BIOORGANIC CHEMISTRY 2, 1–10 (1972)

Phosphorylation of Glucose Mediated by Imidazole-Containing Catalysts

WILLIAM STILLWELL, GARY STEINMAN[1], AND RICHARD L. MCCARL

The Pennsylvania State University, University Park, Pennsylvania 16802

Received August 16, 1971

Histidine and some histidine derivatives were found to catalyze the synthesis of glucose phosphate from glucose and inorganic phosphate. A free imidazole ring with the N-1 proton possibly hydrogen bonded to a proximal carboxylate anion was required for catalysis in this system. A free amine group located near the imidazole was also required. Intermediates probably include (1) an imine formed between the amine of histidine and the carbohydrate carbonyl group and (2) phosphohistidine, in which phosphate is attached to N-1 of histidine.

INTRODUCTION

The catalytic function of an enzyme is usually the result of one or several amino acids held in a specific three-dimensional arrangement by a large number of nonparticipating amino acids. In a few cases it has been shown that the enzyme action can be duplicated, although at a much lesser rate, by a single amino acid or certain functional groups. For example, while chymotrypsin catalyzes the hydrolysis of *p*-nitrophenyl acetate (*1*), imidazole, histidine, polyhistidine, and several short peptides containing histidine were also found to catalyze *p*-nitrophenyl acetate hydrolysis, but at a much slower rate than does chymotrypsin (*2–4*).

Histidine is found at the active site of many enzymes catalyzing reactions involving transfer of phosphate to and from a carbohydrate (*5*). Kundig has discovered a transferase system in which a phosphoimidazole in a heat-stable protein is used to phosphorylate several sugar acceptors (*6*). Furthermore, N-phosphohistidine has been found at the active site of succinate thiokinase (*7,8*) and nucleoside diphosphokinase (*9*).

In this project it was discovered that histidine alone can catalyze the phosphorylation of glucose. The mechanism of catalysis probably involves a phosphohistidine intermediate. The role of carboxyl and amine functional groups in relation to the imidazole was studied. The mechanism of catalysis in this model system could shed light on the mechanism of enzymatic catalysis. The catalysis of glucose phosphate formation by simple imidazole-type compounds also may be significant in primitive earth studies.

EXPERIMENTAL

Materials

[U-^{14}C]Glucose was purchased from Schwarz BioResearch. [U^{14}C]L-histidine came from New England Nuclear and International Chemical and Nuclear Corporation.

[1]Present address: Ames-Yissum Ltd. (Division of Miles Laboratory), Jerusalem, Israel.

This latter compound was purified by electrophoresis in a 0.1 *M* ammonium acetate–0.001 *M* EDTA buffer at pH 8.25. The [^{32}P]inorganic phosphate, received as the disodium salt, $Na_2H^{32}PO_4$, was supplied by New England Nuclear. The peptides serylhistidine, serylhistidylaspartic acid, and histidylaspartic acid were purchased from Fox Chemicals. Sigma Chemical Company was the source of glucose-1-P, glucose-6-P, phosphoserine, and acid phosphatase. Dihydrourocanic acid, histamine, histidine methyl ester, and carnosine were obtained from Calbiochem. Calcium imidazole phosphate was a gift of Dr. D. E. Hultquist of the University of Michigan. Amino acids were purchased from Nutritional Biochemical Corporation. The potassium phosphamate was synthesized by the method of Strokes (*10*). All chemicals were reagent grade.

Methods

The production of glucose phosphate from glucose and inorganic phosphate in the presence of different imidazole containing catalysts was followed using ^{14}C-labeled glucose or ^{14}C-labeled histidine or ^{32}P-labeled inorganic phosphate. In most experiments both a 0.1 *M* KH_2PO_4 buffer of pH 7.8 and a solution containing the imidazole compounds were made fresh daily. At time zero equal volumes of each were mixed with a small volume of [U-^{14}C]glucose. All experiments were run numerous times using various concentrations of reactants. The specific activity of the added glucose was 240 mCi/mmole. For some experiments [^{14}C]L-histidine (sp act 198 mCi/mmole) and nonlabeled glucose were mixed at time zero. In other experiments both the imidazole-containing compound and the carbohydrate were nonlabeled and to this mixture was added [^{32}P]inorganic phosphate (sp act 100 mCi/mmole). Samples (5 μl) were taken to determine the total radioactivity. Samples were spotted on Whatman No. 1 paper at predetermined time intervals and the chromatograms were developed in various solvent systems and by electrophoresis. The solvent systems used were; *t*-butyl alcohol: butanone: water: concentrated ammonium hydroxide (4:3:2:1, v/v/v/v), 0.1 *M* potassium carbonate: ethanol (35:65, v/v), methanol:ethanol:water (9:9:2, v/v/v), 95% ethanol: 0.1 *M* sodium borate buffer pH 9.0 (68:32, v/v), methyl cellosolve: methyl ethyl ketone: 3.0 *N* ammonium hydroxide (7:2:3, v/v/v), and ethyl acetate: acetic acid: water (3:3:1, v/v/v). The first two solvent systems were used mainly for separation of imidazole-containing compounds, the next two for separation of glucose and its derivatives, and the last two for separation of glycerol and glyceraldehyde and their phosphorylated derivatives. The electrophoresis buffer was 0.1 *M* ammonium acetate: 0.001 *M* EDTA pH 8.25, and separation was achieved by applying 7 V/cm across the electrodes for several hours.

After the chromatograms were run they were dried, placed in the dark on the emulsion side of Kodax Medical X-ray Film, and after 3 to 5 days the films were developed. The bands of interest on the papers were cut out and placed in scintillation vials with 15 ml of scintillation fluid (4.0 g PPO and 100 mg of dimethyl POPOP to 1 liter of toluene). The samples were counted in a Beckman LS-200 B scintillation counter. The percentage yeilds with respect to labeled compounds added were determined.

Reaction products were identified by running appropriate standards cochromatographically or side by side and were located on either chromatograms or electropheretograms by means of specific color sprays. Imidazole compounds were located with a

sulfonilamide spray, glucose and glucose phosphates by a benzidine spray, and phosphate by a molybdate spray. Glucose phosphates were further identified by phosphatase degradation.

Phosphohistidine was synthesized via the method of Ratlev and Rosenburg (*11*), which uses potassium phosphamate as the phosphorylating agent. (The movement of histidine and 1- and 3-phosphohistidine in an electric field has been extensively studied by Boyer and Hultquist (*12*, *13*).) Phosphoimidazole was obtained as the insoluble calcium salt. It was converted to the sodium salt by mixing with the sodium form of Dowex 50 at 0°C for 30 min.

RESULTS

When histidine and ^{14}C-labeled glucose were mixed in a phosphate buffer of pH 7.8, several labeled products were formed in small yields. A product with an R_f of 0.38 in the methanol: ethanol: water solvent was formed in about 1% yield with respect to the [^{14}C]glucose added (Table 1). This compound, later shown to be an imine, was

TABLE 1

RATE OF PRODUCTION OF IMINE USING DL-HISTIDINE AND [^{14}C]GLUCOSE[a]

Time after mixing (min)	% Yield relative to [^{14}C]glucose added
0.5	0.72
2	0.96
30	0.97
75	1.0
135	1.1
255	1.1

[a] Three μCi of [^{14}C]-glucose (sp act 240 mCi/mmole) were added to 0.5 ml of 0.5 *M* KH_2PO_4 buffer at pH 7.8 and 0.0451 *M* in histidine. Samples of 30 μl were removed at various times and chromatographed. The radioactive compounds appearing in the methanol:ethanol:water solvent were cut out and counted. Zero time was when labeled compound was added to the reaction mixture. The percentage yield is expressed as the average of three experiments. Results for this type of experiment vary by up to 20%.

formed in direct relation to amount of histidine present (Fig. 1) but was independant of the phosphate concentration. This ^{14}C-labeled compound was cut out, eluted, lypholized to dryness, taken up in water, and rechromatogramed. Ninety-nine percent of the label on the chromatogram appeared as free glucose, implying that the basic

glucose structure was not changed during the first reaction. Similar results were noted when [^{14}C]histidine and unlabeled glucose were allowed to react in a phosphate buffer (*i.e.*, the same compound with an R_f of 0.38 was seen). When this compound was treated as before and placed in water, the label on the resulting chromatogram coincided with histidine. These experiments indicate that the compound of R_f 0.38 is in equilibrium with free glucose and free histidine in solution. The compound did not form when [^{14}C]glucose was added to imidazole or dihydrourocanic acid but did form when [^{14}C]glucose was allowed to react with histamine. This result implicates the α-amine of histidine in the reaction. Both the compound formed from ^{14}C-labeled histidine and

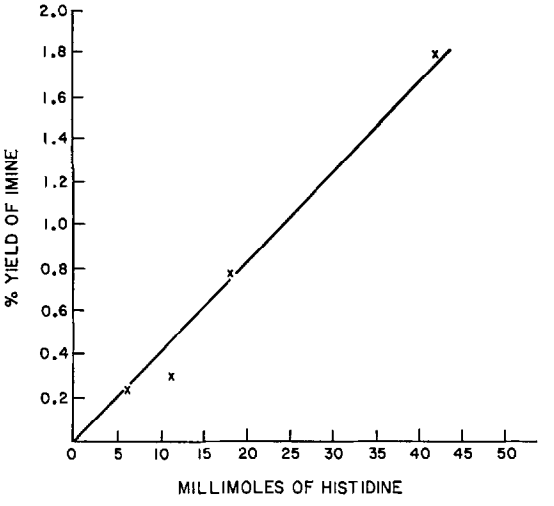

FIG. 1. Imine production as a function of histidine concentration when DL-histidine is mixed with [^{14}C]glucose (sp act 240 mCi/mmole) in phosphate buffer pH 7.8. The percentage yield is expressed relative to the [^{14}C]glucose added.

unlabeled glucose, and that formed from ^{14}C-labeled glucose and unlabeled histidine, were reduced with sodium borohydride. After reduction both compounds were stable to hydrolysis, and both gave the same R_f values in numerous solvent systems and by electrophoresis. On the basis of these data the compound appears to be an imine formed between the α-amine group of histidine and the carbonyl of glucose.

In addition to the imine two other products were formed in smaller yields from glucose and histidine in phosphate buffer. These compounds have R_f values of 0.26–0.31 in the methanol: ethanol: water solvent and were stable in aqueous solution. Both compounds, when cochromatographed with standard glucose 1- and -6 phosphate in several solvent systems and coelectrophoresed in ammonium acetate buffer, migrated with R_f values identical to the standards. These two compounds were subjected to acid phosphatase degradation, yielding free glucose. Consequently, these two compounds were designated glucose phosphates, most likely glucose 1- and -6 phosphate.

A study was made of the reaction mechanism for the production of glucose phosphate from glucose and inorganic phosphate using histidine or histidine-like catalysts. Potential catalysts containing each of the three functional groups present in histidine (amine,

carboxyl, and imidazole groups) was studied in relation to their role in glucose phosphate formation. These potential catalysts were added to the ^{14}C-labeled glucose in phosphate buffer system, and the production of imine and glucose phosphate was noted. Dihydrourocanic acid has the carboxyl and imidazone groups of histidine but possesses no α-amine; glycine has the α-amine and the carboxyl but no imidazole; histamine has the α-amine and the imidazole but no carboxyl; and imidazole contains neither the amine nor the carboxyl. From these studies the requirements for specific functional groups necessary to produce glucose phosphate could be studied. In several histidine like catalysts all three functional groups remained intact but in derivative form. A copper complex of histidine served to chelate the free amine and carboxyl groups of histidine (*14*). The tripeptide serylhistidylaspartic acid which has been found at the active site of phosphoglucomutase (*5*) was included in the system in place of histidine. Also replacing free histidine in the system were serylhistidine, which has a free carboxyl

TABLE 2

LIST OF THE COMPOUNDS CHECKED FOR THEIR CATALYTIC EFFORT ON PRODUCTION OF IMINE AND GLUCOSE PHOSPHATES[a]

Potential catalyst	Imine	Glucose phosphate
Imidazole	–	–
Histidine	+	+
Histimine	+	–
Dihydrourocanic acid	–	–
Histidine methyl ester	+	–
Histidine copper complex	–	–
Histidine (no phosphate)	+	–
Phosphoimidazole	–	+
Phosphohistidine	+	+
Phosphoserine	–	–
Phosphoserine and histidine (no phosphate)	+	–
Phosphoserine and imidazole (no phosphate)	–	–
Glycine	+	–
Histidylaspartic acid	+	+
Serylhistidylaspartic acid	+	–
Carnosine	+	–
Imidazole and glycine	+	+
Histidylaspartic acid (no phosphate)	+	–
Aspartic acid	+	–
Histidine and aspartic acid	+	+

[a] All experiments represented in this table were performed several times with various concentrations of the reactants (particularly the labeled glucose). In all experiments the individual components (phosphate, histidine derivatives, and labeled glucose) were prepared separately and mixed at time zero. Volumes were always less than 250 μl. Samples were removed 5 and 10 min after mixing. The + represents formation of the compound and – represents failure to detect the compound.

adjacent to the imidazole moiety, and histidylaspartic acid, which still has a free amine group adjacent to the imidazole group. The amine group was also included in a peptide bond in the compound carnosine (β-alanylhistidine). In one reaction histidine methyl ester was employed. In all cases, the effect of these potential imidazole-containing catalysts on the production of imine and glucose phosphate was noted (Table 2).

From the data summarized in Table 2 some important conclusions about the reaction mechanism for the formation of glucose phosphate can be made. All three functional groups of histidine are required for catalysis. Glucose phosphate did not appear unless the imine was formed; thus the free amine group on histidine was necessary for the appearance of glucose phosphate. Also, the amine group was required to be adjacent to the imidazole unit, as shown by the failure of carnosine, serylhistidylaspartic acid, and serylhistidine to catalyze glucose phosphate production. These peptides have the free amine group located several atoms away from the imidazole moiety.

Secondly, a free carboxyl in histidine is required for the catalysis—the methyl ester will not suffice. If the α-amine group is present but not the carboxyl group, as in histamine, an imine is formed, but no glucose phosphate. In histidylaspartic acid, where the free amine group of histidine is available and two free carboxyl groups are near by, there is a large increase in the catalytic effect on the production of glucose phosphates (Table 3). The fact that the imidazole moiety of histidine is required for catalysis is shown by the failure of glycine to effect formation of glucose phosphate, although the imine is formed. The methyl ester of histidine gave a much smaller yield of imine

TABLE 3

PRODUCTION OF IMINE AND GLUCOSE PHOSPHATES FROM GLUCOSE AND PHOSPHATE IN THE PRESENCE OF SEVERAL CATALYSTS[a]

Compound added as catalyst	Concn. of catalyst (*M*)	Quantity used (μl)	0.1 *M* Phos added	μCi Glu (μl)	Imine	% Yields glucose phosphate
Imidazole	0.155	50	50	2.0	0	0
Imidazole + glycine	0.155 each	50	50	2.0	0.1	Small
Histidine	0.0645	50	50	0.45	0.76	0.38
Histidine	0.077	50	50	2.0	1.41	1.55
Dihydrourocanic acid	0.238	50	50	1.0	0	0
Histidine methyl ester	0.232	50	50	1.0	0.44	0
Histamine	0.126	50	50	2.0	1.50	0
Serylhistidine	0.077	25	25	0.4	26.8	0
Serylhistidyl aspartic acid	0.077	25	25	0.4	26.7	0
Histidylaspartic acid	0.077	25	25	0.45	16.7	2.89
Aspartic acid	0.077	25	25	0.45	0.19	0
Histidine and aspartic acid	0.077 each	25	25	0.45	1.17	0.41

[a] In these experiments the designated quantity of freshly made imidazole derivatives was mixed with the same quantity of 0.1 *M* phosphate buffer and labeled glucose at time zero. Samples of 25 μl were removed 5 min after mixing and spotted for chromatography. Yields were based on the amount of labeled glucose added.

than did histidine, probably because of the greater neighboring group participation of the carboxylate as opposed to the less nucleophilic ester function. The structure of histidine in aqueous solution probably involves an intramolecular hydrogen bond between the free carboxylate and the N-1 hydrogen of the imidazole ring. Neutralizing the carboxylate by ester formation decreases both the neighboring group participation and hence the amount of imine formed and also decreases the hydrogen bond effect. Both result in a decrease in glucose phosphate production.

From these data it can be concluded that glucose phosphate production from glucose, phosphate buffer, and some catalyst, requires that the catalyst possess a free amine group adjacent to an imidazole ring. Furthermore, the amine group must form an imine with the carbohydrate and the catalyst must possess a free carboxyl group also adjacent to the imidazole ring.

It seemed possible that the observed histidine-mediated catalysis of glucose phosphate synthesis could involve a phosphorylated histidine intermediate. Phosphohistidine has recently come under scrutiny because of many of its unusual properties. Several workers have shown that phosphorylated imidazole or histidine can under suitable conditions phosphorylate several acceptor molecules. For example, Kundig (*6*) has discovered a low molecular-weight phosphorylated protein containing phosphohistidine at the active sites which will phosphorylate carbohydrates. Boyer (*7,8*) has demonstrated the transfer of phosphate from phosphohistidine to guanosine diphosphate in succinic dehydrogenase, and Wang (*15–19*) has demonstrated in his model systems phosphate transfer from 1-phosphoimidazole to adenosine diphosphate. In the studies presented here both N-phosphorylated imidazole and N-phosphorylated histidine were shown readily to phosphorylate glucose (Table 2).

Many enzymatic reactions involving phosphate and carbohydrates (*e.g.*, mutases and phosphatases) have been thought to involve a phosphoserine reactive site (*5*). In all cases histidine is located in the immediate vicinity of serine. The exact relationship between serine and histidine in these systems is unknown. In the case of mitochondria undergoing oxidative phosphorylation, Wadkins (*20*) has shown that it would be possible to transfer a phosphate from phosphohistidine to serine to form relatively stable and hence detectable phosphoserine. Since phosphoserine is proposed to be the active site compound, it might exhibit some phosphorylation properties when mixed with glucose. In this laboratory it was noted, however, the phosphoserine did not phosphorylate glucose. Since both histidine and serine are located at the active site of numerous phosphate–carbohydrate enzymes, it seemed possible that the phosphate could be transferred from phosphoserine to histidine and then to the carbohydrate. After phosphoserine, glucose, and either imidazole or histidine were mixed, no glucose phosphate could be detected. In the system described here phosphohistidine and phosphoimidazole (but not phosphoserine) do effect glucose phosphate formation.

Two possibilities for the transfer of phosphate from the imidazole ring of histidine to the carbohydrate exist: the reaction could proceed inter- or intramolecularly. A competitive reaction was run to resolve the possibilities. $^{32}P_i$ was added to a solution containing equimolar concentrations of glycerol, glyceraldehyde, and histidine. The production of phosphorylated glyceraldehyde and phosphorylated glycerol was checked by comparison with appropriate standards. If the reaction is intermolecular, both glyceraldehyde phosphate and glycerol phosphate should have been produced;

while if the reacion is intramolecular, only glyceraldehyde phosphate should have appeared. In fact only glyceraldehyde phosphate was produced, which is consistent with an intramolecular mechanism.

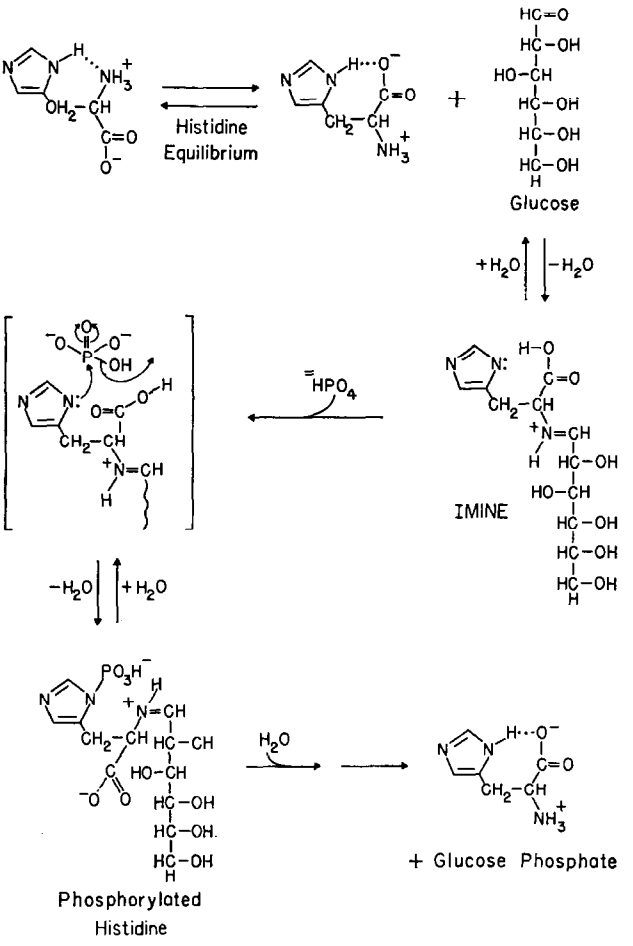

FIG. 2. Proposed mechanism for the production of glucose phosphates from glucose and inorganic phosphate using a histidine catalyst.

The mechanism presented in Fig. 2 is consistent with the experimental data, and the imine intermediate could be further stabilized by means of the reactions shown in Fig. 3. This imine type was proposed by Boyer (*21*) on the basis of pH and concentration studies involving phosphohistidine in mitochondria.

The effect on this system of having the functional groups held together in one molecule instead of being distributed among several different molecules can be readily seen from Table 3. Histidine catalysed the production of glucose phosphates. However, imidazole plus glycine, which combination possesses the three functional groups, did

not give the yield of phosphorylated products histidine did. The best catalyst was histidylaspartic acid, which gave a much larger yield of the glucose phosphates than did any other catalyst. Histidine plus aspartic acid gave a yield about the same as that provided by histidine. Having the amine and carboxyl groups held adjacent to the imidazole unit resulted in a large stimulation in the catalytic properties.

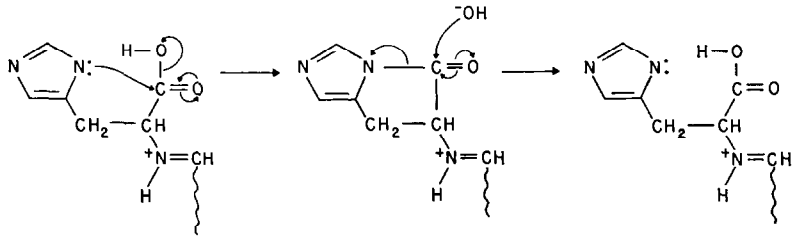

FIG. 3. Stabilization of imine intermediate through a Boyer-type intermediate.

DISCUSSION

If model systems, incorporating appropriate functional groups are chosen correctly, it should be possible to duplicate any enzyme action. In this paper it has been shown that the minimum requirements for catalysis of sugar phosphate production are a free amine and free carboxyl group adjacent to an imidazole ring. It was also possible to study several of the relationships between functional groups and catalytic properties. From these studies it appears that histidine might play an active role in catalysis by serving as the acceptor molecule for inorganic phosphate. Serine has no specific role in the model system. However, in the enzymatic case serine may serve as a secondary attachment site for phosphate or may serve in some other unknown capacity.

Demonstration of glucose phosphate synthesis in aqueous solutions mediated by a simple catalyst could be significant for primitive earth studies. The compounds reported in this paper (in particular, imidazole containing compounds and carbohydrates) were synthesized under presumed primitive earth conditions. Such biogenesis could have occurred on or near hydroxyappitite, and the glucose phosphate synthesized could be used as an energy source to drive thermodynamically unfavorable reactions. Such an energy-producing system could serve as a nucleus around which an incipient organism could be built.

ACKNOWLEDGMENTS

This work was supported in part by NASA Grant NGL 39-009-015.

REFERENCES

1. T. C. BRUICE AND S. J. BENKOVIC, "Bioorganic Mechanisms," Vol. I. Benjamin, New York 1966.
2. E. KATCHALSKI, G. D. FASMAN, E. SIMONS, E. BLOUT, F. GURD, AND W. KOLTUN, *Arch. Biochem. Biophys.* **88**, 361 (1960).

3. P. CRUICKSHANK AND J. C. SHEEHAN, *J. Amer. Chem. Soc.* **86**, 2070 (1964).
4. J. C. SHEEHAN, G. B. BENNETT AND J. A. SCHNEIDER, *J. Amer. Chem. Soc.* **88**, 3455 (1966).
5. M. DIXON AND E. C. WEBB, "Enzymes," Academic Press, New York, 1964.
6. W. KUNDIG, S. GHOSH AND S. ROSEMAN, *Proc. Nat. Acad. Sci. U.S.* **52**, 1067 (1964).
7. R. A. MITCHELL, L. G. BUTLER AND P. D. BOYER, *Biochem. Biophys. Res. Commun.* **16**, 545 (1964).
8. G. KREIL AND P. D. BOYER, *Biochem. Biophys. Res. Commun.* **16**, 551 (1964).
9. A. W. NORMAN, R. T. WEDDING AND M. K. BLACK, *Biochem. Biophys. Res. Commun.* **20**, 703 (1965).
10. H. M. STOKES, *Amer. Chem. J.* **15**, 198 (1893).
11. T. RATHLEV AND T. ROSENBERG, *Arch. Biochem. Biophys.* **65**, 319 (1956).
12. D. E. HULTQUIST, R. W. MOYER AND P. D. BOYER, *Biochemistry* **5**, 322 (1966).
13. D. E. HULTQUIST, *Biochem. Biophys. Acta* **153**, 329 (1968).
14. B. SARKER AND J. WIGFIELD, *J. Biol. Chem.* **242**, 5572 (1967).
15. J. H. WANG, *Proc. Nat. Acad. Sci. U.S.* **58**, 37 (1967).
16. T. A. COOPER, W. S. BRINIGAR AND J. H. WANG, *J. Biol. Chem.* **243**, 5854 (1968).
17. W. S. BRINIGAR AND J. H. WANG, *Proc. Nat. Acad. Sci. U.S.* **52**, 699 (1964).
18. W. S. BRINIGAR, B. B. KNOFF AND J. H. WANG, *Biochemistry* **6**, 36 (1967).
19. S. TU AND J. H. WANG, *Biochemistry* **9**, 4505 (1970).
20. C. L. WADKINS, *Biochem. Biophys. Res. Commun.* **13**, 411 (1963).
21. P. D. BOYER, *Science* **141**, 1147 (1963).