The Preparation and Characterization of 1-Phosphohistidine and 3-Phosphohistidine*

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ABSTRACT: 1-Phosphohistidine and 3-phosphohistidine have been synthesized by reaction of histidine with phosphoramidate. The two isomers have been separated by paper electrophoresis and anion-exchange chromatography. The synthetic phosphohistidine isomer which is identical with the compound isolated from mitochondria and from a succinate thiokinase preparation has been identified as 3-phosphohistidine. 3-Phosphohistidine has been isolated as the solid lithium salt and has been characterized by composition, ultraviolet and nuclear magnetic resonance spectra, titration curve, and rate of hydrolysis over a wide range of pH values. α-*N*-Acetyl-3-phosphohistidine, 1-methyl-3-phosphohistidine, α-*N*-acetyl-1-methyl-3-phosphohistidine, and the isomers of phosphohistidine methyl ester have been prepared and some properties reported.

Histidine was first phosphorylated by Severin and Yudelovich (1947) by reaction with $POCl_3$ in alkaline solution. Attachment of the phosphoryl group to an imidazole N was deduced from the product's alkaline stability, acid lability, and failure to react with a diazonium compound. Phosphohistidine[1] was later prepared by Gustafson and Wagner-Jauregg (1954) using $POCl_3$, by Müller *et al.* (1956) using phosphoramidate, and by Rosenberg (1964) using diphosphoimidazole. Only partial purification of the products has been reported. Except for determinations of hydrolytic rate in acidic and neutral solution (Severin and Yudelovich, 1947; Gustafson and Wagner-Jauregg, 1954; Müller *et al.*, 1956; DeLuca, 1963) and for a determination of the heat of hydrolysis (Sakhatskaya, 1947), little has been reported on the physical and chemical properties of phosphohistidine. In none of the studies has any evidence been obtained to indicate whether the synthetic product under study was 1-phosphohistidine, 3-phosphohistidine, or a mixture of the two isomers.

In addition to these reports on phosphohistidine, preparations and characterizations have been reported for the ring-substituted, mono- and diphosphoryl derivatives of imidazole (Gustafson and Wagner-Jauregg, 1954; Rathlev and Rosenberg, 1956; Cramer *et al.*, 1961; Rosenberg, 1964; Christensen, 1964), histamine (Rathlev and Rosenberg, 1956), carnosine (Severin *et al.*, 1947; Rozhanskii, 1952; Goodall, 1956), and 2-iodohistidine (Perlgut and Wainio, 1964). Various imidazoyl phosphate esters have also been studied (Wagner-Jauregg and Hackley, 1953; Baddiley *et al.*, 1956; Chambers and Moffatt, 1958; Theodoropoulos *et al.*, 1960; Asinger *et al.*, 1961; Schaller *et al.*, 1961; Cramer and Schaller, 1961).

Interest in the chemistry of phosphohistidine has been stimulated by the finding that a protein-bound phosphohistidine moiety is involved in the formation of adenosine triphosphate (ATP)[2] by mitochondria and is closely associated with the succinate thiokinase reaction (Suelter *et al.*, 1961; DeLuca *et al.*, 1963; Mitchell *et al.*, 1964; Kreil and Boyer, 1964). Another phosphohistidine-containing peptide has been shown to be involved in the transfer of phosphoryl groups to sugars in *Escherichia coli* (Kundig *et al.*, 1964). Free phosphohistidine had earlier been shown to serve as the phosphoryl donor of a phosphoramidase-catalyzed reaction (Fujimoto and Smith, 1962).

The work reported here establishes that the phosphohistidine associated with the succinate thiokinase reaction is the 3 isomer and reports the progress made in the synthesis, purification, and characterization of 1-phosphohistidine and 3-phosphohistidine. Preliminary reports of some phases of this work have been made previously (DeLuca *et al.*, 1963; Boyer *et al.*, 1963; Hultquist *et al.*, 1963; Hultquist and Moyer, 1965).

Experimental Procedure

Materials. Monohydrated L-1-methylhistidine, L-3-methylhistidine, monohydrated α-*N*-acetyl-L-histidine,

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[1] In this paper "phosphohistidine" will be used to designate either or both of the ring-substituted compounds, 1-phosphohistidine and 3-phosphohistidine. This designation is analogous to that used with other histidine derivatives such as methylhistidine and with other phospho compounds such as phosphoserine and phosphoarginine. The term phosphorylhistidine would also be appropriate. The designation of *N*-phosphonohistidine used in *Chemical Abstracts* is not regarded as appropriate because it is at variance with much biochemical nomenclature.

[2] Abbreviations used are: ATP, adenosine triphosphate; nmr, nuclear magnetic resonance.

and L-histidine methyl ester–2HCl were obtained from California Corp. for Biochemical Research. L-Histidine and imidazole were Eastman Organic Chemicals.

Monopotassium phosphoramidate was prepared by the method of Stokes (1893). The calcium salt of phosphoimidazole was obtained by the procedure of Rathlev and Rosenberg (1956). α-*N*-Acetyl-1-methylhistidine and α-*N*-acetyl-3-methylhistidine were prepared by reaction of 10 mg of methylhistidine in 1 ml of 0.25 M LiOH with 0.01 ml of acetic anhydride. Adding 20 ml of acetone separated the reaction mixture into two layers. Addition of 20 ml of acetone to the resulting lower layer precipitated the product which was then collected by centrifugation. The acetylated products of both methylhistidine isomers failed to give color on reactions with ninhydrin.

Phosphorylation of Imidazole Compounds. Imidazole compounds were phosphorylated with phosphoramidate at pH 7 and room temperature (Rathlev and Rosenberg, 1956). Histidine, histidine methyl ester, 1-methylhistidine, 3-methylhistidine, α-*N*-acetyl-1-methylhistidine, and α-*N*-acetyl-3-methylhistidine at concentration of 4 mg/ml were treated for varying intervals of time with 40 mg/ml of the monopotassium salt of phosphoramidate.

Paper Electrophoresis. High-voltage paper electrophoresis was carried out on Whatman No. 3MM paper. Electrophoretograms were sprayed with ninhydrin in acetone–acetic acid to detect compounds with free amino groups, with acidic molybdate reagent (Bandurski and Axelrod, 1951) to detect inorganic phosphate and acid-labile phosphates, and with Pauly reagent (Ames and Mitchell, 1952) to detect imidazole compounds with unsubstituted ring nitrogens. Phosphorylated imidazoles were detected with Pauly spray after the dried electrophoretogram had been heated for 30 min at 100°, in order to hydrolyze the N–P bond (DeLuca *et al.*, 1963). Iodine dissolved in ethanol was used to detect α-*N*-acetyl-1-methylhistidine and α-*N*-acetyl-3-methylhistidine (Cowgill, 1955).

The phosphorylation reaction mixtures were subjected to electrophoresis without any purification except in the case of the isomers of α-*N*-acetylmethylhistidine. To detect the phosphorylated derivatives of these compounds, much of the phosphoramidate and inorganic phosphate was removed from the reaction mixtures by the following procedure carried out at 0°. Acetone (20 ml) was added and the resulting precipitate was removed by centrifugation, washed with 20 ml of acetone, and then extracted with 0.5 ml of methanol. Addition of 10 ml of acetone to the methanol extract precipitated the crude product which was dissolved in pH 8.0 buffer.

Isolation of 1-Phosphohistidine and 3-Phosphohistidine. Histidine was treated with phosphoramidate for 50 min under the conditions described in the previous section. The phosphorylated derivatives were separated by Dowex-1 chromatography at pH 8.25 using a 0.1 M Tris, 0.1 M Tris–1.0 M $KHCO_3$ gradient and characterized as to phosphate and histidine content as described previously (DeLuca *et al.*, 1963). The analyses showed that compounds were eluted from the column by the pH 8.25 bicarbonate gradient in the following sequence: histidine, inorganic phosphate, 1-phosphohistidine, 3-phosphohistidine,[3] and diphosphohistidine. Fractions containing each of the monophosphohistidine isomers were pooled and bicarbonate was removed from these solutions by the following procedure, carried out at 0°. Perchloric acid–water (1:1) was added slowly with vigorous stirring until pH 3 was reached. When the evolution of CO_2 ceased (1–3 min), the solution was adjusted to pH 9.0 with KOH. After 1 hr $KClO_4$ was removed by filtration.

3-Phosphohistidine was prepared in large quantities by employing longer reaction times. To 2.0 g of histidine in 50 ml of water was added 3 g of phosphoramidate. After 3 days at room temperature the preparation was diluted with 4 l. of water and placed on a 4.8 × 32 cm Dowex-1-Cl^- column which had been equilibrated with 0.1 M Tris chloride buffer of pH 8.25. The column was eluted at pH 8.25 with a 0.1 M Tris chloride, 0.1 M Tris chloride–1.0 M LiCl linear gradient; 500 ml of each eluent was employed. Fractions giving a strong reaction with ninhydrin were pooled (approximately 120 ml). The solid lithium salt of 3-phosphohistidine was isolated from this solution by the following procedure which was carried out at 0°. A 0.1 M Tris, 0.5 M LiCl buffer of pH 8.25 was added (approximately 600 ml) until little ninhydrin-positive material was precipitated from a small aliquot of the solution by the addition of three volumes of ethanol. Lithium phosphate was then precipitated by the slow addition of three volumes of ethanol and removed by centrifugation. The lithium salt of 3-phosphohistidine was precipitated from the supernatant by slowly adding four additional volumes of ethanol. After 5 min, the amorphous salt was collected on a Büchner funnel using Whatman No. 1 paper and then lyophilized to dryness. The product could be stored in a dessicator at −20° without appreciable hydrolysis. An over-all yield of 30% was obtained. This product was employed in all studies reported here unless stated otherwise.

The potassium salt of 3-phosphohistidine was prepared by dissolving 92 mg of the lithium salt in 1.5 ml of water, adjusting the pH to 10 with KOH, and passing the solution through a column of the potassium form of Dowex-50. Fractions giving ninhydrin reactions were combined, adjusted to pH 9, and lyophilized to dryness.

Preparation and Isolation of α-N-Acetyl-3-phosphohistidine. To 2 ml of water were added 95 mg of calcium hydroxide and 10 mg of the lithium salt of 3-phosphohistidine. Over the course of 2 min, 0.1 ml of acetic anhydride was added with vigorous shaking. After several minutes the basic solution was centrifuged and the insoluble salts were discarded. Addition of 10 ml of ethanol to the supernatant at 0° resulted in a gel which was filtered under suction. The precipitate was washed with cold acetone and then immediately dis-

[3] The isomer bound more tightly to the column is designated 3-phosphohistidine on the basis of subsequent identification.

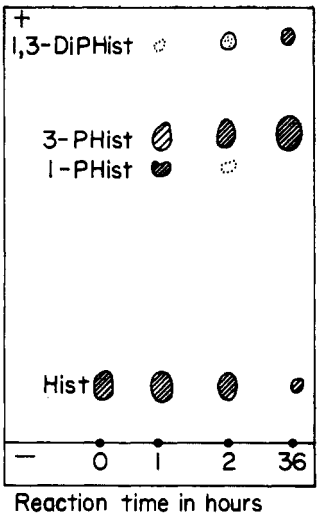


FIGURE 1: Electrophoresis of products resulting from phosphorylation of histidine. The buffer employed was 0.1 M ammonium acetate–0.001 M EDTA, adjusted to pH 8.25 with aqueous ammonia. 39 v/cm were applied for 1 hr at 20°. Compounds were detected with Pauly reagent after heat treatment of the electrophoretogram. 1,3-DiPHist, 1,3-diphosphohistidine; 3-PHist, 3-phosphohistidine; 1-PHist, 1-phosphohistidine; Hist, histidine. Identification of compounds is based on subsequent data.

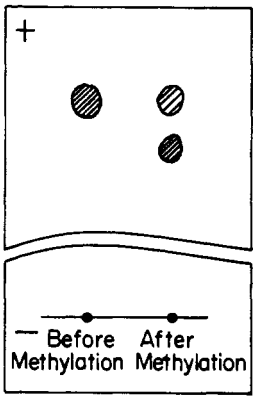


FIGURE 2: Electrophoresis of α-*N*-acetylphosphohistidine before and after reaction with CH_3I. Aliquots of the upper layer from the methylation reaction mixture were subjected to electrophoresis at 10° in 0.1 M ammonium acetate adjusted to pH 8.6 with aqueous ammonia; 45 v/cm were applied for 1 hr. Compounds were detected with acidic molybdate reagent.

solved in a basic solution. This product, the calcium salt of α-*N*-acetyl-3-phosphohistidine, gave negative tests with ninhydrin and Pauly reagents.

Preparation and Isolation of α-N-Acetyl-1-methyl-3-phosphohistidine. The calcium salt of α-*N*-acetyl-3-phosphohistidine was dissolved in 0.75 ml of 0.1 M $KHCO_3$. A concentrated solution of $KHCO_3$ was added until no further precipitation occurred. $CaCO_3$ was removed by centrifugation and to the 0.45 ml of supernatant was added 1.5 ml of methanol and 0.40 ml of methyl iodide. The sample was refluxed for 1 hr. Phosphate compounds were detected in only the upper aqueous layer.

The methyl derivative of α-*N*-acetyl-3-phosphohistidine was isolated from the reaction mixture by preparative paper electrophoresis. One-half of the upper layer was streaked on Whatman No. 3MM paper and subjected to electrophoresis at 10° in 0.1 M ammonium acetate adjusted to pH 8.6 with aqueous ammonia; 45 v/cm was applied for 1 hr. Acid-labile phosphate compounds were located on strips cut from the paper. The methylated derivative was eluted with 10 ml of 7.5 N aqueous ammonia.

Degradation of α-N-Acetyl-1-methyl-3-phosphohistidine to 1-Methylhistidine. The alkaline solution of α-*N*-acetyl-1-methyl-3-phosphohistidine was brought to dryness on a hot plate. Water was then added and the sample again was brought to dryness. The residue was refluxed in 2 ml of 6 N HCl for 5 hr. The hydrolysate was lyophilized to dryness. The residue, dissolved in 0.2 ml of water, was subjected to electrophoresis in buffers ranging in pH from 4.0 to 9.8 as presented in the Results seetion.

Characterization of 3-Phosphohistidine. Ultraviolet spectra were observed using approximately 0.15 mM solutions of the imidazole compounds in pH 2.1 HCl. Absorbancy was proportional to concentration under the conditions employed, even at 200 mμ. Extinction of 3-phosphohistidine was based on quantitative histidine and phosphate determinations following acid hydrolysis.

Hydrolysis studies of 3-phosphohistidine were carried out at 48.5° in HCl solutions ranging from 6 to 0.01 N, at 46° in 0.05 M citrate–0.05 M phosphate buffers ranging in pH from 2.1 to 5.0, and at 80.5° in 0.05 M citrate–0.05 M borate buffers ranging in pH from 2.2 to 9.2. The hydrolyses in HCl solution and in citrate–phosphate buffers were studied by following the decrease in absorbancy in the 230–234-mμ range which occurs on hydrolysis of 3-phosphohistidine. A solution (0.1 ml) of the lithium salt of 3-phosphohistidine in water was added to a cuvet, containing 3 ml of buffer, which had previously been brought to constant temperature in the cell compartment of a Beckman DU spectrophotometer. Initial concentration of 3-phosphohistidine was approximately 0.8 mM. Optical density was followed until the reaction was complete.

Hydrolyses in 0.05 M citrate–0.05 M borate buffers were followed by measuring the appearance of inorganic phosphate. Hydrolyses were carried out in a stoppered vessel at 80.5°. To initiate the reaction an aliquot of a solution of the lithium salt of 3-phosphohistidine was added to buffer preheated to 80.5°. The phosphohistidine concentration was approximately 0.13 mM. At the times indicated, 1-ml aliquots were removed, cooled to 0°, and analyzed for inorganic

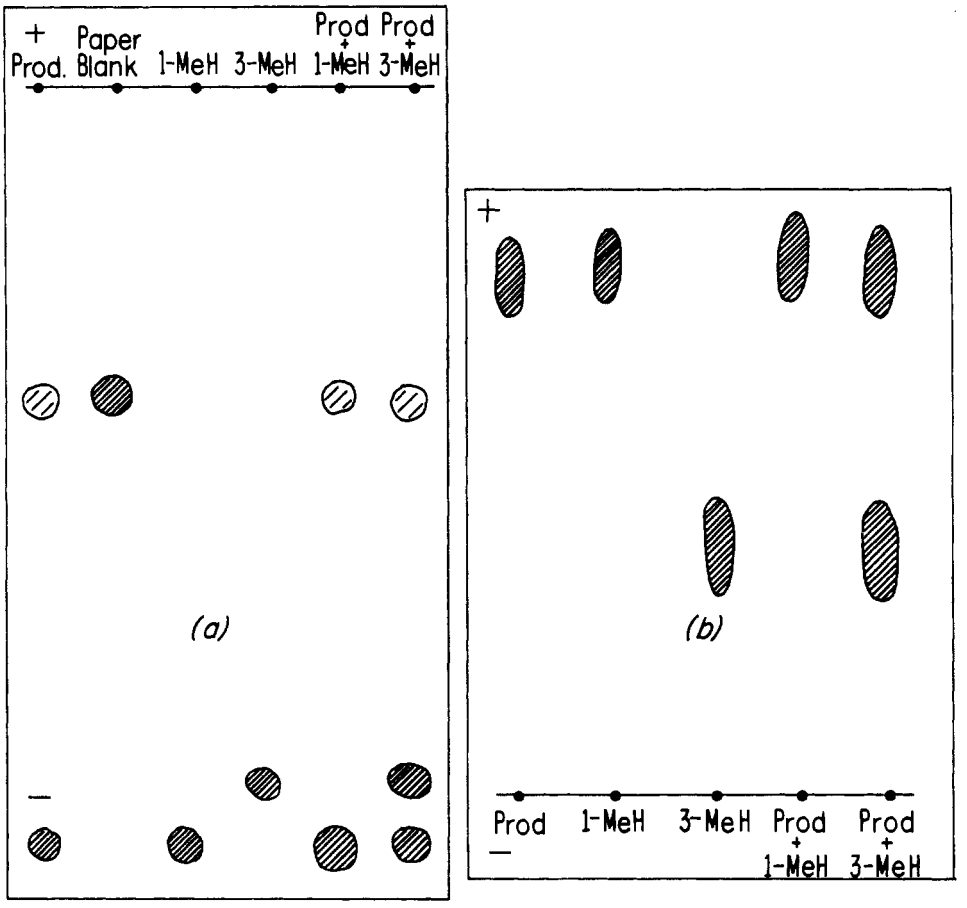


FIGURE 3: Coelectrophoresis of the acid hydrolysis product of α-*N*-acetylmethylphosphohistidine, prepared from 3-phosphohistidine, with 1-methylhistidine and 3-methylhistidine. Prod, acid hydrolysis product; 1-MeH, 1-methylhistidine; 3-MeH, 3-methylhistidine. Electrophoretograms were developed with ninhydrin reagent. (a) 1.35 hr at 45 v/cm in pH 5.5, 0.1 M acetate; (b) 1.0 hr at 45 v/cm in pH 9.8, 0.05 M borate.

phosphate by rapidly extracting the molybdate complex with cold isobutyl alcohol–benzene and subsequently measuring optical density of the extract at 310 mμ (Martin and Doty, 1947). The reaction was carried to completion.

pK' values were obtained by titrating 1.5 ml of 0.05 M solutions of imidazole compounds, previously adjusted to pH 12 with saturated NaOH, to pH 1.5 with 0.5 N HCl. HCl was added from a 5-ml buret, calibrated in 0.01-ml units, directly into the solution by a capillary tube. Samples were titrated at 25° in small test tubes into which were placed magnetic stirring bars. The pH was measured to the nearest 0.05 unit with a Beckman Expanded Scale pH meter using a single probe electrode. The titration curves were corrected for the volume of acid required to titrate a pH 12.0 solution of NaOH. Nuclear magnetic resonance (nmr) spectra were obtained using a Varian A-60 instrument.

Results

Electrophoresis of Phosphorylation Products. The progress of a reaction of histidine with phosphoramidate is shown in Figure 1. At the times indicated, aliquots of the reaction mixture were subjected to electrophoresis at pH 8.25. Each of the anionic products gave a positive test for a free amino group and acid-labile phosphate but gave a positive Pauly reaction only after N–P bonds had been hydrolyzed. Electrophoresis of the phosphorylated derivatives isolated by Dowex-1 chromatography revealed that the three derivatives, in order of decreasing electrophoretic mobility, were diphosphohistidine, 3-phosphohistidine, and 1-phosphohistidine.

Similar separations of the phosphorylated products were achieved by electrophoresis in buffers of pH 7.7, 8.0, and 8.5. 1-Phosphohistidine and 3-phosphohistidine failed to separate in buffers of pH 8.8, 9.1, 9.4, and 9.8. Complexing of 1- and 3-phosphohistidine with Co^{2+} was also demonstrated electrophoretically. The mobility of each isomer in pH 7.5, 0.05 M sodium acetate was approximately twice the mobility observed in the same buffer which contained 5×10^{-4} M Co^{2+}.

Electrophoretic analysis showed that reaction of

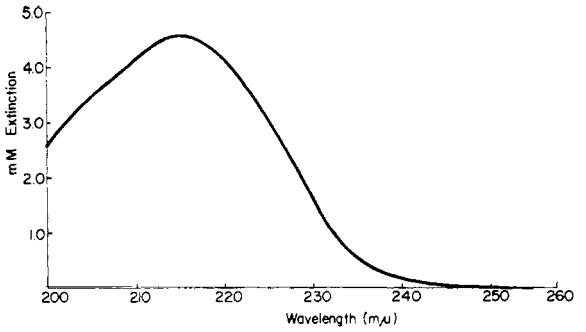


FIGURE 4: Ultraviolet spectrum of 3-phosphohistidine in pH 2.1 HCl.

TABLE I: pK' Values of Derivatives of Histidine.

Compound	pK' of Titratable Groups	
	Imidazolium	Amino
3-Phosphohistidine	6.4	9.5
Histidine	6.0	9.1
α-*N*-Acetylhistidine	6.9	—
1-Methylhistidine	6.4	8.6
3-Methylhistidine	5.7	9.2

histidine methyl ester with phosphoramidate for times less than 1 hr also yielded two phosphorylated products. Both compounds gave positive tests for amino groups and acid-labile phosphate but failed to give a Pauly reaction until N–P bonds had been hydrolyzed. The two derivatives were readily separated by electrophoresis in buffers of pH 6.4, 6.9, 7.1, and 7.5. In these buffers the compounds migrated slower than the isomers of phosphohistidine. With longer times of phosphorylation the faster moving compound increased in quantity while the slower compound disappeared. Small amounts of more negatively charged compounds were detected after longer reaction times.

Electrophoresis in pH 9.35 borate buffer revealed that after 2 days in the presence of phosphoramidate, no phosphorylation of 3-methylhistidine had taken place, while, in the same period, 1-methylhistidine was largely converted to a phosphorylated derivative that reacted with ninhydrin and migrated slightly slower than 3-phosphohistidine. Electrophoresis in pH 8.0 ammonium acetate–aqueous ammonia showed that these same phosphorylation conditions failed to phosphorylate α-*N*-acetyl-3-methylhistidine but partially converted α-*N*-acetyl-1-methylhistidine to a labile phosphate-containing compound.

Identification of the More Negatively Charged Monophosphohistidine as the 3 Isomer. The following reaction sequence established the position of the phosphoryl group of the more negatively charged isomer of phosphohistidine.

3-phosphohistidine + acetic anhydride →
α-*N*-acetyl-3-phosphohistidine $\xrightarrow{\text{methyl iodide}}$
α-*N*-acetyl-1-methyl-3-phosphohistidine $\xrightarrow{\text{HCl}}$
1-methylhistidine

Electrophoretic analysis of the methylation reaction mixture (Figure 2) showed that more than one-half of the α-*N*-acetyl-3-phosphohistidine was converted to a slower moving compound which gave a positive test for acid-labile phosphate but a negative test with ninhydrin reagent. Even after the electrophoretogram had been treated with acid to hydrolyze P–N bonds, the compound failed to give a color with Pauly reagent. This confirms methylation of a ring nitrogen. The compound had the same electrophoretic mobility at pH 8.0 as α-*N*-acetyl-1-methyl-3-phosphohistidine prepared by phosphorylating α-*N*-acetyl-1-methylhistidine. The absence of inorganic phosphate in the methylation reaction mixture shows that little hydrolysis of the phosphohistidine derivatives occurred during the procedure.

Electrophoresis of the product resulting from acid hydrolysis of the α-*N*-acetylmethylphosphohistidine prepared from 3-phosphohistidine revealed two compounds which gave ninhydrin reactions. Neither compound migrated as histidine or gave a positive reaction with Pauly reagent. The minor component comigrated with the single ninhydrin-positive compound obtained by carrying a paper blank through the entire procedure. The predominant product of the hydrolysis comigrated with 1-methylhistidine in pH 4.0 acetate, pH 5.5 acetate, pH 9.0 borate, and pH 9.8 borate. In each of these buffers, with the exception of pH 4.0 acetate, 1-methylhistidine and 3-methylhistidine are readily separable. In coelectrophoresis experiments at pH 5.5 and 9.8 the product and 3-methylhistidine moved as two distinct spots. Figure 3 shows the results of coelectrophoresis in buffers of pH 5.5 and 9.8.

Ultraviolet Spectrum of 3-Phosphohistidine. The spectrum of 3-phosphohistidine in pH 2.1 HCl is shown in Figure 4. The absorption maximum is found at 215 mμ. In this same solution 1-methylhistidine and 3-methylhistidine showed absorption maxima at 214 mμ; histidine, histidine methyl ester, and α-*N*-acetyl histidine at 211 mμ; phosphoimidazole at 210 mμ; and imidazole at 207 mμ.

Rates of Hydrolysis of 3-Phosphohistidine. An example of hydrolytic data obtained by following the decrease in absorbancy at 230 mμ is shown in Figure 5a for a hydrolysis at 48.5° in 0.5 N HCl. An example of data obtained by following the appearance of inorganic phosphate is shown in Figure 5b for a hydrolysis at 80.5° in pH 3.05 citrate–borate buffer. At all pH values studied the plots of logarithm of residual phosphohistidine *vs.* time were linear. The first-order rate constants at 80.5° are plotted *vs.* pH in Figure 6a. The

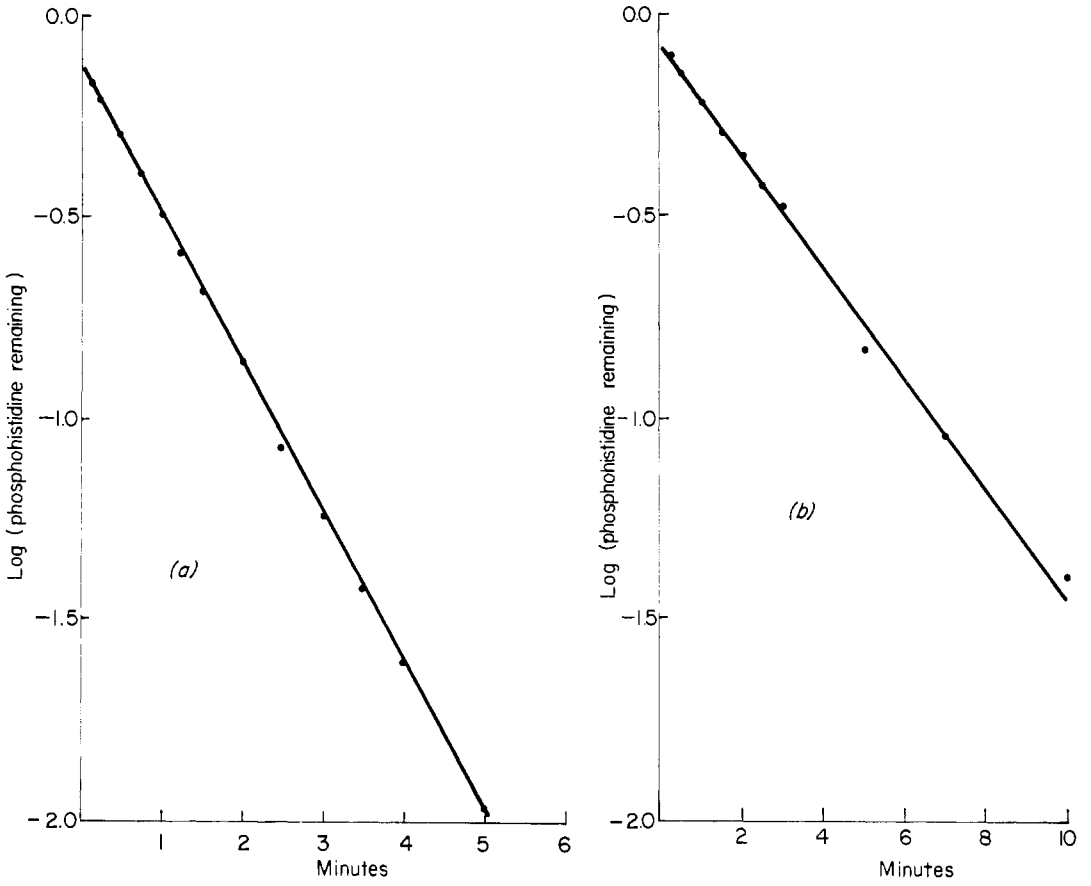


FIGURE 5: Hydrolysis of 3-phosphohistidine. (a) 0.5 N HCl at 48.5°. Decrease in absorbancy at 230 mμ was followed. Absorbancy at a given time minus final absorbancy is a measure of phosphohistidine remaining at that time. The log of this quantity has been plotted *vs.* time. (b) 0.05 M citrate–0.05 M borate buffer of pH 3.05 at 80.5°. Release of inorganic phosphate was measured photometrically at 310 mμ as the phosphomolybdate complex. Final absorbancy minus absorbancy at a given time is a measure of the phosphohistidine remaining at that time and the log of this quantity has been plotted *vs.* time.

first-order rate constants at 46 and 48.5° are plotted against hydrogen ion concentrations in Figure 6b and 6c.

In preliminary studies 1-phosphohistidine showed a faster rate of hydrolysis than 3-phosphohistidine at pH 8.25 and at pH 4.0. 1-Phosphohistidine also hydrolyzed to a large extent after standing for 1 min at 0° in 0.5 N H_2SO_4 in the presence of molybdate, conditions that hydrolyze very little 3-phosphohistidine.

Titration of 3-Phosphohistidine. Titration of 3-phosphohistidine from pH 12.0 to 3.5 resulted in the uptake of two protons per molecule. Additional protons were taken up below pH 3.5. These pK' values and the functional groups to which they are assigned are listed in Table I together with pK' values determined for a number of other imidazole compounds. At 3°, the pK' values of 3-phosphohistidine were found at 10.0 and 6.8. No groups with pK' values between 6.4 and 3.0 were found at 25 or 3°.

Nmr Spectra of 3-Phosphohistidine. The nmr spectra of 3-phosphohistidine in neutral and alkaline solution are similar to those of histidine, 1-methylhistidine, and 3-methylhistidine except for lack of peak splitting and for small chemical shifts. Comparison of the spectra of histidine and the potassium salt of 3-phosphohistidine at 25° in pH 9.0 D_2O demonstrates that the phosphoryl group shifts the C-2, C-4, and β hydrogen peaks downfield 4, 7, and 6 cps, respectively. Spectra of the lithium salt of 3-phosphohistidine in both neutral and alkaline solution show much broader peaks than are observed with the potassium salt.

In pH 2.5 HCl solution the peaks of 3-phosphohistidine were sharp and coupling was observed. As observed with the spectrum of histidine, the signal due to the hydrogens on the β carbon appeared as a doublet with a coupling constant of 7 cps. At this pH, coupling was also observed between the phosphorus atom and the C-2 and C-4 hydrogens. The coupling is revealed by a comparison of the spectra of histidine and 3-phosphohistidine in the region of the C-2 and C-4 peaks (see Figure 7). In 0.1 N HCl at 0°, 3-phosphohistidine showed broad peaks due to turbidity of the solution.

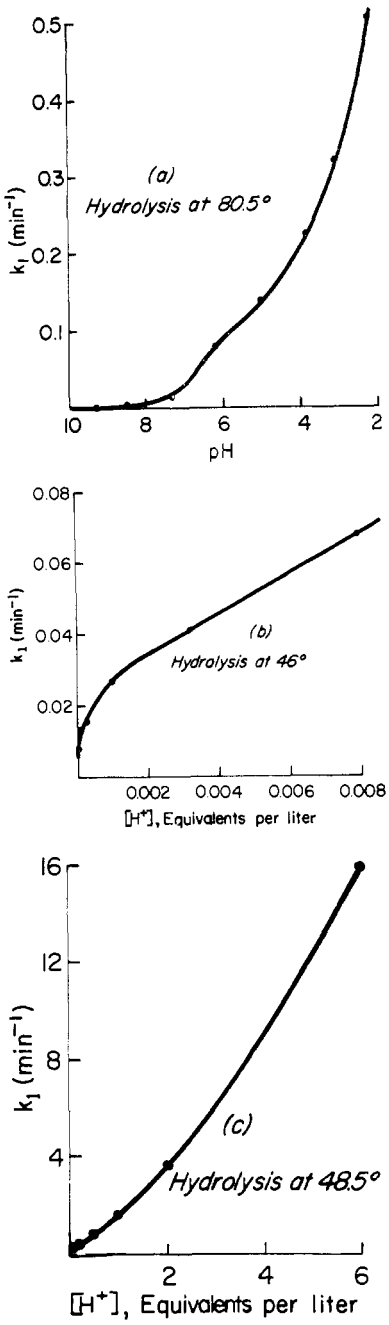


FIGURE 6: Effect of hydrogen ion concentration on rate constant k_1 for 3-phosphohistidine hydrolysis. (a) 0.05 M citrate–0.05 M borate buffers at 80.5°; (b) 0.05 M citrate–0.05 M phosphate buffers at 46°; (c) HCl solutions at 48.5°.

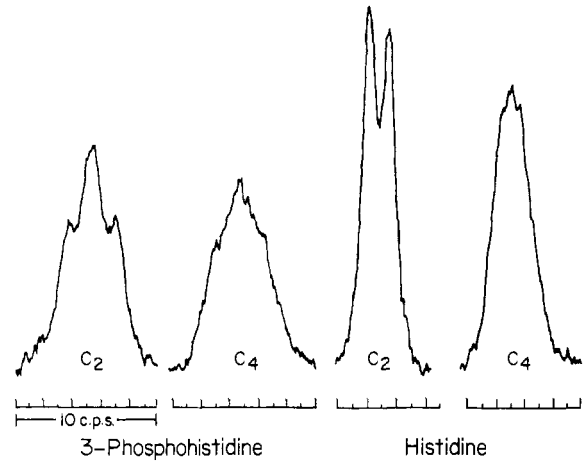


FIGURE 7: C-2 and C-4 hydrogen peaks of the nmr spectra of 3-phosphohistidine and histidine in pH 2.5 HCl. Samples contained 51 mg of the lithium salt of 3-phosphohistidine in 0.45 ml of pH 2.5 HCl and 31 mg of histidine in 0.50 ml of pH 2.5 HCl, respectively. Samples were prepared in HCl solution at 0° and spectra were observed immediately.

Splitting of the C-2 and C-4 peaks was observed, however, and the splitting constants were in agreement with those observed at pH 2.5.

Discussion

The phosphorylation of histidine by phosphoramidate, as summarized in Figure 1, follows an unusual reaction pattern. Whereas the 1 isomer is present in slightly greater amounts than the 3 isomer after 15 min of reaction, the isomers are present in approximately equal amounts after 1 hr. With longer times of reaction the amount of 1-phosphohistidine decreases while that of the 3 isomer increases, until after 3 days, 3-phosphohistidine is present in much larger amounts than free histidine, while only a trace of the 1 isomer exists. With time, the amount of diphosphohistidine slowly increases. 1-Phosphohistidine disappears between pH 7 and 9, even at 0°, but does not at pH 11.8.

The phenomenon may result from a reaction between the 1 isomer and phosphoramidate to give 1,3-diphosphohistidine. Since the 1 isomer disappears faster than the diphospho compound appears, the diphospho compound probably hydrolyzes spontaneously with preferential hydrolysis at the 1 position. The net reaction is a conversion of the 1 isomer to the 3 isomer. A faster rate of hydrolysis at the 1 position would be expected since 1-phosphohistidine is more labile than 3-phosphohistidine. Furthermore, our attempts to prepare and isolate 3-methyl-1-phosphohistidine were not successful, but 1-methyl-3-phosphohistidine was readily prepared. The reaction of 1-phosphohistidine with phosphoramidate does not depend on the presence of the free carboxylate ion of the phosphohistidine since the same phenomenon occurs with the isomers of phosphohistidine methyl ester. The reaction of 3-phosphohistidine with phosphoramidate might also be expected to occur. In this case the predominant product resulting from hydrolysis of the diphosphohistidine would be the starting material 3-phosphohistidine and the over-all result would be the hydrolysis of phosphoramidate. This reaction could

be the mechanism of the reported catalysis of phosphoramidate hydrolysis by phosphohistidine (Rathlev and Rosenberg, 1956).

The separation and purification of the isomers of phosphohistidine was made difficult by their lability and by the similarity of their physical properties. Below pH 7.0 or in the presence of heterocyclic nitrogen compounds hydrolysis occurred. At pH values above 8.5 the isomers failed to separate on paper electrophoresis or anion-exchange chromatography. The isolation of 1-phosphohistidine is more difficult because of the greater lability of this isomer and because of its reaction with phosphoramidate which prevents large amounts from accumulating.

The present findings suggest that previously reported preparations of phosphohistidine consisted largely or entirely of 3-phosphohistidine. These preparations were contaminated with histidine, phosphate, and salts (Severin and Yudelovich, 1947; Gustafson and Wagner-Jauregg, 1954; Rathlev and Rosenberg, 1956; Müller *et al.*, 1956; Rosenberg, 1964). The samples of 1- and 3-phosphohistidine obtained in the present study by elution of a Dowex-1 column with bicarbonate were shown by electrophoresis to be free of the other isomer, histidine, diphosphohistidine, inorganic phosphate, and phosphoramidate. The ratio of phosphate to histidine for each isomer was 1.00. Because of the difficulty of removing salts from these preparations and the difficulty of precipitating the sodium or potassium salt of phosphohistidine, the sample of 3 isomer used in most of the characterization studies was obtained by Dowex-1 chromatography with a lithium chloride gradient. This treatment allowed subsequent precipitation of the lithium salt of 3-phosphohistidine without precipitation of lithium chloride. Samples obtained in this manner were often contaminated with traces of 1-phosphohistidine. Nmr spectra demonstrated that even after lyophilization the samples contained a sizeable amount of water and smaller amounts of ethanol and Tris. The product was therefore not satisfactory for elementary analysis. The ethanol and Tris could be removed by passage through a Dowex-50 column (K^+ form).

Identification of 1-methylhistidine as the product of sequential acetylation, methylation, and acid hydrolysis of the more stable and more negatively charged isomer of phosphohistidine conclusively identified that isomer as 3-phosphohistidine. Blocking of the α-amino group was necessary since this group undergoes methylation more readily than the ring nitrogen. A considerable search was necessary to find conditions suitable for the methylation reaction. The pH obtained with $KHCO_3$ in water–methanol–methyl iodide was not low enough to hydrolyze the P–N bond and not high enough to cause hydrolysis of methyl iodide. The solvent solubilized both the polar α-*N*-acetyl-3-phosphohistidine and an adequate amount of the nonpolar methyl iodide. The temperature and time were adjusted so that a large degree of methylation was achieved without appreciable hydrolysis. The new compound detected on electrophoretograms after methylation of acetylphosphohistidine was undoubtedly the α-*N*-acetyl-1-methyl-3-phosphohistidine, since this phosphate-containing compound comigrated with the authentic compound, gave no Pauly reaction before or after acid hydrolysis, gave a negative test with ninhydrin reagent, and yielded 1-methylhistidine on acid hydrolysis.

One still had to consider the possibility that α-*N*-acetyl-1-methylhistidine moved at the same rate as the new phosphate-containing compound, and that the 1-methylhistidine arose from the α-*N*-acetyl-1-methylhistidine on acid hydrolysis. It was demonstrated, however, that under the conditions employed α-*N*-acetyl-1-methylhistidine, α-*N*-acetyl-3-methylhistidine, and α-*N*-acetylhistidine all migrated much slower than the new phosphate-containing compound.

The chemical and physical properties of the more stable isomer of phosphohistidine are in complete agreement with the attachment of the phosphoryl group on N-3 of the ring. The positive reaction with ninhydrin reagent and the ability to form an acetylphosphohistidine which gives no reaction with ninhydrin rule out the possibility of an α-*N*-phosphoryl compound. As discussed below, the titration data also indicate that a free α-amino group is present. Failure to couple with a diazonium compound demonstrates that substitution has occurred on the imidazole ring. Assignment of the phosphoryl group of the more stable isomer to the 3 position agrees with the finding that the phosphorylated derivative of 1-methylhistidine is relatively stable while 3-methylhistidine cannot be shown to undergo phosphorylation. The similarity between the phosphorus C-2 hydrogen and phosphorus C-4 hydrogen coupling constants obtained from the nmr spectrum is in agreement with a 3-phosphoryl derivative.

The properties of the more labile monophosphohistidine demonstrate that the phosphoryl group in this compound is bonded to a ring nitrogen rather than to the α-amino or carboxyl groups. This compound, like 3-phosphohistidine, gives a positive reaction with ninhydrin reagent and does not couple with a diazonium compound if care is taken to avoid hydrolysis. Both the electrophoretic and the column chromatographic studies showed that, for a given pH between 7.0 and 8.5, this molecule possesses a smaller negative charge than 3-phosphohistidine. Above pH 8.5 the two compounds have the same charge. This behavior would be difficult to explain if the phosphoryl group were bonded to either the carboxyl or α-amino group.

The diphosphohistidine eluted from the Dowex-1 column appears to be the 1,3-diphospho derivative. This compound, like the monophospho compounds, reacts with ninhydrin reagent but fails to react with Pauly reagent. The observation was made that diphosphohistidine has an extinction coefficient at 235 mμ three times that of 1- or 3-phosphohistidine. Since the ultraviolet absorption maximum of monophosphohistidine is found at longer wavelengths than that of histidine and histidine derivatives in which the ring nitrogens are unsubstituted, the absorption of 1,3-diphosphohistidine might be expected to be found at even longer wavelengths. Attachment of the second

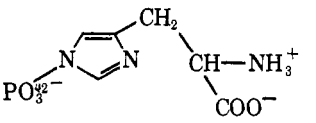
phosphoryl group to the side chain or to the other phosphoryl group by a pyrophosphate bond would not be expected to result in such a marked change. No evidence is available as to the identity or nonidentity of this compound with the diphosphohistidines reported previously (Rozhanskii, 1952; Rathlev and Rosenberg, 1956).

The shift of the ultraviolet spectrum of histidine to longer wavelengths on phosphorylation of the imidazole ring has been used to locate the phosphohistidines in column eluates and to study the kinetics of 3-phosphohistidine hydrolysis. Phosphohistidine, like the other histidine derivatives, has a much sharper absorption peak in pH 2.1 solution than in neutral solution.

The remarkable acid lability and alkali stability of phosphohistidine and other phosphoimidazole compounds have been emphasized since these compounds were first studied. Over the wide range of hydrogen ion concentrations used in the present study, hydrolyses were first order with respect to phosphohistidine. These findings agree with the first-order kinetics observed by DeLuca (1963) at pH 7, pH 4, and in 2 N H_2SO_4. The reported second-order kinetics for hydrolysis of a concentrated phosphohistidine solution at pH 7 and room temperature (Rathlev and Rosenberg, 1956; Müller *et al.*, 1956) may reflect intermediate diphosphohistidine formation.

The effect of hydrogen ion concentration on the hydrolysis of 3-phosphohistidine in acidic solution is distinctly different in some aspects from the effect on phosphoimidazole and acetylimidazole hydrolysis. An increase in hydrogen ion concentration has no effect on the hydrolysis of acetylimidazole after the imidazole ring has been completely converted to its protonated form (Jencks and Carriuolo, 1959). The curve of the rate constant k_1 for hydrolysis of phosphoimidazole plotted against pH shows a plateau between pH 6 and 4.5 (Jencks and Gilchrist, 1965), but k_1 increases rapidly below pH 2. In contrast, the rate of 3-phosphohistidine hydrolysis increases continuously as the hydrogen ion concentration is increased from 10^{-9} to 6 N. Since the plots of the first-order rate constant *vs.* hydrogen ion concentration for both 3-phosphohistidine and phosphoimidazole are essentially linear between 10^{-2} N and 6 N HCl, it would appear that in strongly acidic solution the principal hydrolytic process is a reaction involving a proton and the completely protonated form. The involvement of a proton rules out the spontaneous reaction of the cations with water as the principal mechanism.

The assignment of the pK' values of 3-phosphohistidine to titratable groups is based on the pK' values of model compounds. Only the α-amino group would be expected to have a pK' as high as 9.5. Assignment of pK' = 6.4 to the imidazolium ion of 3-phosphohistidine is based on evidence that phosphorylation of free imidazole has no effect on the dissociation of the imidazolium ion (Jencks and Gilchrist, 1965). Since no additional pK' values are found above pH 3, the pK' values for the phosphoryl and carboxyl groups must be below this pH. The very acidic character of the secondary phosphoryl hydrogen agrees with data obtained for phosphoramidate (Jencks and Gilchrist, 1965). The titration data thus indicate that, at pH 8.0, 3-phosphohistidine exists principally in the form



The electrophoretic mobility at this pH is in agreement with a net charge of -2 on the molecule. The lesser mobility of 1-phosphohistidine than 3-phosphohistidine between pH 7.7 and 8.5 suggests that the pK' of the imidazolium group of 1-phosphohistidine is greater than 6.4.

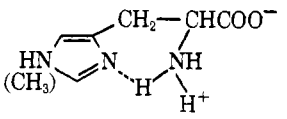
Analysis of the nmr spectra of 3-phosphohistidine shows that only weak spin–spin coupling occurs between the phosphorus atom and the ring hydrogens. Since the C-2 hydrogen of histidine appears as a doublet with $J_{2,4}$ = 1.4 cps, the triplet of the C-2 hydrogen of 3-phosphohistidine can be explained by a coupling constant of 1.6 cps for the phosphorus C-2 hydrogen interaction. The complex C-4 hydrogen peak of histidine results from coupling with the C-2 hydrogen and the hydrogens of the side chain. The change in the C-4 hydrogen peak on phosphorylation can be explained in terms of a P–H coupling with J approximately equal to 1.8 cps. These coupling constants are much smaller than the coupling constants observed for other compounds in which hydrogen and phosphorus atoms are separated by three bonds (Manatt *et al.*, 1963; Kaplan *et al.*, 1963).

The broad peaks of the nmr spectra and chromatographic anomalies suggest that salts of 3-phosphohistidine, especially the lithium salt, readily aggregate. Double zoning occurs on paper chromatography when preparations of 3-phosphohistidine containing lithium chloride are developed with ethanol–propanol–water–ammonia (Ebel and Volmar, 1951) or when the potassium salt of 3-phosphohistidine is developed with ethanol–0.005 M aqueous $CuSO_4$–concentrated aqueous ammonia (60:39:1). In both cases the degree of double zoning is completely dependent on the amount of sample spotted on the paper. Aggregation might also explain the broad peaks of the infrared spectrum exhibited by the lithium salt of 3-phosphohistidine.

In analogy with the isomers of phosphohistidine, the isomer of phosphohistidine methyl ester which is less negatively charged between pH 6.4 and 7.5 and which disappears in the presence of phosphoramidate is probably the 1 isomer. This compound possesses an ester linkage less susceptible to alkaline hydrolysis than the ester bond of 3-phosphohistidine methyl ester.

Molecular models of histidine derivatives show that a large number of intramolecular interactions are possible. Infrared studies of α-*N*-acetylhistamine indicate hydrogen bonding between the side-chain N–H and the ring N-1 (Lukton, 1961). Results from the present study suggest that hydrogen bonding between the α-amino

group and N-1 is responsible for the different physical properties of 3-methylhistidine and histidine relative to 1-methylhistidine. Histidine and 3-methylhistidine were found to have lower imidazolium pK' values and higher amino pK' values than 1-methylhistidine. These results explain the findings that, relative to histidine and 3-methylhistidine, 1-methylhistidine has a greater electrophoretic mobility toward the anode in the pH range 9.0–9.8 and a greater mobility toward the cathode in the pH range 4.5–6.2. Hydrogen bonding of the type



would be expected to make the imidazolium ion of histidine and 3-methylhistidine more acidic and stabilize the protonated form of their amino groups. 1-Methylhistidine would be unable to hydrogen bond in this manner. The finding that 3-methylhistidine acts as a less polar compound than 1-methylhistidine on paper chromatography (ethanol–0.05 M aqueous $CaCl_2$, 7:3) might also be explained by this postulated hydrogen bonding.

Previous hydrolytic, chromatographic, and electrophoretic studies demonstrated that the compound isolated from mitochondria and from a succinate thiokinase preparation is identical with the more stable isomer of phosphohistidine but easily distinguishable from the less stable isomer (DeLuca *et al.*, 1963; Hultquist *et al.*, 1963). The phosphohistidine isolated from a phosphoryl transferring enzyme of *E. coli* (Kundig *et al.*, 1964) has also been shown to be identical with the more stable isomer. The present findings thus establish that the isomer isolated from these sources is 3-phosphohistidine.

References

Ames, B. N., and Mitchell, H. K. (1952), *J. Am. Chem. Soc. 74*, 252.

Asinger, F., Haaf, F., Meisel, H., and Baumgarte, G. (1961), *Angew. Chem. 73*, 706.

Baddiley, J., Buchanan, J. G., and Letters, R. (1956), *J. Chem. Soc.*, 2812.

Bandurski, R. S., and Axelrod, B. (1951), *J. Biol. Chem. 193*, 405.

Boyer, P. D., Hultquist, D. E., Peter, J. B., Kreil, G., Mitchell, R. A., DeLuca, M., Hinkson, J. W., Butler, L. G., and Moyer, R. W. (1963), *Federation Proc. 22*, 1080.

Chambers, R. W., and Moffatt, J. G. (1958), *J. Am. Chem. Soc. 80*, 3752.

Christensen, N. H. (1964), *Arch. Biochem. Biophys. 105*, 643.

Cogwill, R. W. (1955), *Anal. Chem. 27*, 1519.

Cramer, F., and Schaller, H. (1961), *Chem. Ber. 94*, 1634.

Cramer, F., Schaller, H., and Staab, H. A. (1961), *Chem. Ber. 94*, 1612.

DeLuca, M. (1963), Ph.D. Thesis, University of Minnesota.

DeLuca, M., Ebner, K. E., Hultquist, D. E., Kreil, G., Peter, J. B., Moyer, R. W., and Boyer, P. D. (1963), *Biochem. Z. 338*, 512.

Ebel, J. P., and Volmar, Y. (1951), *Compt. Rend. 233*, 415.

Fujimoto, A., and Smith, R. A. (1962), *Biochim. Biophys. Acta 56*, 501.

Goodall, M. C. (1956), *Nature 178*, 539.

Gustafson, C., and Wagner-Jauregg, T. (1954), *Federation Proc. 13*, 222.

Hultquist, D. E., and Moyer, R. W. (1965), *Federation Proc. 24*, 656.

Hultquist, D. E., Moyer, R. W., Kreil, G., and Boyer, P. D. (1963), Abstracts of Papers, 145th National Meeting of the American Chemical Society, New York, N. Y., Sept., p. 61C.

Jencks, W. P., and Carriuolo, J. (1959), *J. Biol. Chem. 234*, 1272.

Jencks, W. P., and Gilchrist, M. (1965), *J. Am. Chem. Soc. 87*, 3199.

Kaplan, F., Singh G., and Zimmer, H. (1963), *J. Phys. Chem. 67*, 2509.

Kreil, G., and Boyer, P. D. (1964), *Biochem. Biophys. Res. Commun. 16*, 551.

Kundig, W., Ghosh, S., and Roseman, S. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1067.

Lukton, A. (1961), *Nature 192*, 422.

Manatt, S. L., Juvinall, G. L., and Elleman, D. D. (1963), *J. Am. Chem. Soc. 85*, 2664.

Martin, J. B., and Doty, D. M. (1947), *Anal. Chem. 21*, 965.

Mitchell, R. A., Butler, L. G., and Boyer, P. D. (1964), *Biochem. Biophys. Res. Commun. 16*, 545.

Müller, T., Rathlev, T., and Rosenberg, T. (1956), *Biochim. Biophys. Acta 19*, 563.

Perlgut, L. E., and Wainio, W. W. (1964), *Biochem. Biophys. Res. Commun. 16*, 227.

Rathlev, T., and Rosenberg, T. (1956), *Arch. Biochem. Biophys. 65*, 319.

Rosenberg, T. (1964), *Arch. Biochem. Biophys. 105*, 315.

Rozhanskii, M. E. (1952), *Biokhimiya 17*, 222.

Sakhatskaya, T. S. (1947), *Biokhimiya 12*, 163.

Schaller, H., Staab, H. A., and Cramer, F. (1961), *Chem. Ber. 94*, 1621.

Severin, S. E., Georgievskaya, E. F., Ivanov, V. I. (1947), *Biokhimiya 12*, 35.

Severin, S. E., and Yudelovich, R. Ya. (1947), *Biokhimiya 12*, 105.

Stokes, H. N. (1893), *Am. Chem. J. 15*, 198.

Suelter, C. H., DeLuca, M., Peter, J. B., and Boyer, P. D. (1961), *Nature 192*, 43.

Theodoropoulos, D., Gazopoulos, J., and Souchleris, I. (1960), *J. Chem. Soc.*, 5257.

Wagner-Jauregg, T., and Hackley, B. E. (1953), *J. Am. Chem. Soc. 75*, 2125.