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Fermentative Phosphorylation of Glucosamine by Yeast

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Phosphorylation of glucosamine was investigated under the conditions of yeast fermentation (dissolution of glucose). Air-dried cell preparation of baker's yeast was used as enzyme source of fermentation and phosphorylation of glucosamine. By use of the energy derived from glucose dissolution, 20 mM of Gm 6-P were formed during 8 hr of incubation.

FBP, an intermediate of glycolysis, was found to be a suitable energy source than glucose in both efficiency of energy transference to glucosamine and rate of Gm 6-P formation.

A higher concentration of phosphate ion was required in the reaction mixture which contains glucose than that required in the reaction mixture containing FBP. Initiation of glucosamine phosphorylation in the reaction mixture containing glucose was closely related to the amount of glucose added. Gm 6-P formation was initiated immediately before the maximum accumulation of FBP and proceeded simultaneously with the degradation of accumulated FBP. Formation of Gm 6-P continued until FBP was consumed.

The remarkable catalytic action of an added ATP was observed in both glucose and FBP systems.

KEY WORDS: FBP accumulation of / Harden-Young effect / Gm 6-P / Glc NAc 6-P / ADP⇄ATP regeneration /

Abbreviations used:

Gm 6-P, glucosamine 6-phosphate; FBP, fructose 1,6-*bis*phosphate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; Glc NAc, N-acetylglucosamine; Glc NAc 6-P, N-acetylglucosamine 6-phosphate; UTP, uridine 5′-triphosphate; UMP, uridine 5′-monophosphate; PPi, inorganic pyrophosphate; UDP-Glc NAc, uridine 5′-diphosphate-N-acetylglucosamine; Pi, inorganic phosphate.

INTRODUCTION

Numerous studies on the metabolism of D-glucosamine by tissue preparations *in vitro* revealed the initial step in its utilization to be conversion to D-Gm 6-P. It would appear that, in most instances, this reaction is carried out by a non-specific hexokinase.[1-8] Another way of Gm 6-P synthesis by amination of fructose 6-phosphate has also been reported.[9-11]

The Gm 6-P is known as a primary product of mucopolysaccharides synthesis *in vivo*, and the pathway of mucopolysaccharides formation is shown in the following equations:

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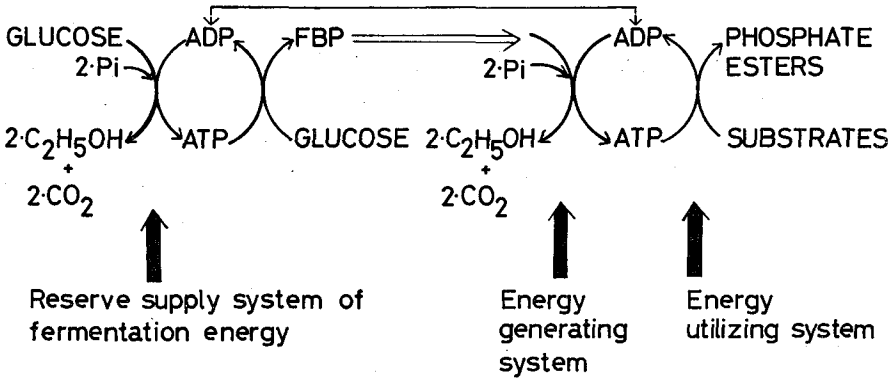

Fig. 1. Principle of Coupled Fermentation with Energy Transfer.

$$\text{Glucosamine} + \text{ATP} \longrightarrow \text{Gm 6-P} + \text{ADP} \quad (1)$$

$$\text{Gm 6-P} + \text{acetyl-CoA} \longrightarrow \text{Glc NAc 6-P} + \text{CoA} \quad (2)$$

$$\text{Glc NAc 6-P} \rightleftarrows \text{Glc NAc 1-P} \quad (3)$$

$$\text{Glc NAc 1-P} + \text{UTP} \longrightarrow \text{UDP-Glc NAc} + \text{PPi} \quad (4)$$

$$\text{UDP-Glc NAc} + \text{acceptor} \longrightarrow \text{Mucopolysaccharide} + \text{UMP} + \text{Pi} \quad (5)$$

The reaction shown by Eq. (1) is catalyzed by hexokinase.

During the course of the studies on the utilization of the energy of yeast fermentation to the phosphorylation processes of various organic compounds,[12–16] we have reported that a high level of inorganic phosphate was required to accumulate energy for ADP⇄ATP regeneration. The form of energy accumulated was FBP. The FBP was first pointed out in the fermentative mixture of glucose by cell-free extract of yeast by Harden and Young[17] as a sugar phosphate which breakdowns to ethanol and CO_2 by further metabolism, releasing 4 moles of ADP⇄ATP regeneration energy. The phenomenon of FBP accumulation in quantities by addition of a high level of inorganic phosphate was called as "Harden-Young" effect.

This paper describes the phosphorylation of glucosamine by cooperative reaction of yeast hexokinase and ATP regenerating system of baker's yeast under fermentative conditions. Figure 1 shows the scheme of transfer of energy of yeast fermentation and phosphorylation mechanism of substrate added.

MATERIALS AND METHODS

Microorganism: The microorganism used was baker's yeast, obtained from Oriental Baker's Yeast Co., Ltd., Osaka. Pressed baker's yeast was crushed and dried using electric fan for 12–24 hr at room temperature. Air-dried cells were completely dried over P_2O_5 *in vacuo* at room temperature and kept at −20°C until use.

Chemicals: Barium salt of FBP was kindly supplied by Kyowa Hakko Kogyo Co., Ltd., Tokyo and all other chemicals were analytical grade commercial products.

Analysis: FBP was determined by the method of Roe.[18] Glucosamine and

Gm 6-P were assayed colorimetrically by the method of Blix,[19] and GlcNAc and GlcNAc 6-P were also assayed colorimetrically by Leloir[10] and Reissig.[20]

Standard reaction mixture: A standard reaction mixture consisted of 50 mM of glucosamine, 200 mM of FBP or 400 mM of glucose 200–400 mM of phosphate buffer (pH 7.4), 10 mM of $MgSO_4 \cdot 7H_2O$ and 100 mg per ml of dried cells of yeast in a total volume of 2.0 ml. The reaction was carried out at 28°C with shaking. The content of each component was varied to find the optimum conditions for glucosamine phosphorylation.

Determination of Gm 6-P and Glc NAc 6-P in the reaction medium: Paper chromatography was employed for the separation of Gm 6-P and Glc NAc 6-P from glucosamine in the reaction mixture. Spun supernatant solution (3000 rpm, 20 min) of 20 to 100 μl of boiled reaction mixture (100°C, 3 min) was spotted on two paper strips (2×40 cm). After chromatography by multiple development (3 times by ascending) using a solvent system of 95% ethanol: 1 M ammonium acetate (7.5 : 3),[21] glucosamine and Gm 6-P were detected as cherry-red colored spots on one of the dried papers by treatment with Ehrlich's reagent, described by Partridge.[22] The spots corresponding to glucosamine and Gm 6-P on the untreated paper were cut out and transferred to individual test tubes, and the content of glucosamine or Gm 6-P was determined colorimetrically by the method of Blix.[19] Glc NAc and Glc NAc 6-P were also detected on the paper chromatogram as purple-red spots according to the method of Salton[23] and assayed colorimetrically from untreated paper by the method of Reissig.[20]

Isolation of Gm 6-P and Glc NAc 6-P from the reaction mixture: To the supernatant of the boiled reaction mixture (45 ml) treated with an equivolume of magnesia mixture,[24] 1/3 volume of 20% barium acetate was added. Precipitates formed were removed by filtration. The filtrate was diluted 4 times with 99% ethanol and allowed so stand for several hr. White precipitates were collected by centrifugation and redissolved in a small portion of distilled water. The solution was applied on to a column of Dowex 50 X-8 (H^+ form, 1.8×20 cm). The Gm 6-P was absorbed on the column and eluted with 0.1 N HCl according to the method of Leloir.[25] Fractions containing Gm 6-P were combined and condensed under reduced pressure at 45°C to dryness and dried again over soda lime in desiccator *in vacuo* to remove HCl. The dried sample was redissolved in a small volume of distilled water and 110 to 130% equivalent amount of solid $BaCO_3$ was added and stirred for 2 hr. Residual precipitates were filtered off, and the filtrate was decolorized with activated charcoal. The charcoal-treated filtrate was diluted with 3 to 4 volumes of 99% ethanol. White precipitates were collected by centrifugation and dried in desiccator *in vacuo*. The Glc NAc and Glc NAc 6-P were found in the unabsorbed fractions of Dowex 50 X-8, H^+ form column. Fractions containing N-acetylated aminosugars were combined and neutralized by addition of 1 N NaOH and applied on the column of Dowex 1, X-2 (acetate form. 1.8×20 cm). The Glc NAc and N-AcGm 6-P were separated by the method of Horecker[26] and Distler.[1] Barium salt of Glc NAc 6-P was obtained from the eluent as the case of Gm 6-P mentioned above.

Chemical N-acetylation of the produced Gm 6-P: The Gm 6-P thus obtained was acetylated by treating with acetic anhydride as described by Distler[1] and

Maley,[27] and the formed Glc NAc 6-P was chromatographed on the column of Dowex 1, X-2 (acetate form) as above stated.

RESULTS

Effects of phosphate ion concentration and ATP addition on the phosphorylation of glucosamine: A comparison of the optimum phosphate concentrations for glucosamine phosphorylation was made in both reaction systems of glucose and FBP.

As shown in Table I, the optimum concentration of phosphate ion was 200 mM in the FBP system and 400 mM in the glucose system, respectively. A higher level of phosphate ion was required for the glucose system than that required for the FBP system. However, the presence of an excess of phosphate ion resulted in a decrease of Gm 6-P formation. The amount of Gm 6-P formed in the reaction system of FBP within 2 hr incubation corresponded to about 7 to 8 times formed in the glucose system within 4 hr incubation without addition of ATP.

Addition of 2.5 mM of ATP to the reaction mixture increased the rate of formation of Gm 6-P by a factor of 1.5–2 times in either the FBP system or glucose system. A remarkable effect of added ATP on glucosamine phosphorylation was observed in the glucose systems containing 200–400 mM of phosphate ion. Inhibition of Gm 6-P formation by higher concentration of phosphate was released by addition of 2.5 mM ATP in glucose and FBP systems.

Effect of glucose concentration on glucosamine phosphorylation: Since a difference was observed in the rate of glucosamine phosphorylation between the

Table I. Effects of Phosphate Concentration and ATP Addition on Phosphorylation of Glucosamine

Energy source	Pi conc. (mM)	ATP (mM)	Gm 6-P formed (mM)			
			Incubation time (hr)			
			2	4	6	8
FBP	200	0	41.0	40.0		
	200	2.5	48.4	49.5		
	400	0	28.0	28.0		
	400	2.5	38.0	42.0		
Glucose	200	0		5.7	6.3	4.6
	200	2.5		12.2	10.0	8.0
	400	0		5.2	8.2	13.3
	400	2.5		15.2	21.4	28.8
	600	0		4.5	6.0	7.2
	600	2.5		8.3	12.2	18.6
	800	0		3.0	4.6	5.0
	800	2.5		4.3	5.3	7.2

The reaction mixture contained (mM): K-phosphate buffer (pH 7.4), indicated; FBP, and glucose, indicated; glucosamine 150; $MgSO_4 \cdot 7H_2O$, 10; ATP, indicated. Dried baker's yeast, 100 mg per ml. Total volume 2.0 ml. Shaken at 28°C.

glucose system and the FBP system, the chemical changes in the reaction system of the glucose were examined.

Figure 2 shows the chemical changes of the reaction mixture containing 200–800 mM glucose with fixed phosphate concentration at 400 mM. It is evident from the figure that phosphorylation of glucosamine was hindered to some extent by elevation of the amount of glucose added, and that phosphorylation of glucosamine was initiated immediately before maximum accumulation of FBP was attained. Phosphorylation of glucosamine continued until FBP was consumed.

The amount of FBP accumulated in the reaction mixture with low glucose content was less than that in the reaction mixture with high concentration of glucose, in which a longer incubation time of more than 2 hr was required to attain the maximum accumulation. About 40 mM of FBP was accumulated within 1 hr incubation in the reaction mixture containing 200 mM or 400 mM of glucose. While, more than 75 mM of FBP was accumulated in the reaction mixture with more than 800 mM of glucose added, and the maximum accumulation was attained in 2 hr or more incubation. Longer incubation was required to form a relatively large amount of Gm 6-P when high concentration of glucose was added. In the reaction mixture containing low concentration of glucose, a comparable amount of Gm 6-P was formed at an early incubation time, but it was degraded soon after the FBP was consumed. It can be concluded that a suitable concentration of glucose is between 400 and 600 mM.

Supplementation of the reaction mixture with a hexokinase preparation did not show stimulant effect to elevate the rate of glucosamine phosphorylation.

Optimum concentration of glucosamine: The rate of phosphorylation of glucosamine in the glucose system was compared with that in FBP system.

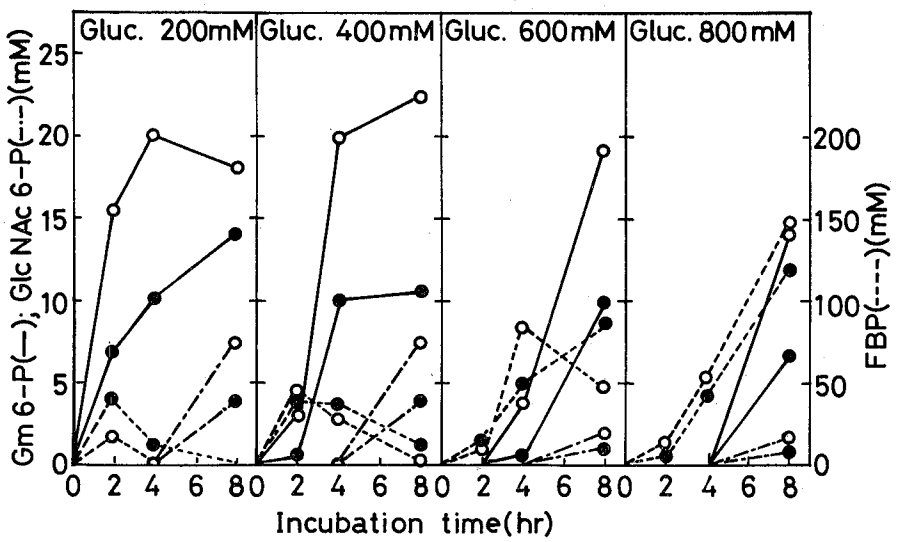

Fig. 2. Relationship between FBP Accumulation and Glucosamine 6-P Formation as a Function of Glucose Concentration.
The reaction mixture (2 ml) contained (mM): potassium phosphate buffer (pH 7.4), 400; glucosamine, 50; $MgSO_4 \cdot 7H_2O$, 10; ATP, 0 (—●—) or 5 (—○—). Dried baker's yeast, 100 mg per ml. Glucose content is indicated in the figure.

Table II. Effect of Glucosamine Concentration on Glucosamine 6-P Formation

Glucosamine conc. (mM)	Gm 6-P formed (mM)	
	FBP* system	Glucose** system
50	24.2	18.6
100	43.0	27.4
150	50.0	28.8

The reaction mixture contained (mM): K-phosphate buffer (pH 7.4), 200 in FBP system; 400 in glucose system; FBP, 100; glucose, 700; $MgSO_4 \cdot 7H_2O$, 10. Dried baker's yeast, 100 mg per ml in a total volume of 2.0 ml. Shaken at 28°C.
* 3 hr incubated; ** 8 hr incubated.

Table II shows the rate of glucosamine phosphorylation decreases in inverse proportion to the concentration of glucosamine added. The percentage of Gm 6-P formed from 50 mM, 100 mM, and 150 mM of glucosamine was 48%, 43%, and 33% respectively in the FBP system within 3 hr incubation, and 37%, 27%, and 19% in the glucose system within 8 hr incubation. The overall yield of Gm 6-P amounted to 50 mM from 150 mM of glucosamine in the FBP system and 28.8 mM from 100 mM of glucosamine in the glucose system. Further incubation was ineffective for increasing the amount of Gm 6-P formed.

From the results obtained, the optimum condition for glucosamine phosphorylation under the fermentative condition can be summarized as follows: Potassium phosphate buffer (pH 7.4), 200 mM in FBP system or 400 mM in glucose system; FBP, 100 mM, or glucose, 400–600 mM; glucosamine, 100–150 mM; $MgSO_4 \cdot 7H_2O$, 10 mM and dried cells of baker's yeast, 100 mg per ml.

Side product: As shown in Figs. 2 and 3, derivatives of N-acetylated gluco-

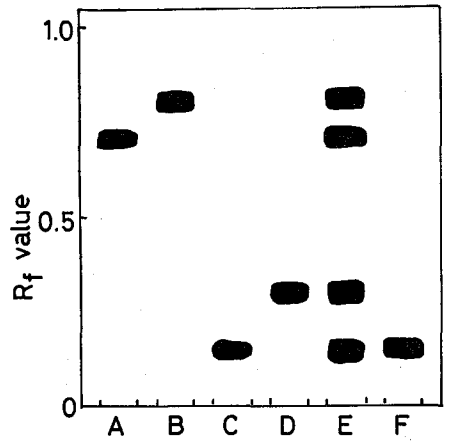

Fig. 3. Detection of Hexosamine and N-acetylated Hexosamine by Paper Chromatography.
Solvent system used was composed of 95% ethanol: 1 M ammonium acetate (pH 7.5), 7.5: 3. A, glucosamine; B, Glc NAc; C, Gm 6-P; D, Glc NAc 6-P; E, reaction mixture; F, product as barium salt.

samine were detected in the reaction mixture. They were identified as Glc NAc and Glc NAc 6-P by paper chromatography and their amounts were about 5 to 10 mM within 4 to 6 hr incubation.

Isolation and identification of Gm 6-P and Glc NAc 6-P: Elution profiles of Gm 6-P and Glc NAc 6-P on column chromatography are shown in Fig. 4. Behavior of these compounds on paper chromatograms[26] was identical to that of authentic Gm 6-P and Clc NAc 6-P, respectively.

Infra-red spectra of both Gm 6-P and Glc NAc 6-P, as presented in Fig. 5, matched those of authentic samples.

Results of elementary analysis were consistent with values calculated for Gm 6-P barium salt and Glc NAc 6-P barium salt.

Anal. Found: C, 18.20; H, 3.05; N, 3.62. Calcd. for $C_6H_{12}O_5NPO_3Ba$: C, 18.28; H, 3.04; N, 3.55. Found; C, 21.98; H, 3.16; N, 3.16; Calcd. for $C_8H_{14}O_6$–

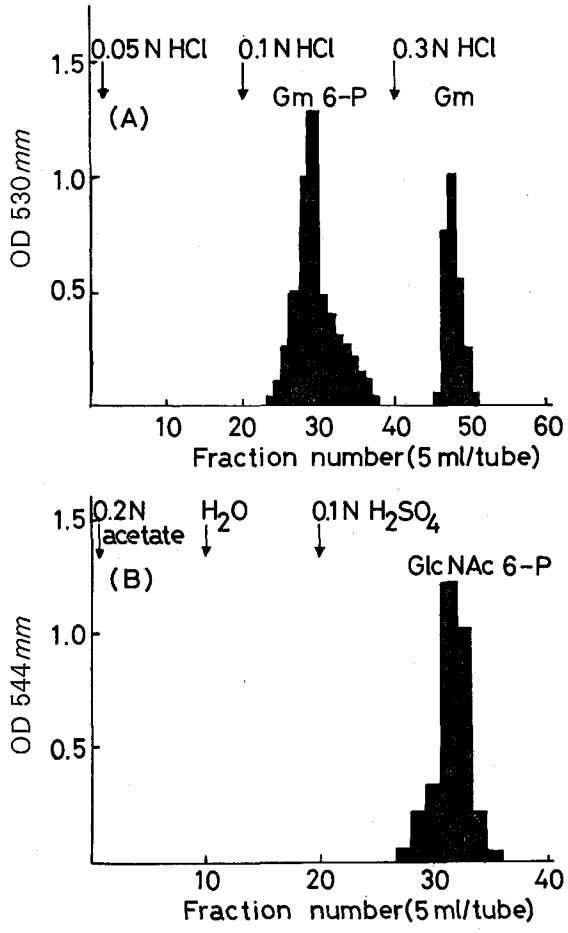

Fig. 4. Separation of Glucosamine 6-P and N-acetylglucosamine 6-P by column Chromatography.
(A): Separation of glucosamine 6-P on column of Dowex 50 X-8 (H^+ form, 1.8×20 cm). (B): Separation of Glc NAc 6-P on column of Dowex 1, X-2 (acetate form, 1.8×20 cm).

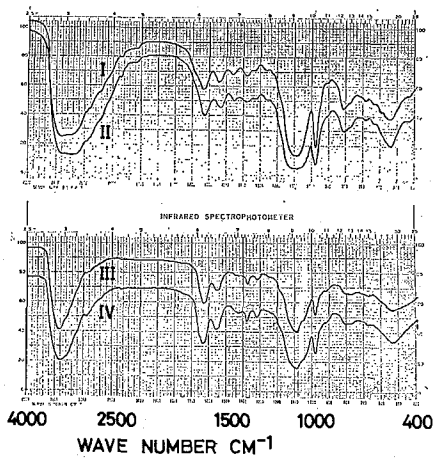

Fig. 5. Infra-red Spectra of Glucosamine 6-P and N-Acetylglucosamine 6-P.
(I): Authentic Gm 6-P (Ba salt), (II): Product; (III): Authentic Glc NAc 6-P; (IV): Product acetylated by chemical means.

NPO_3Ba: C, 22.02; H, 3.21; N, 3.21.

Chemical acetylation of produced Gm 6-P: Chemical acetylation of the produced Gm 6-P was carried out according to the method of Distler[1] by treating with acetic anhydride in methanol. The yeild of Glc NAc 6-P was 100% based on the substrate. Barium salt of the Glc NAc 6-P was identified by color reaction, paper chromatography, IR spectrum, and elementary analysis.

DISCUSSION

Energy of yeast fermentation coupled effectively to the phosphorylation of glucosamine in both reaction systems containing glucose and FBP in the presence of high concentrations of phosphate ion. The fact that a higher concentration of phosphate ion was required in the glucose system than that required the FBP system suggests two functions of the phosphate ion: the one is to regulate the rate of glucose dissolution, which related closely to the accumulation and consumption of FBP, and the other is the fact that phosphate ion itself is to be a substrate to form FBP, accordingly the requirement of phosphate ion was twice as much as FBP system.

In both reaction systems containing glucose and FBP, the presence of an excess of phosphate lowered the rate of phosphorylation of glucosamine. This suggests that energy generating system of yeast fermentation was partially inhibited and then dissolution of FBP to pyruvate was delayed.

The stimulant effect to form Gm 6-P by addition of a catalytic amount of ATP implicates that the relative ATP content of the phosphate donor pool or the reaction system is insufficient to phosphorylate both glucose and glucosamine in same time, and accordingly the addition of a catalytic amount of ATP to the reaction mixture would promote the apparent rate of glucosamine phosphorylation and FBP accumulation. The fact that phosphorylation of glucose was accelerated by addition of a

catalytic amount of ATP, which was inferred from the amount of FBP accumulated, confirmed the above assumption.

Owing to a lower affinity of hexokinase for glucosamine[28,29] than that for glucose and a lower phosphorylation rate of glucosamine than that of glucose,[2,30] the rate of glucosamine phosphorylation would be inhibited considerably if free glucose were presented simultaneously with glucosamine in the same reaction mixture. FBP does not compete with glucosamine in the phosphorylation process, because it does not require any ATP to phosphorylate itself.

It can be concluded that the concentrations of phosphate ion and glucose in the reaction mixture are important factors in the coupling system of glucose dissolution by baker's yeast fermentation and its hexokinase activity.

REFERENCES

(1) J. J. Distler, J. M. Merrick, and S. Roseman, *J. Biol. Chem.*, **230**, 497 (1958).
(2) L. Berger, M. W. Slein, S. P. Colowick, and C. F. Coli, *J. Gen. Physiol.*, **29**, 379 (1946).
(3) D. H. Brown, *Biochim. Biophys. Acta*, **7**, 487 (1951).
(4) S. Ghosh, H. J. Blumenthal, E. Davidson, and S. Roseman, *J. Biol. Chem.*, **235**, 1265 (1960).
(5) R. R. Harpur and J. H. Quastel, *Nature(London)*, **164**, 693 (1949).
(6) A. Sols and R. K. Crane, *J. Biol. Chem.*, **210**, 581 (1954).
(7) E. A. Davidson, *J. Biol. Chem.*, **235**, 23 (1960).
(8) C. F. Lange, Jr. and P. Kohn, *J. Biol. Chem.*, **236**, 1 (1961).
(9) D. G. Comb and S. Roseman, *Biochim. Biophys. Acta*, **21**, 193 (1956).
(10) L. F. Leloir and C. E. Cardini, *Biochim. Biophys. Acta*, **12**, 15 (1953).
(11) D. G. Comb and S. Roseman, *J. Biol. Chem.*, **232**, 807 (1958).
(12) T. Tochikura, Y. Kariya, T. Yano, T. Tachiki, and A. Kimura, *Amino Acid and Nucleic Acid*, **29**, 59 (1974).
(13) T. Tochikura, Y. Kariya, and A. Kimura, *J. Ferment. Technol.*, **52**, 637 (1974).
(14) Y. Kariya, K. Aisaka, A. Kimura, and T. Tochikura, *Amino Acid and Nucleic Acid*, **29**, 75 (1974).
(15) Y. Kariya, A. Kimura, and T. Tochikura, *J. Ferment. Technol.*, **53**, 278 (1975).
(16) Y. Kariya, K. Aisaka, Y. Kaji, A. Kimura, and T. Tochikura, *J. Ferment. Technol.*, **53**, 599 (1975).
(17) A. Harden and W. Young, *Proc. Roy. Soc. London, Ser. B.*, **77**, 405 (1906).
(18) J. H. Roe and N. M. Papadopoulos, *J. Biol. Chem.*, **210**, 703 (1954).
(19) G. Blix, *Acta Chem. Scand.*, **2**, 467 (1948).
(20) J. L. Reissig, J. L. Strominger, and L. F. Leloir, *J. Biol. Chem.*, **217**, 959 (1955).
(21) R. Caputto, L. F. Leloir, C. E. Cardini, and A. C. Paladini, *J. Biol. Chem.*, **184**, 333 (1950).
(22) S. M. Partridge, *Biochem. J.*, **42**, 238 (1954).
(23) M. R. J. Salton, *Biochim. Biophys. Acta*, **34**, 308 (1959).
(24) L. F. Leloir and C. E. Cardini, Methods in Enzymol., Vol. 3, ed. by S. P. Colowick and N. O. Kaplan, Academic Press. Inc., New York, 1957, pp. 850.
(25) L. F. Leloir and C. E. Cardini, *Biochim. Biophys. Acta*, **20**, 33 (1956).
(26) B. L. Horecker and J. E. Seegmiler, *J. Biol. Chem.*, **192**, 175 (1951).
(27) F. Maley and H. Lardy, *J. Am. Chem. Soc.*, **78**, 1393 (1956).
(28) A. Sols, G. DeLa Fuente, C. Villar-Palasi, and C. Asensio, *Biochim. Biophys. Acta*, **30**, 92 (1958).
(29) J. F. McGarrahan and F. Maley, *J. Biol. Chem.*, **237**, 2458 (1962).
(30) P. T. Grant and C. Long, *Biochem. J.*, **50**, xx (1952).