THE BIOSYNTHESIS OF HISTIDINE

IMIDAZOLEGLYCEROL PHOSPHATE, IMIDAZOLEACETOL PHOSPHATE, AND HISTIDINOL PHOSPHATE

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(Received for publication, August 2, 1954)

In a previous communication on the genetics of some histidine-requiring mutants of *Neurospora crassa* (1), it was reported that the order of the mutants in the biosynthetic pathway is

$$\rightarrow X \xrightarrow{\text{C84}} Y \xrightarrow{\text{C141}} Z \xrightarrow{\text{T1710}} \text{histidine}$$

It was later shown (2) that the mutant C84 accumulates imidazoleglycerol[1] in the growth medium, that mutant C141 accumulates imidazoleglycerol and imidazoleacetol,[2] and that T1710 accumulates L-histidinol.[3] It was also observed that none of these compounds will replace histidine in promoting the growth of these mutants or other mutants blocked earlier in the sequence. This suggested that these imidazole compounds might be derived from biosynthetic intermediates such as phosphate esters to which *Neurospora* mycelium is impermeable. Preliminary evidence that certain of these mutants do accumulate phosphorylated imidazoleglycerol and imidazoleacetol has been presented (2).

This is a report on the isolation and characterization of D-*erythro*-imidazoleglycerol phosphate (IGP), imidazoleacetol phosphate (IAP), and L-histidinol phosphate, which are accumulated in the mycelia of several of these mutants.

EXPERIMENTAL

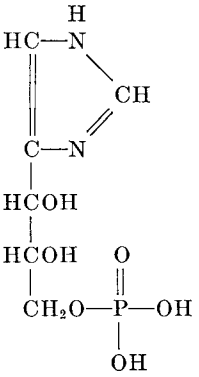
Isolation of Phosphate Esters—Mutant C141 was grown in 16 liter carboys of the Fries minimal medium, supplemented with 450 mg. of L-histidine monohydrochloride monohydrate as described previously (2). After

* Part of this work was done as a Predoctoral Fellow of the Atomic Energy Commission at the California Institute of Technology, and it was supported in part by a grant from the Williams-Waterman Fund for combat of dietary diseases. Postdoctoral Fellow of the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

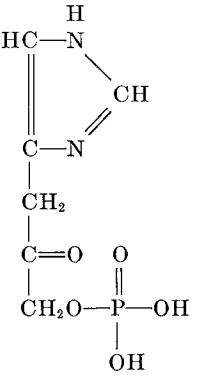
[1] 4-(D-*erythro*-Trihydroxypropyl)imidazole.

[2] 4-(2-Keto-3-hydroxypropyl)imidazole.

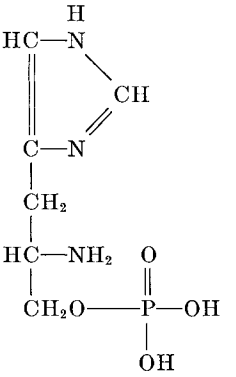
[3] L-4-(2-Amino-3-hydroxypropyl)imidazole.



Imidazoleglycerol phosphate (IGP)



Imidazoleacetol phosphate (IAP)



Histidinol phosphate

4 days growth, the culture was filtered through cheese-cloth, and the mycelium washed with distilled water, squeezed dry, and frozen in dry ice. The frozen mycelium was broken up with a hammer, and a mixture of the mycelium pieces and dry ice was reduced to a fine powder in a Waring blendor. The still frozen powder was then slowly added to boiling water (about 200 ml. per 100 gm. of squeezed mycelium) to extract the esters. *Neurospora* is rich in phosphatases, and it is important to keep the mycelium powder frozen until it is added to the boiling water. After simmering for several minutes to destroy the phosphatases and to coagulate the protein, the mixture was centrifuged and reextracted with half again as much boiling water. The combined supernatant solutions were brought to pH 9 with ammonia, filtered, and put on a quaternary ammonium salt ion exchange resin (Dowex 1 formate) which had been washed with dilute aqueous ammonia to insure the binding of all the histidinol phosphate. A typical elution diagram is given in Fig. 1. The phosphate esters were held back on the Dowex column while the dephosphorylated imidazoles, which make up the bulk of the accumulated imidazole compounds, passed through. The Pauly test, used for assaying the imidazoles, is only semiquantitative, as each imidazole gives a slightly different color. Tyrosine gives a Pauly test and appears as a small peak in front of the IGP-IAP peak. Imidazoleacetol phosphate is eluted directly after the imidazoleglycerol phosphate, and, as is the case in the experiment shown in Fig. 1, sometimes these appear as a single peak.

Further purification of the compounds was accomplished on Dowex 1 chloride columns. The fractions comprising each peak in the formate column eluate were combined and then lyophilized until all of the ammonium formate was removed. Each fraction was taken up in water, brought to pH 9 with ammonia, and adsorbed on an 82 × 2.5 cm. Dowex 1 chloride

column which had been previously washed with water until the effluent was less than 0.0002 N in acid. Gradient elution was used with 0.02 N HCl in the reservoir and 2 liters of water in the mixing chamber. The IAP-IGP peak was fairly well resolved on this column, and the two esters were concentrated by lyophilizing, and each further freed of traces of the other ester by repeating the chromatography on Dowex 1 chloride. The elution may be followed with either the quantitative diazo test, as in Fig. 1, or with the same reagent in a spot plate. Specific quantitative methods for the determination of imidazoleacetol phosphate and imidazoleglycerol phosphate

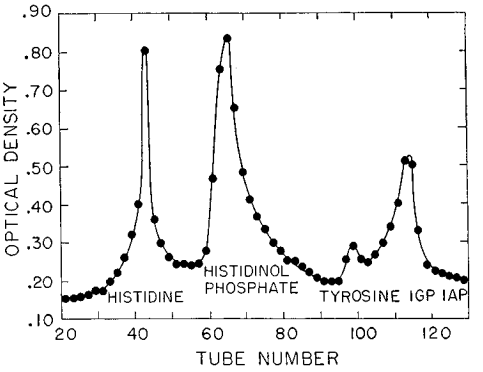


FIG. 1. Separation of the acidic components of an extract of 235 gm. of C141 mycelium. The resin column used was a 3.5 × 42 cm. Dowex 1 (10 per cent cross-linked) formate column, which was prewashed with a liter of aqueous ammonia (pH 9). The eluted fractions (28 ml. per tube) were assayed for imidazole compounds by the Pauly test. To an 0.5 ml. aliquot in a colorimeter tube were added 0.5 ml. of 5 per cent sodium carbonate and then 0.5 ml. of diazosulfanilic acid reagent (3). The reaction was allowed to proceed for 5 minutes, and then 5 ml. of 5 per cent sodium carbonate were added and the tubes read in a Coleman junior spectrophotometer at 480 mμ.

are described in subsequent sections, and these methods were also used in following the purification.

Mutant C141 accumulates all three esters while mutant C84, which is blocked earlier in the sequence, accumulates only imidazoleglycerol phosphate. No phosphate esters were observed in the mycelium of mutant T1710, perhaps because of very active phosphatases. A double mutant C141-T1710 was found, like C141, to accumulate all three esters.

Paper Chromatography of Accumulated Imidazole Compounds

The paper chromatography of imidazoles and the use of a diazosulfanilic acid indicator spray have been described previously (3) and the R_F values of some of these compounds in propanol-ammonia and propanol-acetic acid

solvents have been listed (2). In Table I are the R_F values for the various isolated phosphate esters and dephosphorylated compounds. After a chromatogram has been sprayed with the diazosulfanilic reagent and sodium carbonate, a 5 per cent copper sulfate spray changes the red spots of imidazoleacetol and imidazoleacetol phosphate to dark green. None of the other compounds will reduce copper.

Imidazoleglycerol Phosphate—The ester was purified by chromatography on two Dowex 1 chloride columns. The column effluent containing the ester was lyophilized and the residue dissolved in a small amount of water. This solution was used in further studies.

TABLE I

R_F Values of Various Diazo-Reacting Compounds from Neurospora

Substance	Formic acid solvent	Formix solvent
Histidinol phosphate	0.26	0.12
Imidazoleglycerol phosphate	0.27	0.15
Histidine	0.37	0.16
Imidazoleacetol phosphate	0.39	0.19
Histidinol	0.55	0.38
Imidazoleglycerol	0.63	0.39
Tyrosine	0.70	0.53
Imidazolepropanediol	0.72	0.51
Imidazoleacetol	0.73	0.47

Ascending chromatograms in methanol-chloroform-10 per cent formic acid (3:3:1) and in *t*-butanol-50 per cent formic acid (7:3) (Formix).

A barium salt was made from part of this solution by neutralizing it to a brom thymol blue end-point with a saturated barium hydroxide solution. The salt was precipitated with 4 volumes of ethanol and 2 volumes of acetone. The barium salt had a ratio of 1.03 P to 2.00 N atoms.[4]

On paper chromatography of the ester (Table I), only one spot was apparent with the diazosulfanilic spray or a phosphate ester spray (4). On treatment with a highly purified potato phosphatase[5] for 10 minutes at pH 5, or on hydrolysis in 6 N HCl at 100° overnight, a new compound appeared with the same R_F value as imidazoleglycerol in the propanol-ammonia, propanol-acetic acid solvents (2), and in the formic acid solvent (Table I). The stability of this compound to acid hydrolysis suggested that the phosphate group was esterified on the primary hydroxyl group. This was es-

[4] Analysis carried out in the Microanalytical Laboratory under the direction of Dr. W. Alford.

[5] Prepared in conjunction with Dr. A. Weissbach and Dr. G. Ashwell according to an unpublished procedure of Dr. A. Kornberg.

tablished by treating the imidazoleglycerol phosphate with sodium metaperiodate. Both imidazoleglycerol and imidazoleglycerol phosphate, on treatment with periodate, yield imidazoleformaldehyde,[6] which was identified by its absorption maxima at 237 mμ (ϵ = 7100) in 0.1 N HCl, 256.5 mμ (ϵ = 11,800) at pH 7.0, and 280 mμ (ϵ = 14,900) in 0.01 N NaOH (5). Neither imidazoleglycerol nor imidazoleglycerol phosphate has any absorption above 240 mμ. The imidazoleformaldehyde was also identified by its R_F value in several solvents (3). Periodate attacks a glycol only if it is unesterified (6), and consequently the phosphate cannot be esterified with the secondary hydroxyl groups. In the periodate oxidation of imidazoleglycerol phosphate, unlike that of imidazoleglycerol, no formaldehyde is liberated, indicating that it is the primary hydroxyl group which is esterified.

A sensitive assay for imidazoleglycerol and its phosphate ester has been based on the formation of imidazoleformaldehyde in the periodate reaction. To 0.05 ml. of sample (0.01 to 0.1 μM) in 4 per cent perchloric acid is added 0.5 ml. of 0.1 N sodium metaperiodate. The oxidation is complete after 30 minutes at room temperature, at which time 1.0 ml. of 10 per cent ethylene glycol is added to remove the excess reagent and thus the ultraviolet absorption caused by the periodate. After 10 minutes, 2 ml. of 0.05 N NaOH are added and the absorption read at 280 mμ.

The configuration of the asymmetric carbon atoms in imidazoleglycerol was not known when the previous paper on the structure of the unesterified imidazoles was written (2). L-*erythro*-Imidazoleglycerol had been synthesized from L-arabinose but could not be crystallized. The D-*erythro* isomer has now been synthesized from D-arabinose by the same procedure. A syrup of the synthetic D-*erythro* isomer crystallized when a seed crystal of imidazoleglycerol isolated from *Neurospora* was added. The crystals were triturated with cold acetone and recrystallized from absolute ethanol. Only about 50 mg. were obtained from 10 gm. of arabinose. No attempt was made to try to improve the synthesis so that a better yield might be obtained. In the analogous reaction with hexoses, the expected optically active imidazole compound is obtained in each case, despite the low yields reported (7). The compound isolated from *Neurospora* was judged, on the basis of the following criteria, to be identical with the synthetic compound and thus to have the D-*erythro* configuration. The data are for the monohydrochlorides.

Analysis of the synthetic compound,

$C_6H_{10}N_2O_3 \cdot HCl$.	Calculated.	C 37.10, H 5.67, N 14.43
	Found.	" 37.15, " 6.09, " 14.24

[6] A sample of this compound was kindly obtained from Dr. H. Tabor.

Neurospora compound, m.p., 103–103.5°; synthetic D-*erythro* compound, m.p., 102.5–103°; mixed m.p., 102.5–103°.

The optical rotation of the *Neurospora* compound was $[\alpha]_D^{25.6}$ +13.3° (*c* 7.5, water); of the synthetic compound, $[\alpha]_D^{25.6}$ +13.0° (*c* 7.5, water). The order of magnitude and the sign of the molecular rotation are consistent with Richtmyer's benzimidazole rule for D-*erythro* compounds (8).

Both compounds formed rosettes of prismatic needles upon crystallization. The R_F on paper chromatograms of the synthetic compound was identical with that of the isolated compound in each of the several solvents used. The two compounds show the same behavior on the Dowex 50 column (2).

Imidazoleacetol Phosphate—Following isolation from the Dowex 1 formate column, this ester was purified by passage through two Dowex 1 chloride columns as described. The compound gave a single spot on chromatograms when sprayed with diazosulfanilic acid, with the copper sulfate reagent, or with the Bandurski-Axelrod phosphate ester spray (4). On treatment with purified potato phosphatase, or with 4 N HCl for 30 minutes at 100°, the compound is completely hydrolyzed to imidazoleacetol, as indicated by its R_F in the solvents described and its absorption spectrum in alkali. Imidazoleacetol has no ultraviolet spectrum in neutral or acidic solution above 240 mμ. However, when alkali is added, a peak appears at 370 mμ (ϵ = 10,400). The appearance of the 370 mμ peak is presumably due to enolization of the carbonyl function and consequent conjugation with the imidazole ring. Compounds which have this conjugation, such as imidazoleacrylic acid (urocanic acid), show strong ultraviolet absorption (9). The time curve for appearance of this 370 mμ absorption is given in Fig. 2. The slow disappearance after the maximum is reached may be due to cyclization or polymerization. Imidazoleacetol phosphate does not give this 370 mμ peak in alkali, but, following removal of the phosphate group by acid hydrolysis, a typical imidazoleacetol behavior in alkali can be shown (Fig. 2). The ease of hydrolysis of the phosphate ester is as would be expected from a phosphate ester with an adjacent carbonyl function. Methyl tetrose-1-phosphate, for example, is half hydrolyzed after 30 minutes at 100° in 1 N HCl (10).

The appearance of ultraviolet absorption has been used for a specific quantitative method for imidazoleacetol phosphate. To a test-tube containing 0.2 ml. of sample in 4 per cent perchloric acid (or in water) is added 0.1 ml. of concentrated HCl. The tube is capped with a marble and heated for 30 minutes in a steam bath to hydrolyze the ester. Then 3 ml. of 3 N sodium hydroxide[7] are added to the cooled tube, and readings at 370

[7] Weak alkali does not give as strong an absorption, presumably because a proton is still present on one of the nitrogen atoms. The imino group has a pK of 13 in urocanic acid (9).

mμ are taken every minute until the absorption reaches a maximum (Fig. 2). A sodium hydroxide blank is used.

The ester contains one phosphate group for each imidazoleacetol group, as determined from a hydrolysis curve in dilute acid (Fig. 3). The ratio of inorganic phosphate to free imidazoleacetol was 1.0 at 1.5 hours, 1.1 at 2.5 hours, 1.0 at 5.5 hours, and 1.1 at 9.5 hours, at which time the compound was completely hydrolyzed.

Histidinol Phosphate—This compound was purified on several Dowex 1 chloride columns as described. Its early elution from Dowex 1 columns is

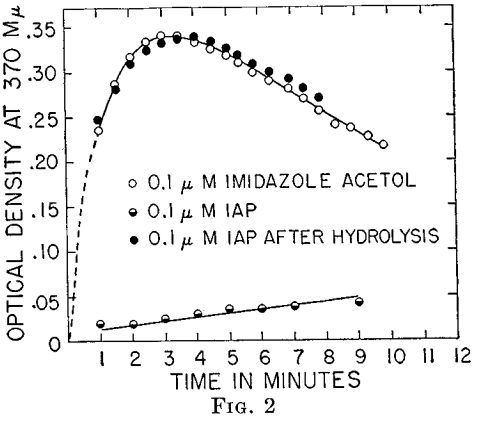


FIG. 2

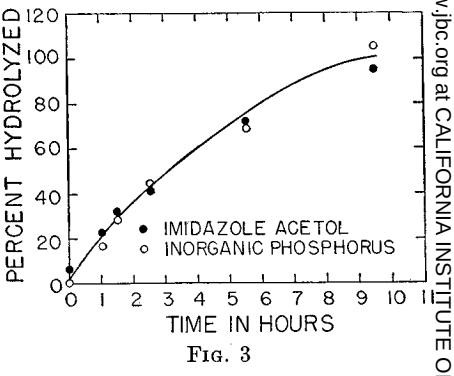


FIG. 3

FIG. 2. Enolization of imidazoleacetol in strong alkali. At zero time, 3.0 ml. of 3 N sodium hydroxide were added to 0.1 μmole of imidazoleacetol or imidazoleacetol phosphate in 0.1 ml. of water. Imidazoleacetol phosphate (0.1 μM) in 0.2 ml. of water and 0.1 ml. of concentrated HCl were heated for 30 minutes on a steam bath to hydrolyze the phosphate ester. 3.0 ml. of sodium hydroxide were added at zero time.

FIG. 3. Hydrolysis of imidazoleacetol phosphate (0.11 μmole per ml.) in 0.1 N HCl at 100°. Inorganic phosphate was measured by the Fiske-Subbarow procedure (11) and imidazoleacetol by the 370 mμ absorption in alkaline solution.

consistent with the behavior expected from an α-amino phosphate ester. A single spot is apparent with ninhydrin, the diazo reagent, or the phosphate ester reagent. On hydrolysis by acid or phosphatase, a compound having the same R_F as histidinol appears. Treatment with nitrous acid gave a new compound which, when hydrolyzed with phosphatase, had the same R_F as imidazolepropanediol (2). This indicates that it is the hydroxyl group rather than the amino group which is esterified.

The phosphate ester is not active with Adams' purified L-histidinol dehydrogenase (12). On HCl hydrolysis, however, a compound is formed which does reduce DPN in the presence of this enzyme.[8] This enzyme is apparently specific for L-histidinol and is inactive with D-histidinol.

[8] We are indebted to Dr. E. Adams for testing these compounds with his enzyme preparation and for a generous sample of synthetic L-histidinol.

Histidinol phosphate has been synthesized by reaction of L-histidinol with polyphosphoric acid[9] by the method used by Cherbuliez and Weniger (13) for synthesizing ethanolamine phosphate. Approximately 50 per cent yield of a hygroscopic glass was obtained. Most of the unchanged histidinol could be recovered on passage through a Dowex 1 chloride column. This synthetic compound has the same R_F as the isolated compound in the solvents used. Both the synthetic and isolated phosphate esters can be almost completely converted to imidazoleacetol phosphate by an enzyme from *Neurospora* in the presence of α-ketoglutarate. This reaction is reversible and does not work with the unphosphorylated compounds (14). This serves as further proof of structure.

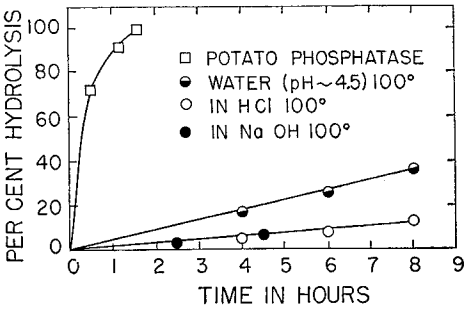


FIG. 4. Hydrolysis of histidinol phosphate. Each solution contained 4.2 μmoles of histidinol phosphate per ml. The alkaline hydrolysis was carried out in nylon test-tubes, as silicate interferes in the Fiske-Subbarow procedure (11) for inorganic phosphate, which was used in each case. The incubation of potato phosphatase was carried out in acetate buffer at pH 5.0 in the presence of 0.01 M $MgCl_2$.

The hydrolysis curves of histidinol phosphate in 1 N NaOH, 1 N HCl, and in water (pH 4.5) (Fig. 4) correspond closely with those reported by Cherbuliez and Bouvier (15) for ethanolamine phosphate. Both of these compounds are extremely resistant to acid and alkaline hydrolysis, but are more readily hydrolyzed at pH 4.5.

DISCUSSION

The finding that imidazoleglycerol, imidazoleacetol, and histidinol are accumulated by various histidine mutants of *Neurospora*, combined with information on the order of the mutants in the biosynthesis scheme, implicated these compounds in the biosynthesis of histidine (1, 2). Consequently, the phosphate esters of these compounds which have been identified and shown to be accumulated in the mycelia of the mutants are likely

[9] Sample obtained from the Monsanto Chemical Company as tetraphosphoric acid.

biosynthetic precursors of histidine. This cannot be tested by growth experiments, since *Neurospora* is impermeable to phosphate esters. However, evidence has been obtained for an enzyme in cell-free extracts of *Neurospora* which converts imidazoleglycerol phosphate to imidazoleacetol phosphate, and for a second enzyme which transaminates imidazoleacetol phosphate to histidinol phosphate in the presence of pyridoxal phosphate and glutamate (14). Thus, it appears that these phosphate esters are actually intermediates. The inactive non-phosphorylated imidazole compounds which appear in the growth medium are probably formed from these accumulated phosphate esters by the action of various phosphatases present in *Neurospora* mycelium.

A path of conversion of histidinol phosphate to histidine is suggested by the work of Adams (12) and of Vogel, Davis, and Mingioli (16), which indicates that histidinol is on the biosynthetic pathway in *E. coli*. Vogel and coworkers showed L-histidinol to be accumulated by one *E. coli* histidineless mutant and utilized by another. Adams has purified a diphosphopyridine nucleotide (DPN) enzyme from yeast, from *Arthrobacter*, and from *E. coli*, which will oxidize L-histidinol to histidine. This enzyme is not found in those histidine-requiring *coli* mutants which are unable to use histidinol for growth. Histidinol phosphate probably precedes histidinol on the pathway of histidine synthesis. A biosynthetic pathway consistent with the *Neurospora* and *E. coli* data would be imidazoleglycerol phosphate → imidazoleacetol phosphate → histidinol phosphate → histidinol → histidine. However, none of the *Neurospora* mutants will use histidinol to replace their histidine requirement. This might be a question of permeability, or it may be that in *Neurospora* the phosphate group is removed after the final oxidation.

The D-*erythro* configuration of imidazole glycerol is the same as D-ribose, and this suggests that a compound such as ribose-5-phosphate may be the early histidine precursor in *Neurospora*. The ease of chemical synthesis of imidazoleglycerol and of other polyhydroxy imidazoles from sugars is of interest in this regard (2, 14).

It would appear that mutant C141 is blocked at a stage between histidinol phosphate and histidine and that mutant C84 is blocked between imidazoleglycerol phosphate and imidazoleacetol phosphate. This question will be discussed more fully in connection with the experiments on enzymatic interconversion of these compounds.

SUMMARY

Three phosphate esters accumulated by several histidine-requiring mutants of *Neurospora* have been characterized as D-*erythro*-imidazoleglycerol phosphate, imidazoleacetol phosphate, and L-histidinol phosphate. Spe-

cific methods for the determination of the first two of these esters are given, as well as isolation procedures for all the esters. L-Histidinol phosphate has been synthesized from L-histidinol, and D-*erythro*-imidazoleglycerol has been synthesized from D-arabinose.

The properties of these esters are discussed, as well as the evidence pointing to their being intermediates in histidine biosynthesis.

The senior author wishes to express his appreciation to Dr. B. L. Horecker, in whose laboratory much of this work was carried out, for his encouragement, hospitality, and many helpful suggestions.

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