₃ requires C, 56.5; H, 4.8; N, 22.0; acetyl 22.5% (for 1)]. The high value of the acetyl determination is thought to be due to the opening of the imidazole ring].

Identity of products from fungal culture with synthetic acetyl derivatives. Each of the three products from fungus culture was found by paper chromatography on at least three solvent systems to contain the corresponding synthetic acetyl derivative (see Table 2).

Infrared spectra showed that the fungus product from 5-amino-2-oxo-benzimidazole contained either the acetyl derivative or a structurally very similar compound. Infrared spectra showed that other two fungus products contained the corresponding acetyl derivatives.

SUMMARY

1. The fungus *Cordyceps militaris* converts exogenous adenine into cordycepin.

2. When grown on suitable media, all the eight fungi tested, including *C. militaris*, converted 4-aminopyrazolo[3,4-d]pyrimidine into a riboside and, with the exception of *Scopulariopsis brevicaulis*, into 4-hydroxypyrazolo[3,4-d]pyrimidine riboside.

3. *C. militaris* was grown on media containing 2,6-diaminobenzothiazole, 5-amino-1,2-dimethylbenzimidazole and 5-amino-2-oxobenzimidazole, and it converted these compounds into the corresponding monoacetyl derivatives.

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