

A NEW RIBOSE NUCLEOSIDE FROM *NEUROSPORA*:  
"OROTIDINE"\*

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A series of investigations using mutants of the fungus *Neurospora* has provided ample evidence that the pyrimidine orotic acid has a significant role in the biological synthesis of the ribose nucleosides uridine and cytidine.<sup>1-4</sup> By use of isotopically labeled orotic acid several investigators<sup>5-8</sup> have recently demonstrated that a similar conclusion may be drawn concerning a function of this pyrimidine in the biosynthesis of nucleic acids in animal tissues. In addition, an apparently more specialized biochemical activity of orotic acid has been shown<sup>9-12</sup> for the organism *L. bulgaricus*. Thus, it now seems evident that this pyrimidine is an important substance in biological systems in general. However, results of the earlier work with *Neurospora* mutants in this laboratory suggested that the principal pathway of biosynthesis of uridine in the mold consists of the building of the pyrimidine component stepwise onto the carbohydrate rather than of a direct coupling of ribose and uracil. As a consequence of this hypothesis, several attempts have been made to synthesize glycosides and especially a ribofuranoside of orotic acid. None of these experiments yielded a carbohydrate derivative of this pyrimidine.<sup>13</sup> However, it now appears that the desired product has been obtained by isolation from one of the uridine-requiring mutants of *Neurospora* (strain 36601). The present report is concerned with this isolation and with some of the properties of the crystalline cyclohexylamine salt of the orotic acid riboside.

*Experimental.—Growth of Mutant 36601:* The mutant strain (*Neurospora crassa*, 36601) was grown from conidial inocula in 35-liter cultures at 25°C. with forced aeration for a period of 4½ days. The minimal medium<sup>11</sup> was supplemented with 500 mg. of cytidine sulfate (per 35 liters). The mycelium thus obtained was filtered off through cheese cloth and after being washed with 500 ml. of distilled water was squeezed out thoroughly by hand. (Wet weight, 200 g.)

*Extraction and Isolation:* The moist mold was chopped into small pieces, suspended in 100 ml. of water and 200 ml. of 95% alcohol and maserated for 10 minutes in a Waring blender. The resulting slurry was heated at 80° for 30 minutes and allowed to stand overnight. After filtering and washing the residual mycelium with 200 ml. of hot 50%

ethanol, 5 g. of barium acetate was added to the filtrate, and the solution was adjusted to pH 8.5 with barium hydroxide. The resulting precipitate was removed and washed by centrifugation, and the supernatant solution (containing approximately 1.2 gm. of glycoside) was evaporated under reduced pressure to 500 ml. The orotidine was then precipitated by addition of a saturated solution containing 10 gm. of lead subacetate. The precipitate was centrifuged, washed with water, suspended in 100 ml. of water and decomposed with  $H_2S$ . The filtrate from the lead sulfide contained 1.05 gm. of orotidine (approximately 60% pure). This material was purified further by fractionation in the chromatopile,<sup>10</sup> using 12.5 cm. S & S filter paper (No. 598) and a developing solvent composed of 3 parts of propanol to 1 part of water. After location of the riboside by

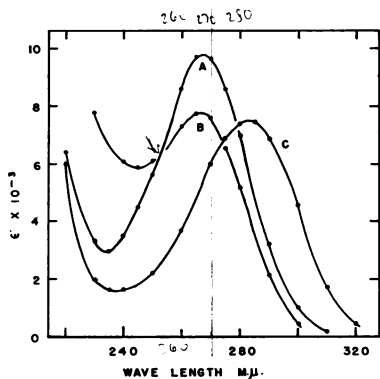


FIGURE 1

Absorption spectra of orotidine (curve A) and orotic acid (curve C) in 0.1 *N* HCl and orotidine in 0.1 *N* NaOH (curve B).

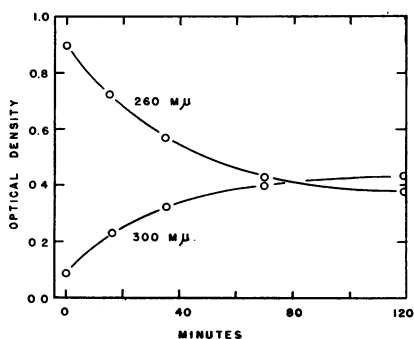


FIGURE 2

Rate of hydrolysis of orotidine in 0.5 *N*  $H_2SO_4$  at 100°C. Aliquots were taken at intervals from the hydrolysis mixture (1 mg./ml. of cyclohexylamide salt) and diluted to 40  $\gamma$ /ml. in 0.1 *N* HCl for the measurements of optical densities.

absorption spectra measurements, the substance was extracted from the paper with hot water. An aliquot of the resulting solution (containing 200 mg. of orotidine) was evaporated *in vacuo* in the presence of 500 mg. of cyclohexylamine. The resulting syrup was dissolved in 3 ml. of ethanol, and 10 ml. of benzene was then added. After two days at 5°C. the white crystalline product (210 mg.) was filtered and air dried. The salt was recrystallized from 10 ml. of hot 95% ethanol plus enough benzene to produce a slightly turbid solution. The product was dried over  $P_2O_5$  at 60°C.

The recrystallized cyclohexylamine salt of orotidine gave the following analyses: Found %: C, 49.54; H, 6.68; N, 11.09. Calculated % for  $C_{10}H_{25}N_3O_8$ : C, 49.66; H, 6.52; N, 10.85. Pentose (orcinol): Found %:

38.7, 38.6, 37.5. Calculated %: 38.8. M.-P.: 183–184°C. (corr.). Absorption spectra: Figure 1.

*Degradation Products:* Unlike the ribosides of uracil and cytosine, the isolated riboside of orotic acid hydrolyzes readily in dilute mineral acid. A rate curve is shown in figure 2. The data were obtained by measurement of the changes in absorption spectrum at wave-lengths of 260 and 300 m $\mu$ . (See Fig. 1.)

In order to identify the degradation products, a sample of the cyclohexylamine salt (120 mg.) was heated at 100° for one hour with 2.5 ml. of 1 *N* H<sub>2</sub>SO<sub>4</sub>. After the solution was allowed to cool, the crystalline precipitate of orotic acid monohydrate was centrifuged, washed 3 times with distilled water (1 ml. each time) and dried at room temperature over CaCl<sub>2</sub>. The product (47 mg., 85% yield) was recrystallized from boiling water and dried over P<sub>2</sub>O<sub>5</sub> at 100°C. This product gave the following analyses: Found %: C, 38.53; H, 2.74. Calculated % for C<sub>6</sub>H<sub>4</sub>N<sub>2</sub>O<sub>4</sub>: C, 38.48; H, 2.56. M.-P.: 343–344°C.<sup>14</sup> The product was identical with a known sample of orotic acid with respect to absorption spectra in acid and alkali and to movement on paper chromatograms in several solvents.

The supernatant solution from the orotic acid isolation was utilized for experiments on paper chromatography of the sugar component. Using Whatman No. 1 paper and a solvent mixture of 5 parts of propanol to 1 part of 1% NH<sub>4</sub>OH the unknown sugar gave an R<sub>f</sub> value of 0.33  $\pm$  0.01. Known sugars tested simultaneously gave the following R<sub>f</sub> values: Ribose, 0.33; xylose, 0.29; arabinose, 0.26; glucose, 0.19; galactose, 0.18, mannose, 0.23. Mixing the unknown with the known sugars did not affect the R<sub>f</sub> values found. Similar results were obtained by chromatography in other solvents. Benzidine<sup>15</sup> and naphthoresorcinol<sup>16</sup> reagents were used to locate the sugars on the paper. Typical pentose reactions were given by the unknown sugar.

Confirmation of the identity of the pentose component was obtained by applying the method of Ikawa and Niemann.<sup>17</sup> Absorption spectra obtained after heating a known mixture of ribose and orotic acid in 80% H<sub>2</sub>SO<sub>4</sub> for 30 minutes were found to be identical with spectra obtained from an equivalent quantity of cyclohexylamine riboside after the same treatment. The orotic acid is nearly destroyed by the treatment and does not seriously interfere with the determination.

*Discussion.*—On the basis of the data presented here, there remains little doubt that the substance isolated from *Neurospora* is a riboside of orotic acid although some further work is necessary to make the ribose identification unequivocal. In connection with the problems of structure, the unexpected lability of the glycoside to acidic hydrolysis merits consideration. It is possible that this property indicates the presence of an

oxygen rather than a nitrogen (position 3) glycoside, but on the other hand, this relative instability may be due to the presence of the adjacent carboxyl group on the pyrimidine ring. The latter explanation seems the more probable, and it is consistent with the fact that attempts to couple dialkoxy orotic acids and esters with acetobrom sugars have failed to yield glycosides under conditions that are satisfactory with diethoxyuracil.

Further questions concerning structure, synthesis and biological activity of the isolated orotic acid glycoside will be considered in subsequent communications.

*Summary.*—1. Some of the properties of a new glycoside of orotic acid and its isolation from *Neurospora* are described. The pure substance was obtained as a cyclöhexylamine salt. 2. Evidence is presented to show that the substance is a pentoside and that the pentose is ribose.

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