THE BIOSYNTHESIS OF HISTIDINE IN *NEUROSPORA CRASSA*

Thesis by

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ABSTRACT

The biosynthesis of histidine has been investigated using a series of histidine requiring mutants of Neurospora and Penicillium. A general method is given for the isolation of a number of imidazole derivatives that are accumulated by these mutants. Methods of degradation and chromatography have been used to identify with reasonable certainty three crystalline products as 4-(D-erythro-trihydroxypropyl)-imidazole, 4-(2-keto-3-hydroxypropyl)-imidazole and L-4-(2-amino-3-hydroxypropyl)-imidazole (L-histidinol). Evidence is presented to show that two other non-crystalline products are phosphate esters of the trihydroxy and the ketohydroxy compounds. Chromatographic evidence is presented which indicates two other compounds are imidazole acetic acid and 4-(2,3-dihydroxypropyl)-imidazole. A chemical synthesis of isomers of 4-(trihydroxypropyl)-imidazole is described.

The trihydroxy, ketohydroxy, and aminohydroxy compounds have been placed in that order leading to histidine, on the basis of the sequence of the mutants and the accumulations by the double mutants. Evidence is discussed suggesting that the phosphate esters of these compounds are more likely to be the true precursors of histidine. A scheme is postulated for the relation of all these substances to histidine synthesis and the possibility that a pentose-5-phosphate may be an early precursor of histidine is discussed.

A method for the paper chromatography of imidazoles is presented and the Rf values of numerous synthetic and natural imidazole derivatives are given.

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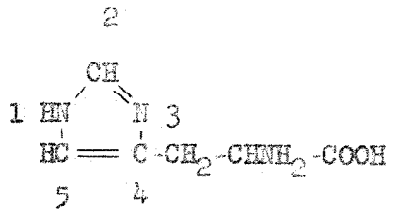
I. INTRODUCTION

Almost all plants and some animals have the ability to synthesize L-histidine, one of the amino acid components of protein. This study was undertaken in an attempt to obtain evidence regarding the mechanism of this synthesis. The use of mutant organisms has proved to be very useful in studies of this type and in this investigation a series of histidine-requiring mutants of Neurospora crassa was used to help elucidate the normal pathway of synthesis.

II. THE METABOLISM OF HISTIDINE

Chemistry

L-Histidine was first isolated from a protamine hydrolysate by Kossel (1) in 1896. He prepared the compound from the fraction precipitated by mercuric chloride. Hedin (2) independently isolated the silver salt of histidine from a hydrolysate of another protein the same year. Pauly (3) subsequently demonstrated that histidine is a 4(5)* substituted imidazole of the structure:



He also discovered that imidazoles form azo dyes with diazobenzenesulfonic acid, and this reaction has been widely used as a basis for the colorimetric determination of histidine. The first synthesis of histidine was accomplished by Pyman (4), by condensing 4-chloromethyl imidazole with the sodium derivative of the diethyl ester of chloromalonate, followed by hydrolysis, decarboxylation, and treatment of the α-chloro imidazole propionic acid intermediate with ammonia.

L-Histidine as a nutritionally essential amino acid.

The early chemical work on histidine was given new impetus when histidine was discovered to be an essential amino acid for many

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*By convention the numbering starts with the hydrogen-bearing nitrogen atom. Since the ring undergoes tautomerism, the two nitrogens are equivalent in imidazole, and an imidazole with a substituent at the 4 position is thus often described as 4(5)-substituent-imidazole.

of the higher animals. Numerous investigators tested all types of histidine derivatives and imidazoles for activity as a substitute for histidine. One of the earliest of these studies was that of Ackroyd and Hopkins (5) who reported that either arginine or histidine is required in the diet of rats. They concluded the two amino acids are interchangeable in metabolism. Rose and Cox (6, 7) completely confirmed the finding that histidine is an indispensable growth factor for the rat. They discovered, however, that arginine will not serve as a substitute for histidine. Following this lead various investigators showed that histidine is a nutritional essential in many higher animals as well as in various microorganisms. These include the mouse (8), the chick (9), the dog (10), Tetrahymena (11), and Streptococcus faecalis (12). Man, in contrast to the other higher animals tested, has been shown by Rose et al. (13, 14), to maintain normal nitrogen equilibrium even when histidine is not included in the diet. This unexpected finding indicates that either man or his intestinal flora can synthesize a sufficient amount of this amino acid for normal metabolic processes. Work by Levy and Coon (15) suggests that probably man himself has at least some of the enzymes necessary for histidine synthesis. They found that human liver slices incorporate radioactive formate into histidine.

As the rat was the first animal shown to require histidine in the diet and is one of the most common experimental animals, it has been the animal of choice in most experiments on the structural specificity of histidine as a growth factor. Neuberger and Webster (16), using young rats on a histidine-deficient diet, studied a number of compounds to see if they would restore normal growth. Ornithine and α-keto ornithine, two compounds which they thought might be biological precursors, were fed

to these rats, but they did not serve as substitutes for histidine. Various ring substituted imidazoles were also tried. 2-Thiolhistidine, which is easily oxidized to histidine by ferric chloride, and ergothioneine, its betaine, were found to be inactive, as was N^1-methyl histidine (17), which is present in vertebrate muscle as anserine, a peptide of β-alanine. It is apparent that the rat's enzymes exhibit a remarkable specificity for an unsubstituted imidazole ring in histidine. The side chain, however, can be modified considerably without destroying the growth factor activity. D-Histidine, imidazole pyruvic acid, and imidazole lactic acid will support growth of rats on a histidine-free diet (18, 19, 20). It has been shown that in mice a small amount of L-histidine is necessary for the utilization of imidazole lactic acid or D-histidine (21). These compounds are probably all involved in the pathway of conversion of the D to the L amino acid. In general certain substituents on the alpha amino group of amino acids do not destroy growth factor activity. For example the N^{α}-acetyl (16) and N^{α}-methyl (22) derivatives of histidine are equivalent to the parent compound in the diet. Urocanic acid (imidazole acrylic acid), an intermediate in the degradation of histidine, is inactive, as are imidazoles with shorter side chains.

Hogg and Elliott (23) have investigated various compounds as substitutes for histidine in the basal medium of Tetrahymena geleii, a protozoan. L-carnosine (β-alanyl histidine) is transformed into histidine by this organism as it is in higher animals. Imidazole lactic acid is not active alone, but will spare histidine when the latter is fed at low levels.

The biosynthesis of histidine.

Through the feeding of histidine derivatives to higher animals, much information has been collected on the ability of various animals to convert related compounds to the amino acid. The information gathered, however, has not helped appreciably in understanding how this amino acid is synthesized in organisms not requiring an external source of histidine. In more recent years micro-organisms have been used with greater success in studying the problem. Broquist and Snell (12) investigated histidine synthesis in the lactic acid bacteria, Lactobacillus arabinosus, Streptococcus faecalis, and L. casei. They found that imidazole pyruvic acid will replace histidine when pyridoxal is added to the basal medium, but not when the vitamin is absent. Purines or histidine will stimulate L. arabinosus in the presence of pyridoxal, while only histidine will in the absence of pyridoxal. The requirement of L. casei for purine bases is increased when grown in the absence of histidine. They concluded that pyridoxal is involved in the conversion of imidazole pyruvic to histidine, presumably by transamination, for which pyridoxal phosphate is the coenzyme. They also concluded that purines are precursors of histidine. Imidazole pyruvic acid was believed to be a precursor of histidine and to be on the path of conversion of purines to histidine. Imidazole lactic does not replace histidine for these bacteria, whether they are grown with or without pyridoxal.

A partial clarification of the role of purines in histidine synthesis as well as the origin of the amidine carbon of the ring (carbon 2) resulted from the experiments of Levy and Coon (24) using C^{14} labeled substrates.

These investigators grew yeast (Sacc. cerevisae) on a medium containing glucose and a small amount of formate-C^{14} as essentially the only carbon sources. The yeast was grown for twelve hours, harvested, and then its protein was isolated and hydrolyzed. The hydrolysate was fractionated on an ion exchange column from which the histidine, glutamic acid, arginine, aspartic acid, and tyrosine were isolated. Only histidine had appreciable radioactivity and this was all in the 2 carbon atom. The experiment was repeated twice more, with glycine-1-C^{14} in place of the formate in one case, and bicarbonate-C^{14} in the other. In the experiment with labeled glycine all of the above amino acids had negligible radioactivity. When labeled bicarbonate was used only the isolated aspartic acid had any radioactivity, and that was very slight. These experiments indicate that the 2 carbon atom of histidine comes fairly directly from formate, and that glycine is not involved in the synthesis of the imidazole ring in histidine. Yeast has been shown to incorporate glycine directly into the purine imidazole ring. The non-incorporation of glycine into the histidine imidazole ring indicates that this ring is synthesized by a different mechanism and that the purine imidazole ring is not a precursor of the histidine imidazole. Tabor et al. (25) working with Torulopsis utilis, a yeast, have found that, as in Saccharomyces, formate-C^{14} is incorporated into the 2 carbon of histidine. Purine catabolism is known to furnish formate and a possible explanation of the results of Broquist and Snell is that purines serve as formate donors. It would be of interest to see whether formate is equivalent to purines in sparing histidine for L. arabinosus, and whether pyridoxal is still required if this is the case.

Levy and Coon (15) have presented a preliminary report on their experiments designed to find the origin of the five carbon chain of histidine. They grew Saccharomyces on uniformly labeled glucose and non-labeled glutamic acid in an attempt to find out if glutamic acid is a fairly direct precursor of the five carbon chain. The isolated histidine was highly radioactive in the five carbon chain, indicating that histidine does not come from glutamic acid or its metabolic products, and might come fairly directly from glucose. They also did a similar experiment with methyl labeled acetate. There was some label in the carbon 2 of histidine, presumably via formate, but none was found in the five carbon chain. Acetate apparently is also not directly involved in the synthesis of the principal carbon chain of histidine.

Another approach to the problem of histidine biogenesis has been through biochemical genetics. This has been the method of choice in this study, using a series of histidineless mutants of Neurospora. Vogel et al. (26) have used several histidine requiring mutants of E. coli in a similar study. They found that one of these histidine requiring mutants accumulates a substance in its culture medium which is utilized slowly by another mutant as a substitute for histidine. A variant of this second strain was selected which utilizes this accumulated compound 70% as well as histidine. This was used as an assay organism for isolating the substance. It was isolated from the culture medium by adsorption on charcoal, elution, and crystallization as the picrate. The compound was identified as 4-(2-amino, 3-hydroxypropyl)-imidazole (L-histidinol), a substance which had previously been synthesized by Karrer et al. (27) by the reduction of histidine methyl ester. As will be described this

compound was also isolated from the culture medium of one of the Neurospora mutants used in the present study. Vogel and Davis (28) investigated various other amino alcohols as substitute growth factors for E. coli amino acid requiring mutants, but found L-histidinol was the only α-amino alcohol which substituted for the related amino acid. They concluded that histidinol is a true intermediate on the pathway of histidine biosynthesis.

The catabolism of histidine.

The catabolism of histidine has been studied in a variety of organisms and is of interest in relation to the biosynthetic pathway. The major degradative pathway in mammalian liver and in Pseudomonas fluorescens is one in which histidine is transformed to L-glutamic acid via urocanic acid. The literature pertaining to this pathway of histidine degradation has been reviewed in the recent papers of Mehler and Tabor and their collaborators (29, 30, 25).

The first step in this catabolic sequence is conversion of histidine to urocanic acid with the liberation of the alpha amino group of histidine as ammonia. It is not yet known whether this is catalyzed by a single enzyme. Urocanic acid is further degraded by other enzymes to yield L-glutamate, formate, and ammonia. The alpha amino group of the glutamate is derived from the N^3 atom, and the formate from the C^2 atom of histidine. An intermediate in the conversion of urocanic acid to glutamate has been isolated by Tabor et al. (31). This compound yields a mole each of glutamate, formate, and ammonia on hydrolysis. It has also been isolated from the urine of folic acid-deficient rats fed large amounts of histidine or urocanic acid (32). Borek and Waelsch (33)

have isolated a compound with similar properties from a cat liver digest of histidine. On the basis of titration data they believe this compound to be α-formamidinoglutaric acid rather than N-formylisoglutamine which has the same empirical formula, and which has been suggested as the intermediate. Experiments of Mehler and Tabor (29) indicate this pathway accounts for at least 80% of the degradation of histidine in guinea pig liver.

A second pathway of degradation has been studied by Tabor and Mehler (34, 35) and Schayer (36). This involves the decarboxylation of histidine to give histamine, a hormone in higher animals. Histamine is oxidized by rats to give imidazole acetic acid and further products, as yet unidentified. This pathway seems to be mainly a method of detoxifying histamine and probably only accounts for a small amount of the histidine catabolized.

A discussion of the degradative pathways in relation to the synthetic one will be presented after the experimental section.

III. THE HISTIDINELESS MUTANTS OF NEUROSPORA

Origin.

The genetics and physiology of the histidineless mutants of Neurospora have been described by Haas et al. (37). Although histidine mutants are now known to occur at a fairly high frequency among irradiated Neurospora conidia, none was isolated until the special selection technique of Lein et al. (38) was used. This method uses histidine alone as a growth supplement rather than a complex mixture of amino acids. Mutant C84, the first histidine-requiring mutant isolated, was obtained in the original experiments of Lein et al. Using the same method of selection, Mitchell and Mitchell isolated mutant C94, and Haas isolated mutants C140 and C141. Mutant T1710 was obtained by Dr. C. E. Harrold. All of the other histidineless mutants that have been obtained in this laboratory seem to be genetically identical with one of these types (37).

Physiological properties of the mutants.

The fact that histidineless mutants could be readily isolated from platings of ascospores on minimal medium supplemented with histidine, yet had never previously been isolated in many experiments in which mutants were obtained by plating of ascospores on complex media, suggested that the histidineless mutants were inhibited by something in complex medium. This inhibition was investigated in mutants C84 and C94 by Mitchell and Mitchell (37). It was found that arginine or lysine in combination with any one of a large number of other amino acids will completely inhibit the growth of the mutants even though histidine is present in the medium. This has been found

to be true for all of the histidine-requiring mutants tested. A typical experiment, done in collaboration with Dr. Felix Haas, is presented in table I. The amino acids were added to 20 ml. of Fries minimal medium (39) in a 125 ml. Erlenmeyer flask. All cultures were grown at 25° for four days. Growth in each flask is expressed as mg. of dried mycelium.

Table I

addition to minimal	mg/flask	C84	C140	C141	T1710
none	-	0	0	1	1
histidine.HCl.H_2O	0.5	35	36	30	65
L-arginine	0.5	0	0	1	1
L-tyrosine	2.0	0	0	1	1
histidine, arginine	0.5, 0.5	36	41	1	67
histidine, tyrosine	0.5, 2.0	40	42	4	69
arginine, tyrosine	0.5, 2.0	0	0	1	1
hist., arg., tyros.	0.5, 0.5, 2.0	0	0	1	1

Mutants C141 and T1710, as is apparent from table I, will grow a trace on minimal medium; the growth of T1710 approaches that of wild type on minimal after a lag period of about a week. The histidine requirement for half maximal growth, under the conditions used above, is about 5 µg./ml. for T1710, 30 µg./ml. for C141, and 20 µg./ml. for mutants C84, C94, and C140.

Activity of various compounds in substituting for histidine.

A study of the specificity of histidine as a growth-promoting

substance was carried out early in the biochemical investigation of the Neurospora mutants. Numerous compounds, including many synthetic and natural imidazole derivatives, were tried as histidine substitutes. All of the amino acids commonly found in protein were tested on C84 and C94 by Mitchell and Mitchell (37), and found to be inactive. These amino acids, in addition to many other compounds, were tested on all of the mutants with no positive results. Those compounds found to be inactive are the purine and pyrimidine bases of nucleic acids, imidazole, methyl imidazole*, hydroxymethyl imidazole, imidazole formaldehyde, imidazole carboxylic acid, imidazole glyoxylic acid, imidazole acetic acid, histamine, urocanic acid, imidazole lactic acid, the various optical isomers of trihydroxypropyl imidazole, D-arabino-tetrahydroxybutyl imidazole, N^1-methyl histidine, 2-thiolhistidine, 4-amino-5-carboxamide imidazole, 4-amino-5-carboxamidine imidazole, creatine, creatinine, ribose, glucosamine, allantoin, formic acid, and pyridoxamine.

Cross-feeding experiments.

A characteristic of some biochemical mutants which makes them very useful for elucidation of biosynthetic pathways is the accumulation of precursors which pile up because of the blocked reaction. Accumulations have been demonstrated in certain tryptophane, purine, pyrimidine, choline, thiamine, aromatic, and other biochemical mutants (40, 41). These precursors can often be assayed by their activity for other mutants blocked in an earlier reaction step. The information gained from

*Unless otherwise indicated the substituent is at the 4(5) position of the imidazole ring.

accumulations is not always clear, however, as accumulated precursors are often modified by normal metabolic reactions. For example a Neurospora nicotinic acid mutant blocked in the conversion of kynurenine to hydroxykynurenine accumulates N^{α}-acetylkynurenine and kynurenic acid, two metabolically inactive derivatives of kynurenine (40). Undoubtedly the equilibrium between various accumulated precursors is also affected by numerous secondary metabolic factors.

In collaboration with Dr Haas, an attempt was made to demonstrate the accumulation of active intermediates by the histidine mutants (37). This was done by incorporating the filtrate of a mutant grown on half its maximum requirement of histidine into flasks of Fries minimal medium and inoculating each flask with one of the other mutants. To insure against the possible interference of inhibiting substances in the filtrates, another test for accumulations of growth supporting compounds was made. Filtrates of the mutants were concentrated in vacuo and chromatographed. The chromatograms were divided into sections and these were eluted with minimal medium. The eluate fractions were then examined for growth-promoting activity for each of the mutants. None of the mutants was observed to accumulate any substance in the medium which would stimulate the growth of any other histidine mutant.

Imidazoles accumulated by the mutants.

In the early work with mutant C84, Mitchell and Mitchell (42) found that a substance was accumulated in the culture medium that reacted to form a red dye with diazosulfanilic acid, a reagent which is used for detecting imidazoles. When the culture media from mutants C141 and T1710 were tested these mutants were also found to accumulate

substances, presumably imidazoles, which would react with diazosulfanilic acid. Mutants C1h0, C94, and wild type were found not to produce any diazo-reacting substances. These results suggested that the imidazole ring might be formed early in the biosynthetic pathway. An investigation was undertaken to isolate and establish the structures of the various accumulated compounds, even though the evidence from the cross feeding experiments seemed to indicate that they were probably inactive.

Chromatographic methods.

The separation and isolation of the accumulated compounds could not be followed by means of their biological activity, and it was necessary to use chemical assay methods. Paper chromatography seemed an ideal way for following purification of these compounds and therefore the diazosulfanilic test for imidazoles was modified so that it could be used as a spray reagent for paper chromatograms. Numerous imidazoles were chromatographed, using several solvents, and the chromatograms were developed with the diazo spray reagent. These known imidazoles served as useful references when working with the unknown compounds. The Rf values of these imidazoles as well as some of the properties of the reagent are described in the following paper from the Journal of the American Chemical Society.

[Reprinted from the Journal of the American Chemical Society, **74**, 252 (1952).]

The Paper Chromatography of Imidazoles

By Bruce N. Ames and H. K. Mitchell[1]

During the course of investigations on the accumulation of imidazole-like substances by histidineless mutants of *Neurospora*, methods have been developed for paper chromatography and detection on the paper of compounds of this type. Dent[2] reported the use of the Pauly reagent (diazosulfanilic acid) for this purpose. Using the modification of this reagent described below and the method of Jorpes[3] in which the acidic diazo reagent is mixed with the imidazole before developing the color with Na_2CO_3, excellent results have been obtained with a variety of imidazole derivatives.

Procedure and Results.—Air-dried chromatograms are sprayed so as just to wet the paper with the diazosulfanilic acid reagent, and then the color is developed by a light spray of 5% sodium carbonate solution. The diazo reagent is made by adding slowly and with stirring 25 ml. of a freshly made up 5% sodium nitrite solution to 5 ml. of a stock sulfanilic acid solution (0.9 g. sulfanilic acid and 9 ml. of concentrated HCl made up to 100 ml. with distilled water). Both the nitrite and sulfanilic acid solution must be brought to 0° before mixing.

The diazo reagent will keep for about 4–5 days at 0° but decomposes rapidly at higher temperatures. The stock sulfanilic acid solution keeps indefinitely at room temperature.

This method is considerably more sensitive than the ninhydrin method for histamine and histidine. The diazo reagent will indicate 0.3 of a microgram of histamine or histidine on a chromatogram. The sensitivity of the reagent is about the same for other imidazoles.

Using this procedure with known imidazoles several differently colored spots have been observed. In general imidazoles with a side chain at the 4-position, such as histidine, give a red color with the reagent. Substituents in addition to a side chain may alter the color considerably. Guanine gives a bright orange color and 4(5)-amino-5(4)-carboxamide imidazole gives first an orange color with the acid diazo solution and then a bright blue when the sodium carbonate is sprayed on.[4] This blue color is unique[5] and probably can be used for the quantitative estimation of this natural base.

The coupling reaction is not general for all imidazoles. Imidazoles with alkyl substituents on one of the ring nitrogens do not give the reaction[7]; *e.g.*, 1-methylhistidine and anserine. Some imidazoles with a carbonyl group next to the ring at the 4 position do not react; *e.g.*, [imidazolyl-4-(5)]-formaldehyde and [imidazolyl-4(5)]-glyoxylic acid. A weak yellow color is given by 4(5)-carboxyimidazole. Some condensed ring systems containing an imidazole nucleus also do not give the Pauly reaction; *e.g.*, most purines and benzimidazole.

In addition to imidazoles other compounds have been encountered in biological materials which react with diazosulfanilic acid giving colored dyes. Ammonium chloride as well as other ammonium salts gives yellow spots in fairly high dilution. Tyrosine has been found to give a red color under the conditions stated but only at much greater concentrations. Many phenols other than tyrosine are also known to couple with diazosulfanilic acid. This reaction has been used for their identification on paper chromatograms by Evans, *et al.*,[8] who reported various orange, brown and yellow colors from biochemically important phenols. A non-imidazole base that gives a red diazo reaction has been encountered by Hunter[9] in maize.

The R_f values and diazo colors of a number of imidazoles of biochemical and preparative importance are listed in Table I. Absorption spots under 2537 Å. ultraviolet and ninhydrin reaction color are also listed as they are useful in characterization.

Table I

R_f Values on 3.5 Hours, Ascending,[10] Whatman #1 Chromatograms at 25°

Compound	Diazo spot color	R_f with 3:1 propyl alcohol–0.2 *N* ammonia	R_f with 3:1 propyl alcohol–1 *N* acetic acid[11]	2537 Å. absorption	Ninhydrin color
Histidine	Red	0.22	0.12	−	Violet
4(5)-Carboxyimidazole[a]	Yellow	.23	.29	−	.....
Carnosine	Red	.24	.06	−	Violet
2-Thiol histidine[b]	Red	.25	.15	+	Violet
[Imidazolyl-4(5)]-lactic acid	Red	.26	.27	−	.....
Ergothionine	Red	.27	.26	+	.....
Guanine	Orange	.27	.35	+	.....
[Imidazolyl-4(5)]-pyruvic acid[c]	Red	.31	.31	−	.....
[Imidazolyl-4(5)]-acrylic acid	Red	.34	.69	+	.....
Ammonium chloride	Yellow	.39	.41	−	.....
4(5)-Amino-5(4)-carboxamide imidazole[d]	Blue	.52	.51	+	Yellow-Orange
Histamine	Red	.65	.10–0.28	−	Violet
Histidinol[e]	Red	.65	.10– .28	−	Violet
Histidine methyl ester[b]	Red	.75	.45	−	Violet
4(5)-Hydroxymethylimidazole[f]	Red	.75	.56	−	.....
Imidazole	Red-Orange	.88	.58	−	.....

The compounds indicated were kindly supplied by [a] Dr. R. G. Jones, [b] Dr. M. Fling, [c] Mr. P. Thayer, [d] Dr. E. E. Snell, [e] Dr. B. Davis,[12] [f] Dr. P. M. Ruoff.

Chromatograms have been made from the reaction mixture of the Parrod[13] synthesis of imidazoles from glucose.[14]

(1) This work was supported in part by funds from the Rockefeller Foundation and by funds from the Atomic Energy Commission administered through contract with the Office of Naval Research, U. S. Navy, Contract N6 onr-244, Task Order V.

(2) C. E. Dent, *Biochem. J.*, **43**, 169 (1948).

(3) E. Jorpes, *ibid.*, **26** 1507 (1932).

(4) A darker color is obtained if the sodium carbonate is sprayed on before the diazo reagent.

(5) A blue diazotization product has also been reported by Hunter[6] with 4(5)-guanidinoimidazole.

(6) G. Hunter, *Biochem. J.*, **30**, 1183 (1936).

(7) M. Guggenheim, "Die Biogenen Amine," S. Karger, Basel, 1940, p. 407.

(8) R. A. Evans, W. Parr and W. Evans, *Nature*, **164**, 674 (1949).

(9) G. Hunter, *Biochem. J.*, **48**, 265 (1951).

(10) R. J. Williams and H. Kirby, *Science*, **107**, 481 (1948).

(11) Histamine and histidinol give streaks with this solvent.

(12) H. J. Vogel, B. D. Davis and E. S. Mingioli, This Journal, **73**, 1897 (1951).

(13) J. Parrod, *Ann. Chim.*, **19**, 233 (1933).

(14) A modification of the Parrod procedure is listed in ref. 15 for the synthesis of 4(5)-hydroxymethylimidazole, an intermediate in histidine synthesis.

(15) *Org. Syntheses*, **24**, 64 (1944).

Parrod reported imidazole and 4(5)-(*D-arabino*)-tetrahydroxybutaneimidazole as the products. The chromatograms indicate that fair quantities of 4(5)-hydroxymethylimidazole and two other diazo reacting substances were obtained as well.

Chromatograms have been made of many other complex materials, such as deproteinized liver fractions, the products of the action of L-aminoacid oxidase on DL-histidine, and culture filtrates from *Neurospora* and *Penicillium* histidineless mutants, with good separation of imidazoles and reproducible colors and R_f values.

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When the medium from each mutant was chromatographed it was found that T1710 accumulated one compound, C84 another, and C141 accumulated the C84 compound plus still another. C94 and C140 resemble wild type in that they do not produce any imidazoles. Several histidine-requiring Penicillium mutants were available as a result of the wartime penicillin research and these mutants were also tested for accumulations. Two of them formed an imidazole that was different from the major compounds from Neurospora, but which was later shown to be accumulated in small amounts by C141.

None of these compounds corresponded chromatographically to any of the known imidazoles which were first tried. Later when a sample of L-histidinol, isolated by Vogel et al. from an E. coli histidineless mutant, was obtained, it was found to have the same Rf value as the T1710 imidazole. The Rf values and other properties of these imidazoles as determined from the chromatograms, are given in table II. The procedures and solvents are as described previously.

Table II. Chromatographic Properties of the Accumulated Imidazoles

compound	mutants accumulating compounds	Rf in propanol-ammonia	Rf in propanol-acetic	diazo spot color	nin-hydrin color	reduces alkaline copper sulfate
A	C84, C141	.46	.46	red-purple	--	-
B	C141	.66	.45	rose	yellow-orange	+
C	T1710	.62	.10-.28	red	violet	-
"penicillium imidazole"		.66	.55	red	--	-

Genetics and the order of the mutant blocks

Previous investigations in this laboratory have made use of double mutants to provide evidence for a biochemical sequence for Neurospora mutants that have the same growth requirement (43, 44). A compound accumulated by a mutant because of the genetic block will no longer be produced if a double mutant is made by introducing a gene that blocks a reaction step earlier in the biosynthetic sequence. If the five histidineless mutants are blocked in five steps in a linear sequence of biochemical reactions that lead to histidine, and if the accumulated substances are intermediates or are derived from intermediates in this pathway, then any double mutant carrying genes that eliminate two metabolic reactions on the pathway should accumulate the same compounds as the single mutant with the earlier block. If the order of steps for the Neurospora histidine mutants is:

$$\xrightarrow{C94} \text{cpd. A} \xrightarrow{C84} \text{cpd. B} \xrightarrow{C141} \text{cpd. C} \xrightarrow{T1710} \text{histidine}$$

then C94 C141, like C94, should not accumulate any imidazoles, and the C141 T1710 double mutant should behave like C141 in accumulating imidazoles A and B, but not C.

The genetics of these mutants has been investigated in detail by Dr. Haas and Mrs. M. B. Mitchell (37). The mutant gene of C84 is in linkage group E, that of C141 is in linkage group D, while the histidineless genes of C140, T1710, and C94 are in linkage group A. T1710 appears to have a translocation and because of this the non-appearance of wild types from a T1710 X C140 cross is not necessarily an indication that these genes are allelic. T1710 is also physiologically different

from C140 in several respects. C94 is about six units from the C140 locus.

All the double mutants that were genetically possible (The C140 T1710 combination could not be made) were made by Dr. Haas. The imidazoles produced by these double mutants were examined both quantitatively and chromatographically in collaboration with Dr. Haas and the Mitchells (37). No imidazoles were found to be accumulated by C94, C140, C94 C140, C94 C84, C94 C141, C94 T1710, C140 C84, C140 C141, or wild-type 7A. Strains C84, C84 C141, and C84 T1710 accumulate only compound A. Strains C141, and C141 T1710 accumulate A and B. These data are consistent with the scheme presented above. Mutant C84 is placed before C141 in the sequence as the double mutant C84 C141 shows the phenotype of C84 rather than that of C141. The rest of the analysis follows in a similar manner. As a further check Haas outcrossed all the double mutants to wild type and the six histidineless-two wild type asci obtained were examined. Each ascus of this type provides one wild-type culture, one double mutant culture, and cultures of each of the parental single mutants. The same tests as previously carried out with the double and single mutants were performed on each of the four spore pairs of the asci saved from the outcrosses. The results obtained demonstrate that the strains tested as double mutants did have the expected genetic constitution. The data on accumulation from each culture was as expected from the above results.

Growth experiments with the double mutants are not inconsistent with this order. T1710 has a much lower histidine requirement than the other mutants. None of the double mutants having T1710 as a component had this lower requirement for histidine.

The C94 C140 double mutant resembles both C94 and C140 in that it does not accumulate imidazoles, and therefore C94 and C140 can not be placed in serial order with respect to each other, but can be placed before C84 in the sequence. The analysis is not changed if it is assumed that A, B, and C are inactive products derived from the actual intermediates in the biosynthesis.

Isolation and identification of the accumulated compounds.

After this order was postulated, the actual isolation and proof of structure of these accumulated compounds was undertaken with the hope that knowledge of their structures would give some clue as to the biosynthesis of histidine. Mitchell and Mitchell in their early work with compound A from C84 were able to oxidize it to the known compound imidazole glyoxylic acid, a compound which can also be prepared by the oxidation of histidine. They isolated compound A from C84 culture medium by means of a mercury salt precipitation. An elementary analysis of the sirupy product obtained indicated that it contained an imidazole ring with a three carbon side chain bearing two or three hydroxyl groups. The compound reacted with sodium metaperiodate, a reagent that reacts with compounds having adjacent hydroxyl groups. They also prepared the phenyldiazo derivative of the compound, though the exact structure of the derivative was uncertain at that time.

These general outlines were followed in isolating and establishing the structures of all of the imidazole derivatives. The main addition to the isolation procedure was the use of ion exchange columns for separating and purifying the imidazoles. The procedures for the isolation of the compounds, the proofs of their structures, and the synthetic methods developed are described in the following paper.

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[CONTRIBUTION FROM THE KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Some New Naturally Occurring Imidazoles Related to the Biosynthesis of Histidine[1]

BY BRUCE N. AMES,[2] HERSCHEL K. MITCHELL AND MARY B. MITCHELL

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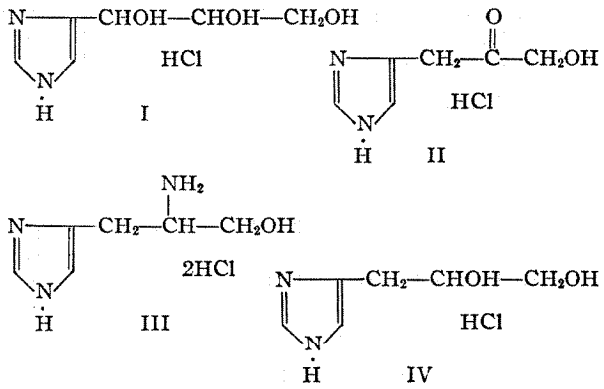
A general method is given for the isolation of a number of imidazole derivatives that are accumulated by mutants of *Neurospora* and *Penicillium*. Methods of degradation and chromatography have been used to identify with reasonable certainty three crystalline products as 4-(trihydroxypropyl)-imidazole, 4-(2-keto-3-hydroxypropyl)-imidazole and L-4-(2-amino-3-hydroxypropyl)-imidazole (L-histidinol). Evidence is presented to show that two other non-crystalline products are phosphate esters of the trihydroxy and ketohydroxy compounds while a sixth product is 4-(2,3-dihydroxypropyl)-imidazole. These findings are discussed in relation to the problem of the biological synthesis of histidine. A chemical synthesis of isomers of 4-(trihydroxypropyl)-imidazole is described.

Previous investigations in this Laboratory on the histidine requiring mutants of *Neurospora*[3,4] have demonstrated that several of these mutants accumulate imidazole derivatives in the medium that are related in some way to the biological synthesis of histidine. Although none of the accumulated imidazoles will support the growth of any of the neurospora mutants, it was hoped that their isolation and identification would provide a clue to the biosynthetic pathway that yields this amino acid. Evidence is presented here that supports the contention that histidine is derived from carbohydrate, probably pentose, in quite a direct manner. This conclusion is in agreement with the results from tracer experiments reported by Levy and Coon.[5]

By means of mercuric chloride precipitations followed by chromatography on Dowex-50, 4-(trihydroxypropyl)-imidazole hydrochloride (I) has been isolated from mutant C84,[6] I and 4-(2-keto-3-hydroxypropyl)-imidazole hydrochloride (II) from mutant C141, and L-4-(2-amino-3-hydroxypropyl)-imidazole dihydrochloride (L-histidinol) (III) from mutant T1710. In addition, two compounds which appear to be the phosphate esters of I and II have been obtained from the mycelium of C141.



Another imidazole accumulated by two penicillium histidine mutants and in small amounts by C141 is chromatographically identical with 4-(2,3-dihydroxypropyl)-imidazole (IV).

Various isomers of I have been synthesized from L-arabinose, D-arabinose, D-ribose and D-xylose by the Parrod reaction of sugars with cuprammonium solution and formaldehyde.

Experimental

Isolation.—Mutant C141 was grown with forced aeration in two 15-liter cultures of Fries minimal medium[7] supplemented with 450 mg. of L-histidine monohydrochloride monohydrate per culture. This is sufficient histidine for half-maximal growth. The mycelium was collected by filtration through cheese-cloth. It was then homogenized in a blendor with several portions of boiling water, filtered and the 3 liters of filtrate obtained was combined with the

(1) This work was supported in part by funds from the Rockefeller Foundation and by funds from the Atomic Energy Commission administered through contract with the Office of Naval Research, U. S. Navy, Contact N6 onr-244, Task Order V.

(2) A. E. C. Predoctoral Fellow in the Biological Sciences.

(3) J. Lein, H. K. Mitchell and M. B. Houlahan, *Proc. Natl. Acad. Sci.*, **34**, 437 (1948).

(4) F. Haas, M. B. Mitchell, B. N. Ames and H. K. Mitchell, *Genetics*, **37**, 217 (1952).

(5) L. Levy and M. J. Coon, *Federation Proc.*, **11**, 248 (1952), (abstract).

(6) Genetic and physiological data for these mutants are given in reference 4.

(7) G. W. Beadle and E. L. Tatum, *Am. J. Bot.*, **32**, 678 (1945).

30 liters of medium in which the mold had been grown. The extracted mycelium was discarded.

The solution (*p*H 4) was then brought to *p*H 8.5 with barium hydroxide. The resulting precipitate, which was allowed to settle overnight, was then removed by filtration and discarded.[8] The alkaline filtrate was treated with 100 g. of mercuric chloride dissolved in 400 ml. of ethanol to give a precipitate containing all the imidazoles. The precipitate was collected and dried. The imidazoles were extracted from the dried precipitate with 800 ml. of 0.5 *N* hydrochloric acid. This extract was treated with hydrogen sulfide and then filtered to remove the mercuric sulfide. The resulting solution was concentrated *in vacuo* to 40 ml. and utilized for chromatography on a Dowex-50 column.

A 2 × 70 cm. column of Dowex-50 resin (250–500 mesh) was prepared and equilibrated with 1.5 *M* hydrochloric acid following the method of Stein and Moore.[9] The sample was eluted with 800 ml. of 1.5 *M* hydrochloric acid followed by 800 ml. of 2.5 *M* hydrochloric acid; 20-ml. samples were obtained during the elution by use of an automatic fraction collector.[10] Samples (0.001 ml.) from each fraction were chromatographed on paper[11] in order to follow the elution process. Appropriate fractions were then combined and evaporated over sodium hydroxide pellets in a desiccator.

The same general procedure as described above has been utilized for isolation of the imidazoles from the mutants other than C141 with the exception that preliminary purifications of histidinol from the culture medium of strain T1710 were carried out by the method of Vogel, *et al.*[12]

A summary of the data on isolations of six natural and one synthetic imidazole is given in Table I.

TABLE I[a]

THE VARIOUS ISOLATED IMIDAZOLES

Substance	Mutant	*Rf* in 3:1 propanol–0.2 *N* NH_3 Whatman no. 1	*Rf* in 3:1 propanol–1 *N* acetic acid Whatman no. 1	Dowex eluate-fraction at which substance starts to appear: Ml.	HCl, *N*
Phosphate ester of I	C141	0.04	0.16	200	1.5
Phosphate ester of II	C141	.07	.17	350	1.5
I	C141 also C84	.46	.46	400	1.5
Isomer of I synthesized from L-arabinose		.46	.46	400	1.5
"Penicillium imidazole"	C141	66	55	800	1.5
				50	2.5
II	C141	.66	.45	800	1.5
				100	2.5
III	T1710	.62	.10–0.28	500	2.5
				20	4

[a] For details of chromatography on paper see reference number 11.

Identification. I from Mutants C141 and C84.[13]—A colorless sirup was obtained when the Dowex fractions containing I, from the C141 isolation, were pooled and evaporated. All attempts at crystallization failed during the first few months of work with the compound. The picrate is extremely soluble and the usual base precipitants were equally unsatisfactory. The compound crystallized spontaneously, however, after a month in the refrigerator. It was triturated with acetone, then with cold absolute ethanol and recrystallized twice from absolute ethanol. Large white rosettes of broad needles were obtained; m.p. 102.5–103° (cor.), $[\alpha]^{23}D$ 11.6° (*c* 12.1, in water).

Anal. Calcd. for $C_6H_{10}O_3N_2 \cdot HCl$: C, 37.10; H, 5.67; N, 14.43. Found:[14] C, 37.53; H, 5.85; N, 14.57.

Periodate Oxidation of I.—One ml. of 0.26 *M* sodium metaperiodate was added to 20 mg. of crystalline I (from C141) in a 10-ml. volumetric flask and the solution was diluted to the mark with distilled water. The volumetric flask was placed in an ice-bath and 2-ml. aliquots were withdrawn 0.5, 1.5 and 2.5 hours after mixing. Periodate consumed was determined by the method of Forrest and Todd.[15] The reaction was found to be at an end within the first half-hour with the consumption of 1.8 moles of periodate per mole of compound I.

A second aliquot of the reaction mixture was titrated with 0.01 *M* sodium hydroxide in order to determine acid produced in the reaction (formic acid). An acid equivalent *of 0.9 mole of acid per mole of I was found.*

A third aliquot (1 ml.) from the periodate reaction was used for the determination of formaldehyde using a modification of the method of Boyd and Logan.[16] The 1-ml. sample was diluted to 150 ml. and a 3-ml. aliquot was mixed with 0.2 ml. of sodium arsenite solution (25%) and 2.5 ml. of filtered chromotropic acid solution (720 mg. of chromotropic acid in 20 ml. of water mixed gradually in an ice-bath with 30 ml. of concd. sulfuric acid). The mixture was heated with occasional stirring at 100° for 30 min. Known amounts of formaldehyde were treated in the same way and after cooling, optical densities were determined in the Beckman spectrophotometer at a wave length of 580 mμ. The standard curve is linear in the range of 2 to 10 μg. of formaldehyde per sample. By use of this method it was found that the periodate reaction mixture yielded an equivalent of 0.94 mole of formaldehyde per mole of compound I.

A fourth aliquot of the periodate reaction mixture was chromatographed on paper.[11] An ultraviolet absorbing, non-diazo reacting substance corresponding to imidazole aldehyde was observed. Authentic imidazole aldehyde[17] was run alongside and the R_f values were identical.[18] No other imidazole was observed.

Degradation of I to Imidazoleglyoxylic Acid.—A sample of I (150 mg.) from C84 was refluxed for 3.5 hours with 10 ml. of concd. nitric acid. Nitric acid was removed by evaporating the solution to dryness in a stream of air, adding water and evaporating again. The residue was then taken up in a small amount of water and the *p*H adjusted to 3.0 with sodium carbonate solution. The resulting precipitate of imidazoleglyoxylic acid was recrystallized from hot water; yield 57 mg., m.p. 280–290° (dec.); R_f in 3:1 1-propanol–0.2 *N* ammonia solvent, 0.28; absorption spectra: 20 μg./ml. in 0.1 *N* hydrochloric acid, maximum at 285 mμ, optical *density 1.92; 20 μg./ml. in* 0.1 *N* sodium hydroxide, maximum at 252 mμ, optical density, 0.81.

Anal. Calcd. for $C_5H_4O_3N_2$: C, 42.90; H, 2.86; N, 20.00; neut. equiv., 140. Found: C, 42.95; H, 2.99; N, 20.40; neut. equiv., 141.

The degradation was repeated on a small scale with I from C141 and imidazoleglyoxylic acid was identified chromatographically.

Imidazoleglyoxylic Acid from Histidine.—L-Histidine was oxidized with nitric acid to imidazoleglyoxylic acid[19] and isolated as described above. As demonstrated by chromatography of samples from the oxidation mixture, histidine is first converted to imidazoleacetic acid at a high rate but it is necessary to reflux about 10 hours to effect the oxidation of this compound and a number of side reactions occur. Compounds I and II do not give rise to this resistant intermediate. Calcd., neut. equiv., 140; found, 139; m.p. *280–290° (dec.). Knoop reported 290° (dec.); R_f in* 1-propanol–ammonia, 0.28. Absorption spectra are the same as for imidazoleglyoxylic from I.

The Bis-(phenyldiazo) Derivative of I.—A cold solution of phenyldiazonium chloride (from 100 mg. of aniline, 0.3

(8) The barium precipitation removes tartrate and other substances *in the medium that would otherwise come down later with the mercury* precipitation of the imidazoles. Barium does not precipitate any of the imidazoles or imidazole phosphates.

(9) W. H. Stein and S. Moore, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 179 (1950).

(10) The authors are indebted to Drs. G. Keighley and H. Borsook for use of this equipment.

(11) B. N. Ames and H. K. Mitchell, THIS JOURNAL, **74**, 252 (1952).

(12) H. J. Vogel, B. D. Davis and E. S. Mingioli, *ibid.*, **73**, 1897 (1951).

(13) Compound I was isolated from C84 several years before the Dowex technique became available. It was never obtained crystalline but was investigated as a sirup.

(14) All microanalyses were done by Mr. G. Swinehart.

(15) H. S. Forrest and A. R. Todd, *J. Chem. Soc.*, 3295 (1950).

(16) M. J. Boyd and M. A. Logan, *J. Biol. Chem.*, **146**, 279 (1942).

(17) A sample was generously supplied by Dr. Peter Lowy of the California Institute of Technology.

(18) An R_f value of 0.75 was obtained in 3:1 *n*-propanol–0.2 *N* ammonia.

(19) F. Knoop, *Beitr. Chem. Physiol. u. Path.*, **10**, 116 (1907).

ml. of concentrated hydrochloric acid, 80 mg. of sodium nitrite and 1.5 ml. of water) was added to 150 mg. of I, (from C84) in 5 ml. of water. The coupling was done in an ice-bath. A concentrated sodium carbonate solution was added to the solution until a red precipitate of the bis-(phenyldiazo) derivative was formed. This precipitate was washed with water and recrystallized from aqueous methanol; m.p. 173–180° (dec.).

Anal. Calcd. for $C_{18}H_{18}N_6O_3$: C, 59.00; H, 4.97; N, 22.94. Found: C, 59.08; H, 5.39; N, 22.30.

Synthesis of 4-(L-*erythro*-Trihydroxypropyl)-imidazole.—Parrod[20] described a method for the preparation of 4-(D-*arabino*-tetrahydroxybutyl)-imidazole from glucose or fructose. This method as modified by Huebner[21] has now been utilized for the preparation of the various optical isomers of 4-(trihydroxypropyl)-imidazoles from different pentoses.

L-Arabinose (10 g.) was dissolved in a solution of 45 g. of copper acetate in 75 ml. of concd. aqueous ammonia. After the addition of 16 ml. of 37% formaldehyde solution the mixture was heated on a water-bath for 30 min. The copper–imidazole precipitate from the cooled reaction mixture was removed, resuspended and treated with hydrogen sulfide. The products were then separated on a Dowex-50 column as described above for the natural imidazoles. The desired product (approx. 0.5 g.) was eluted from the column in the same fractions as compound I. Similar preparations were made on a smaller scale from D-arabinose, D-ribose and D-xylose. None of these products has been obtained in a crystalline form but all have the same chromatographic and chemical properties as I. A comparison of the optical properties of the various products has not yet been carried out since the synthetic compounds have not yet crystallized.

II from Mutant C141.—On evaporating the pooled fractions containing II from the C141 Dowex column, the compound crystallized as white needles. These were recrystallized three times from 95% ethanol; m.p. 171–174° dec. (cor.). The crystals turn brown at 171°.

Anal. Calcd. for $C_6H_8O_2N_2 \cdot HCl$: C, 40.86; H, 5.11; N, 15.90. Found: C, 40.79; H, 4.85; N, 15.58.

II reduces alkaline copper sulfate and Tollens reagent and reacts with phenylhydrazine.

Periodate Oxidation of II.—The periodate oxidation was done by the same method as described for I. Aliquots taken at 1.5, 6.5 and 20 hours all gave a value of 1.1 moles of periodate taken up per mole of II. 1.0 mole of formaldehyde was found. Imidazoleacetic acid[22] was the only imidazole apparent on chromatographing the reaction mixture. The isomeric hydroxyaldehyde, which would also reduce alkaline copper sulfate, would, unlike II, yield imidazoleacetaldehyde and formic acid on periodate oxidation.

Degradation of II to Imidazoleglyoxylic Acid.—Compound II (200 mg.) was refluxed with 20 ml. of concentrated nitric acid. With this compound the maximum yield was obtained within 1 hour. No imidazoleacetic acid could be detected on the chromatograms run at intervals during the oxidation. The carbonyl group apparently activates the methylene group next to the ring with the resultant rapid formation of imidazoleglyoxylic acid in high yield. The isolation was done in the same way as for the product from I; m.p. 280–290° (dec.); R_f value in *n*-propanol–ammonia, 0.28. Absorption spectra are as given for imidazoleglyoxylic from I.

III (L-Histidinol) from Mutant T1710.—On evaporation of the hydrochloric acid eluate from the T1710 Dowex run, III crystallized. It was recrystallized three times from 95% ethanol; m.p. 194–195°. A sample mixed with authentic L-histidinol dihydrochloride[23] (m.p. 193.5–194.5°) melted at 193.5–194.5°. The R_f values of the two samples were identical.

Anal. Calcd. for $C_6H_{11}ON_3 \cdot 2HCl$: C, 33.78; H, 6.10; N, 19.70. Found: C, 33.42; H, 5.87; N, 20.04.

Phosphate Ester of I.—Evaporation of the first fraction from the Dowex chromatogram of imidazoles from mutant C141 (Table I) yielded 500 mg. of sirup. Chromatography of this material on paper gave rise to a spot which reacted with both the diazo reagent and the phosphate ester reagent of Hanes and Isherwood (as utilized by Bandurski and Axelrod[24]). Hydrolysis of the material with 6 *M* hydrochloric acid at 100° for 1 hour or with alkaline phosphatase (Armour) at *p*H 7.8 for 2 hours at 35° yielded products containing orthophosphate and an imidazole that is chromatographically identical with I in several solvents.

II Phosphate Ester from Mutant C141.—The fractions containing the second imidazole from the C141-Dowex run also gave a sirup when evaporated. The substance gave a diazo reaction and a phosphate ester test at the same R_f value on a chromatogram (Table I). Warming this material in dilute hydrochloric acid solution caused complete hydrolysis to yield a substance that is chromatographically identical with II. In addition both II and the hydrolysis product gave an orange ninhydrin test, reduced alkaline copper sulfate and gave a slightly rose colored diazo reaction at the same R_f value on a chromatogram.

This ester hydrolyzed spontaneously over a period of several months. An α-keto group is known to labilize phosphate esters of this type, as can be seen by the extreme lability of dihydroxyacetone phosphate ester.

"Penicillium Imidazole Compound" IV.—This imidazole from C141 runs slightly faster than II on the Dowex column. Unlike II, to which it is very similar in chromatographic properties (Table I), it does not reduce copper nor does it give a ninhydrin reaction. It is chromatographically identical both with the imidazole accumulated by two penicillium histidine-less mutants and with 4-(2,3-dihydroxypropyl)-imidazole obtained by nitrous acid treatment of III. Only a small amount is accumulated by C141 and insufficient material for characterization was obtained from the Dowex run. Two of the penicillium mutants accumulate IV in large amounts, but it has not yet been isolated in large quantities.

Discussion

Structural Evidence.—The degradations of I and II to imidazoleglyoxylic acid establishes the imidazole ring and the position of the non-branched side chain at carbon atom 4. The results of the periodate oxidations furnish evidence for structures[25] which are fitted by only I and II. The analyses, derivatives and preliminary synthetic work in the case of I all confirm the structure presented. As yet there is no evidence indicating whether I is the D-*erythro*, L-*erythro*, D-*threo* or L-*threo* isomer. II has no asymmetric carbon atoms and its structure is therefore uniquely determined. III is a known compound, synthesized by Karrer, *et al.*,[26] and isolated from a histidine-less mutant of *E. coli* by Vogel, Davis and Mingioli.[12] The determination of its structure is therefore based on a comparison with authentic III. Agreement of various physical properties, analysis and a mixed melting point seem to establish conclusively the identity of III.

The phosphate esters of I and II have not been crystallized and the evidence for their structure is chromatographic and enzymic. The position of the phosphate group on I is unknown, though by analogy with the ester of II which only has one hydroxyl, the primary hydroxyl group seems likely, but a phospho-enol linkage is possible in II. The relative stability of the ester of I to acid hydrolysis suggests a primary ester.

The evidence for the identity of IV with the imidazole accumulated by two of the penicillium histidine-less mutants and in small amounts by C141 is solely chromatographic. It is mentioned because

(20) J. Parrod, *Ann. chim.*, **19**, 233 (1933).

(21) C. F. Huebner, THIS JOURNAL, **73**, 4667 (1951).

(22) The R_f value in *n*-propanol–ammonia is 0.31.

(23) A generous sample was obtained through the courtesy of Drs. H. J. Vogel and B. D. Davis.

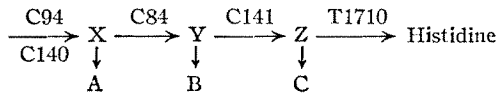
(24) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **183**, 405 (1951).

(25) A discussion of the applicability and specificity of this reagent is given by E. L. Jackson in "Organic Reactions," Vol. 2, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 341.

(26) P. Karrer, M. Suter and P. Waser, *Helv. Chim. Acta*, **32**, 1936 (1949.)

of the apparent structural relationships with the other compounds and its accumulation by both fungi.

The Biosynthesis of Histidine.—A previous publication[4] presented genetic and biochemical evidence that the histidine mutants of neurospora are related from the standpoint of biosynthesis of histidine according to the scheme



A, B and C refer to the compounds accumulated by the various mutants and they were not considered to be intermediates in the biosynthesis. These compounds have now been identified as: A = I, B = II and C = III. These compounds do not support the growth of any of the mutants and the discovery of the phosphate esters of I and II in the mycelium of strain C141 suggests the possibility that these esters correspond to the actual intermediates X and Y indicated in the above scheme. Since phosphate esters in general are not taken up by growing neurospora, it is necessary to use enzymatic methods to determine whether these esters are actually intermediates in histidine biosynthesis. Experiments of this kind are in progress. Since the first compound accumulated in the series contains a trihydroxypropyl side chain, it is a particularly attractive hypothesis that the carbon chain of histidine is derived in quite a direct fashion from pentose phosphate.

Compound III isolated from an *E. coli* histidine mutant by Vogel, *et al.*,[12] provided an important clue as to the nature of the biosynthetic pathway. It was found that his isolated L-histidinol was utilized slowly by another histidineless *E. coli* mutant. By selection from this second mutant, a strain was obtained which utilized L-histidinol 75% as well as histidine. If not histidinol, but its phosphate ester is the true intermediate, results such as this can be easily explained. Perhaps what was being selected for was an organism with a histidinol phosphorylating enzyme.

Acknowledgment.—We are grateful to Dr. Adolph Abrams for his many helpful suggestions concerned with chromatography on Dowex and to Dr. Hugh Forrest for his interest in the work and his aid in the periodate titrations. We are indebted to Dr. Felix Haas for mutants C140 and C141 and to Dr. C. E. Harrold for mutant T1710.

PASADENA, CALIFORNIA

D-erythro-trihydroxypropyl imidazole

In view of the possible relation of the trihydroxypropyl imidazole to pentose it became of interest to determine the exact configuration of this compound. As described previously the L-erythro-trihydroxypropyl imidazole was synthesized from L-arabinose but could not be crystallized. The D-erythro isomer was synthesized from D-arabinose by the same procedure. A sirup of the synthetic D-erythro isomer crystallized when a seed crystal of the Neurospora trihydroxy imidazole hydrochloride was added. The crystals were triturated with cold acetone and re-crystallized from absolute ethanol. Only about 50 mg. were obtained from 10 g. of D-arabinose. No attempt was made to improve the synthesis so that a better yield would be obtained. 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:

1. Analysis of the synthetic compound.

For $C_6H_{10}O_3N_2 \cdot HCl$

	Calc'd	Found
%C	37.10	37.15
%H	5.67	6.09
%N	14.43	14.24

2. Mixed melting point.

m.p. of Neurospora compound	103-103.5° (uncorr.)
m.p. of synthetic D-erythro cpd.	102.5-103° (uncorr.)
mixed melting point	102.5-103° (uncorr.)

3. Optical rotation.

Neurospora compound $[\alpha]_D^{25.6} = +13.3^\circ$ (c 7.5, water)

synthetic compound $[\alpha]_D^{25.6} = +13.0^\circ$ (c 7.5, water)

4. Physical properties.

Both compounds formed rosettes of prismatic needles on crystallization. The Rf of the synthetic compound was identical with that of the compound isolated from Neurospora in each of the several solvents used. The two compounds show the same behavior on the Dowex-50 column.

Other compounds accumulated by the mutants.

During the course of the isolation procedures several other compounds giving diazo tests were eluted from the Dowex columns. They are present in the mutants in small amounts and it is not known whether they are also present in small amounts in wild type. One of these, obtained from C141, is chromatographically identical with imidazole acetic acid. The Rf in 3:1 n-propanol-1N acetic acid is 0.23, in 3:1 n-propanol-1N ammonia 0.31, and in 2:1:1 ethanol-amyl alcohol-1N acetic acid 0.38. It shows approximately the same behavior on a Dowex-50 column as the imidazole acetol (hydroxyketopropyl imidazole).

Enzymatic experiments with some of the imidazoles.

Preliminary experiments have been carried out with some of the imidazoles to see whether enzymatic conversion of one to another could be demonstrated. Paper chromatography was used in the absence of a more sensitive analytical method for detecting a reaction. In one experiment several new diazo-reacting substances appeared on incubation of the phosphate ester of trihydroxypropyl imidazole with ground mycelium.

The enzyme was prepared by grinding 5 grams wild-type mycelium and 2.5 grams sand in 12.5 ml. of 0.05 M phosphate buffer (pH 7) in an iced mortar. This crude enzyme preparation (0.2 ml.) was added to each of three test tubes containing respectively, buffer, trihydroxypropyl imidazole phosphate ester, trihydroxypropyl imidazole, and ribose-5-phosphate. The reaction mixtures were made up to 0.5 ml. with buffer. Controls contained boiled enzyme. These mixtures were incubated aerobically at 35° for 2.5 hours. The reaction was stopped by addition of 0.3 ml. of 20% trichloracetic acid to each tube. The tubes were then centrifuged and the reaction mixtures were extracted with ether. The extracted supernatant was concentrated and chromatographed using the propanol-ammonia solvent. The chromatogram was sprayed with diazosulfanilic acid reagent. The reaction mixture containing the imidazole phosphate ester and fresh enzyme showed, in addition to the diazo spot of the phosphate ester, a spot at Rf 0.46 corresponding to the dephosphorylated compound, and a new spot at Rf 0.27. These were not apparent in the other tubes. The hydrolysis of the phosphate ester was probably caused by non-specific phosphatases. It is planned to investigate this new compound at some future time.

IV. DISCUSSION

In investigating a biosynthetic sequence such as that of histidine, standard enzymatic methods are not of much use in the absence of information on the identity of some of the intermediates. Because of their unique advantages biochemical mutants have come increasingly into favor for investigating this type of metabolism. Mutants of micro-organisms are especially valuable for studying a biosynthesis because of the frequent accumulation of compounds due to the genetic block, the ease of testing compounds for growth-promoting activity, and the ability to establish a sequential order among the mutants in reference to the synthetic scheme. The information obtained from mutants pertaining to the biosynthesis is, however, not strictly comparable to the information that would be obtained by an enzymological study of a metabolic chain, and this difference is worthy of a brief analysis.

In standard enzymological terminology for example, if the phosphate ester of gluconic acid is said to be an intermediate in the conversion of glucose-6-phosphate to ribulose-5-phosphate, in E. coli, it is meant that enzymes are known in E. coli which convert glucose-6-phosphate to to gluconic-6-phosphate, and gluconic-6-phosphate to ribulose-5-phosphate. Gluconic-6-phosphate, then, can be said to be a precursor of ribulose-5-phosphate on this particular pathway, bearing in mind that possibly the enzymes are not associated in any physiological sense in the intact organism. Seldom does one find in papers in biochemical genetics, however, that the words precursor and intermediate are used in even this exact a sense. Evidence bearing on precursors and intermediates can be obtained from an ordered series of mutants by "feeding experiments"

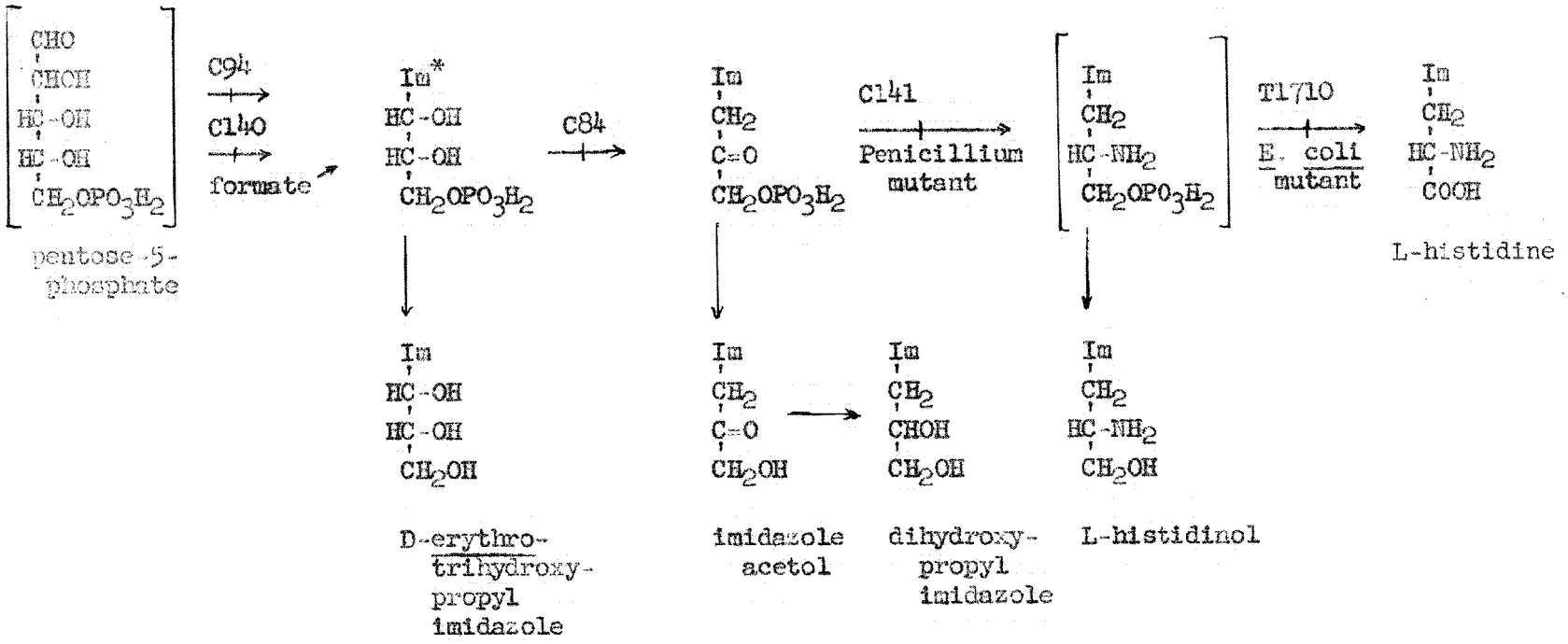
where compounds are examined for their growth-promoting activity for the mutants, or by an accumulation analysis such as has been done for the histidine mutants, or, most elegantly, by a combination of these methods when mutants will "cross feed." In feeding experiments, one considers as "precursors" not only compounds which are precursors in the enzymological sense, but also compounds that can be transformed into such precursors by the mutant. In addition such "feeding" results may be completely misleading if the mutant block is a result of some complicated metabolic interaction. A threonine mutant which will also grow on sulfonamide (45) is a case in point. This mutant seems to have a threonine requirement because of an abnormal catabolism of threonine, the probable cause of which has been traced to the production by the mutant of an excess of p-aminobenzoic acid which catalyzes this excessive destruction of threonine. Sulfonamide enables the mutant to grow by antagonizing the excess p-aminobenzoic acid.

If the cause of the requirement, as in the sulfonamide mutant, is more complicated than apparent, accumulations also may be misleading. Other difficulties in interpreting data on accumulations, such as metabolic transformations, have previously been discussed. If both accumulation and biological activity of a compound can be demonstrated in a series of mutants, then the possibility that the compound is a "precursor" becomes quite likely, but the method still does not distinguish between precursors in the enzymological sense and related compounds that can be transformed into these true precursors. In the histidine mutants, as in other cases, there is no substitute for an enzymological investigation of the compounds suspected of being true precursors.

The information that a compound is not utilized by a mutant can also be misleading, and does not necessarily mean that the compound is not a precursor in the blocked biosynthetic sequence. Neurospora mycelium is apparently permeable to many compounds only in the non-ionized form, e.g., formic acid (46), nicotinic acid (47). Bonner and Beadle (47) have investigated a nicotinicless mutant for which nicotinic acid is as active as nicotinamide when the culture medium is at pH 4.6 but is completely inactive at pH 6.6. Phosphate esters, having a pK of about 1, are not taken up by the mycelium at all (48). The more difficult problem then of identifying possible intermediates which will not enter the cell, such as phosphate esters, must be solved using information gained from accumulations rather than from "feeding experiments" as a guide to enzymatic experiments. It is postulated that the intermediates in histidine synthesis fall into this category.

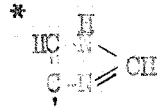
From the order of the mutants as established by the analysis of the double mutants, and the nature of the accumulated compounds, a scheme has been postulated (fig. 1) relating the mutants and their accumulations to histidine synthesis. The phosphate esters of D-erythro-trihydroxypropyl imidazole and imidazole acetol, rather than the unphosphorylated compounds, are presented as likely intermediates. If the unphosphorylated compounds were the actual intermediates then one would expect them to be taken up readily by Neurospora and to show biological activity for the mutants blocked before these compounds in the sequence. The fact that they will not substitute for histidine is more easily explained on the basis of their not being intermediates than on the basis of permeability. The pK of the imidazole ring is about 6.5 for these compounds and therefore they are quite weak bases. Neurospora

Figure 1



histidine → histamine → imidazole acetic acid

histidine → urocanic acid → α-formamidino glutaric acid → L-glutamic acid + formate



is even permeable, however, to such strong bases as choline and arginine. Neurospora is also permeable to imidazole acrylic acid at the pH of Fries medium (5.5), and of course histidine is taken up by the mold. The fact that the accumulated bases diffuse out into the culture medium during growth and are not confined to the mycelium also seems to indicate that Neurospora mycelium is quite permeable to them. If the unphosphorylated compounds were the actual intermediates, it would also be difficult to explain the appearance of their phosphate esters in the mycelium.

On the other hand if the phosphate esters are pictured as possible intermediates these difficulties do not arise. The mold mycelium is impermeable to phosphate esters as has been indicated, so it is not surprising that these substances do not substitute for histidine in growth experiments. The enzyme experiments that were done show that Neurospora has a phosphatase capable of hydrolyzing the phosphate ester of the trihydroxypropyl imidazole, and Neurospora has also been shown by Shuster and Kaplan (49) to be rich in phosphatases. If simple phosphate esters or even more complex dinucleotide-like precursors were accumulated in the mycelium they would be expected to be hydrolyzed very readily by the mold.

A point worth elaborating in the postulated scheme is the relation of the dihydroxypropyl imidazole to the rest of the compounds. The presence of small amounts of dihydroxypropyl imidazole in C141 suggests this compound may arise by some non-specific reduction of imidazole acetol or imidazole acetol phosphate. Analogous to this would be the reduction of dihydroxyacetone to glycerine, and fructose to mannitol, which are well-known microbiological reductions. Lardy (50) has shown

that α-glycerophosphate dehydrogenase is not strictly specific as to substrate and will convert acetol phosphate to propanediol phosphate. This enzyme might be also active on imidazole acetol phosphate in Neurospora. It is of course possible that an ester of the dihydroxy compound is actually an intermediate and the imidazole acetol phosphate is a side product, or that both compounds are intermediates. The accumulation of the dihydroxypropyl imidazole by the two Penicillium mutants examined is of interest in this regard. In figure 1 the dihydroxy compound has been pictured as a side product. This results in a relatively simple chain of reactions to yield histidine. A dehydration of the trihydroxypropyl imidazole phosphate ester could presumably yield the ester of imidazole acetol directly, and a transamination might convert this directly to histidinol phosphate. The L-histidinol or L-histidinol phosphate conversion to histidine probably involves several steps. E. Adams et al. (51) have obtained an unidentified strain of aerobic soil bacteria which adapts to histidinol as a carbon source. It will also adapt to histidine. When the bacteria were adapted to histidinol they were found to be simultaneously adapted to histidine, suggesting that the histidinol is metabolized through histidine. An enzyme extract has been obtained from this organism which converts histidinol to histidine. It has not yet been determined whether this goes through the phosphate ester of histidinol (52).

The structure of the D-erythro-trihydroxypropyl imidazole suggests that earlier precursors are sugar derivatives, especially when one bears in mind that this compound can be synthesized from formaldehyde, ammonia, and pentose under mild conditions. Parrod's original syntheses of D-arabino-tetrahydroxybutyl imidazole were done at room temperature.

The fact that the trihydroxy compound is of D-erythro configuration suggests that D-ribose-5-phosphate or D-arabinose-5-phosphate, the pentoses with this configuration, might well be the starting point for histidine biosynthesis. This indirect evidence pointing to sugars as the precursors of histidine is in agreement with the tracer work of Levy and Coon (15). It is surprising to find that speculations about the biogenesis of histidine from sugars were made many years ago by M. Guggenheim. It seems appropriate to quote from his useful book Die Biogenen Amine (53) concerning the Parrod reaction:

> "Es erscheint nicht ausgeschlossen, dass auch in der lebenden Zelle eine ähnliche Reactionsfolge von einer Pentose oder Hexose aus zur Bildung des Histidins führt."

It is of interest in light of these investigations of the mutants to examine the pathway of histidine degradation for a possible connection with the synthetic pathway. Tracer work on yeast indicated that the degradative pathway was probably not the same as the synthetic pathway; this seems to be true for Neurospora also. Preliminary experiments have been done by Haas (54) showing that, despite their block in the synthetic pathway, the Neurospora histidine mutants will degrade histidine and urocanic acid to non-diazo-reacting substances. It seems likely, therefore, that histidine catabolism in Neurospora is similar to that in other organisms investigated. If this is the case, it is apparent that the proposed synthetic scheme is almost totally dissimilar to the degradative pathway as shown in figure 1. One connection between the pathways, however, may be through a formate pool in the organism. Formate is incorporated into the 2 carbon of the ring during synthesis of histidine, and also is liberated during catabolism of histidine. One wonders whether this formate cycle has any physiological significance

in regulating the biosynthesis or the catabolism, in the manner of a feed-back mechanism. The degradative pathway is also of interest to the present investigation because of the appearance of imidazole acetic acid in small amounts in C14l. This imidazole derivative may arise from the degradation of one of the accumulated imidazoles, or from degradation of histidine through the histamine pathway. As with some of the other unanswered questions, this will probably best be elucidated by means of enzyme experiments.

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