Allosteric Properties of Yeast Glycogen Synthetase. II. The Effect of pH on Inhibition and Its Physiological Implications*

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ABSTRACT: Yeast glycogen synthetase (uridine diphosphate glucose : glycogen α-4-glucosyltransferase) is inhibited at neutral pH by rather high concentrations of several anions, and addition of glucose 6-phosphate reverses this effect. It has now been found that many substances are much more effective inhibitors at pH 6, the reported value for the yeast cell, than at pH 7.5. At the acidic pH, 5 mM adenosine triphosphate, adenosine diphosphate, or guanosine triphosphate inhibits the enzyme about 90%. Glucose 6-phosphate acts as reactivator in this case too. By several criteria these nucleotides are shown to act as allosteric inhibitors. A possible mechanism of regulation of glycogen synthesis is proposed, according to which the combined concentrations of adenosine triphosphate plus adenosine diphosphate would always be sufficient for almost complete inhibition of the enzyme in the absence of glucose 6-phosphate. The concentration of phosphoric ester would then determine the activity of the enzyme. At high adenosine triphosphate:adenosine monophosphate ratios, phosphofructokinase would be inhibited and glucose 6-phosphate would accumulate, thus relieving the inhibition of glycogen synthesis. The opposite effect would take place at low adenosine triphosphate:adenosine monophosphate ratios. Ammonium ion, when present, would also act on the phosphofructokinase reaction, helping to channel the carbon of glucose toward the formation of amino acids and other nitrogenous substances.

In the preceding paper (Rothman and Cabib, 1967), the characteristics of the inhibition of yeast glycogen synthetase (uridine diphosphate glucose : glycogen α-4-glucosyltransferase, EC 2.4.1.11) by anions and of its reversal by glucose-6-P[1] were determined. It was concluded that both, inhibitors and activator, were acting as allosteric modifiers. A further study of the influence of pH on these effects has disclosed that several substances, some of which are common metabolites, become very powerful inhibitors when tested at pH 6. Glucose-6-P maintains its capacity of reversing the inhibition. It is believed that these results may be of significance for the regulation of the enzyme activity *in vivo*.

Experimental Section

The materials and methods were as described in the preceding paper (Rothman and Cabib, 1967).

Results

In a previous study of yeast glycogen synthetase (Algranati and Cabib, 1962), the pH–activity curve was determined, using Tris-maleate as buffer. Since it was later found that maleate is an inhibitor (Rothman and Cabib, 1967), the experiment was repeated with noninhibitory buffers. As shown in Figure 1, in the absence of glucose-6-P, the activity was now higher.[2] In addition, the activity maximum in the absence of the phosphoric ester was broader, extending to pH 6, which has been reported to be the physiological value for yeast (Caldwell, 1956; Eddy, 1958). Thus, it appears that maleate is a stronger inhibitor at pH 6 than at 7.5. This was confirmed, as shown in Table I, where it can be seen that chloride maintains the same inhibitory power at both pH values. A number of other compounds, however, share the property of maleate, and, when tested at pH 5.9 are stronger inhibitors than those hitherto known. For instance, phosphoenolpyruvate, at 10 mM, inhibited as much as chloride at a 20-fold higher concentration. Some analogs of phosphoenolpyruvate inhibited to about the same extent. A survey of several nucleotides uncovered even more powerful inhibitors, as can be seen

* From the Instituto de Investigaciones Bioquímicas "Fundación Campomar" and Facultad de Ciencias Exactas y Naturales, Obligado 2490, Buenos Aires (28), R. Argentina. *Received March 24, 1967.* A preliminary note has been published (Rothman and Cabib, 1966). This work was taken from a thesis to be submitted by L. B. R. to the University of Buenos Aires in partial fulfillment of the requirements for the Degree of Doctor of Chemistry and was supported in part by a research grant (GM 03442) from the National Institutes of Health, U. S. Public Health Service, by the Rockefeller Foundation, and by the Consejo Nacional de Investigaciones Científicas y Técnicas (R. Argentina).

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[1] Abbreviations used: glucose-6-P, glucose 6-phosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; UDP, uridine diphosphate; UTP, uridine triphosphate.

[2] The inhibition by maleate at neutral pH was not very large, because UDP-glucose was present at a high concentration in this experiment.

TABLE I: Effect of Inhibitors at Different pH Values.[a]

Inhibitor	Concn (mM)	pH 7.5 Enzyme Activity −G-6-P	pH 7.5 Enzyme Activity +G-6-P	pH 7.5 Activation Ratio	pH 5.9 Enzyme Activity −G-6-P	pH 5.9 Enzyme Activity +G-6-P	pH 5.9 Activation Ratio
None	—	100	173	1.73	100	145	1.45
KCl	200	40	160	4.0	46	126	2.75
Maleate	60	—	—	—	46	—	—
	100	50	—	—	10.5	—	—
Citrate	60	—	—	—	23	—	—
	100	87	—	—	5.5	83	15
Phosphoenolpyruvate	10	86	—	—	55	146	2.65
	20	—	—	—	26	78	3
	30	—	—	—	11	73	6.6
Chorismate	10	—	—	—	50	140	2.8
3-Deoxy-D-*arabino*-heptulosonic-7-P	10	—	—	—	64	173	2.7
Enolpyruvylshikimate-5-P	10	—	—	—	75	165	2.2

[a] The activity was measured by the standard assay except that 0.1 M succinate–cacodylate was used as buffer at pH 5.9. The activity in the absence of inhibitor and glucose-6-P (G-6-P) is taken as 100 at each pH value.

TABLE II: Effect of Nucleotides at Different pH Values.[a]

Expt	Inhibitor	Concn (mM)	pH 7.5 Enzyme Activity −G-6-P	pH 7.5 Enzyme Activity +G-6-P	pH 7.5 Activation Ratio	pH 5.9 Enzyme Activity −G-6-P	pH 5.9 Enzyme Activity +G-6-P	pH 5.9 Activation Ratio
I	None	—	100	173	1.73	100	145	1.45
	ATP	2.5	—	—	—	28	132	4.7
		5	61	146	2.4	10	95	9.5
	ADP	5	38	128	3.35	13	90	6.9
	AMP	5	64	142	2.2	70	125	1.8
	GTP	5	89	178	2	14	90	6.4
	GDP	5	80	138	1.72	40	65	1.6
	GMP	5	77	158	2.05	65	125	1.9
	UDP	0.5	32	72	2.2	42	59	1.4
II	None	—	100	220	2.2	100	195	1.95
	UTP	0.5	68	150	2.2	57	114	2.0
	UMP	3	18	34	1.9	27	68	2.5

[a] Conditions as in Table I.

in Table II. The best were ATP, ADP, and GTP, while AMP, GMP, and GDP were much less effective. It may be noted, in Tables I and II, that glucose-6-P exhibited a strong capacity to reverse the inhibition, except in the case of the uridine nucleotides, which apparently are true competitive inhibitors (Rothman and Cabib, 1967). It seems therefore that the inhibition by ATP, ADP, and GTP at pH 5.9, is similar in nature to that found with other anions at pH 7.5. To confirm this point, several experiments were carried out. ATP behaved kinetically as a competitive inhibitor with a K_i of 0.62 mM, as shown in Figure 2. As in the case of chloride, the Lineweaver–Burk plots were rectilinear both in the presence and in the absence of ATP. Measurements of the rate as a function of glucose-6-P concentration gave rise to sigmoid curves when ATP was present (see Figure 3). It should be noted that under these conditions, 0.2 mM glucose-6-P was sufficient to give maximal stimulation, which in

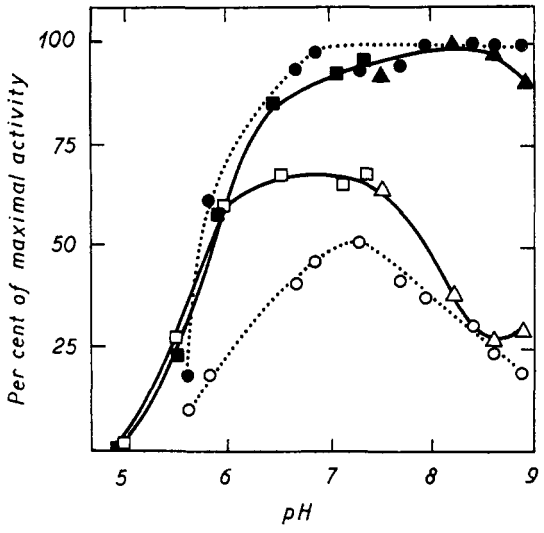

FIGURE 1: pH–activity curve of glycogen synthetase. Dotted lines: as previously obtained (Algranati and Cabib, 1962), using 0.1 M Tris-maleate (●, ○). Full lines: with 0.1 M succinate–cacodylate (■, □) or 0.1 M glycylglycine (▲, △). Open symbols: without glucose-6-P. Closed symbols: with glucose-6-P. Incubation carried out as previously described, but with 5 mM UDP-glucose (sp act. 62,000 cpm/μmole).

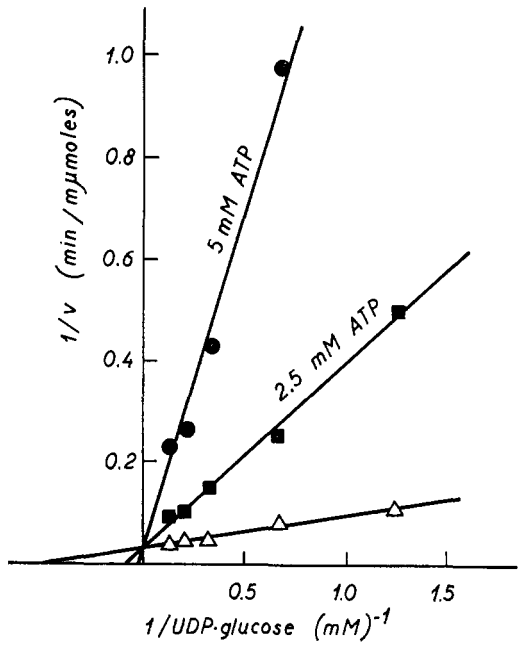

FIGURE 2: Effect of UDP-glucose concentration on reaction rate in the absence and in the presence of ATP. Assay condition as already described (Rothman and Cabib, 1967) but with succinate–cacodylate buffer (0.1 M in each) at pH 5.9.

certain cases amounted to more than 20 times. Cooperative effects were also evident when the rate was represented against ATP concentation, at fixed levels of glucose-6-P (Figure 4). In this case, however, the form of the curve appears to be more complex. Hill plots of the data of Figures 3 and 4 are shown in Figure 5. Dinitrophenylation of the enzyme caused loss of sensitivity to the inhibitor in this case too, although at pH 6 the effect was less marked, as shown in Table III.

The possibility had not been discarded that the enzyme might be converted, by a reaction with ATP, into a glucose-6-P requiring form, as in the case of the muscle synthetase (Friedman and Larner, 1963). To elucidate this point, the enzyme was incubated with 16 mM ATP at pH 6 for 15 min at 30°, and passed through a Sephadex G-25 column. The eluted synthetase did not differ

TABLE III: Inhibition at pH 5.9 of Dinitrophenylated Enzyme.[a]

Inhibitor	Concn (mM)	% Inhibition	
		Native Enzyme	Dinitrophenylated Enzyme
KCl	200	48	2
ATP	2.5	33	13
UDP	2	75	70

[a] Dinitrophenylation and assay of the enzyme were carried out as already reported (Rothman and Cabib, 1967) except that 0.1 M succinate–cacodylate buffer (pH 5.9) was used in the assay.

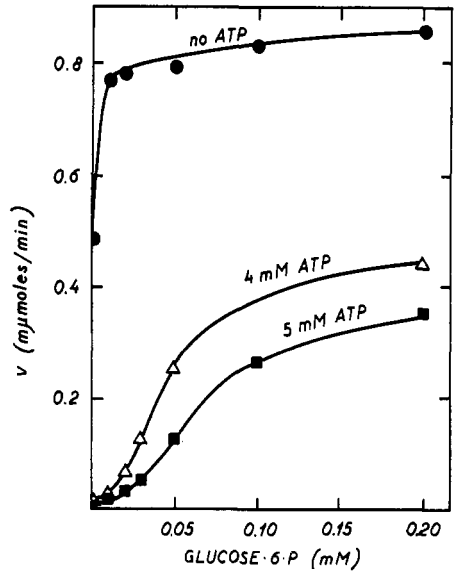

FIGURE 3: Effect of glucose-6-P concentration on reaction rate in the absence and in the presence of ATP. Assay conditions as in Figure 2.

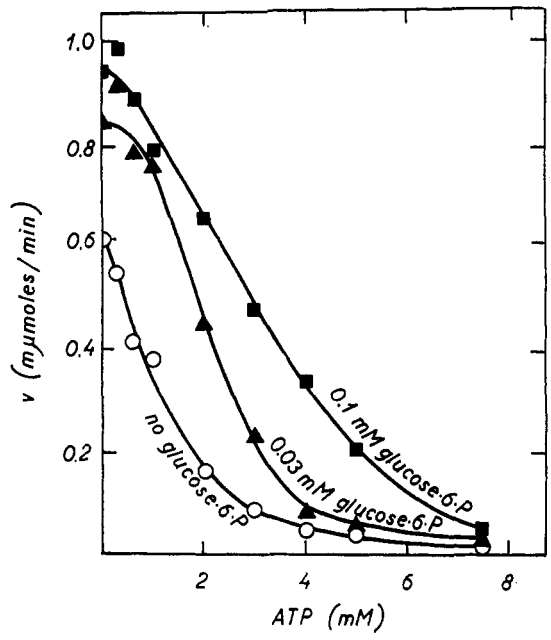

FIGURE 4: Effect of ATP on reaction rate in the absence and in the presence of glucose-6-P. Assay conditions as in Figure 2.

significantly from the untreated enzyme with respect to stimulation by glucose-6-P, inhibition by ATP, and reversal of the latter by glucose-6-P.

The Effect of Magnesium. In a previous study (Rothman and Cabib, 1967) it was demonstrated that Mg^{2+} partially relieved the inhibition of UDP, probably by forming a noninhibitory complex with the nucleotide. As can be seen in Table IV, a similar effect is obtained with ATP. An attempt was made to find out how substances able to form salts with magnesium, and known to occur in the cytoplasm, may affect the inhibition. It is apparent from the results of Table IV that addition of fructose 1,6-diphosphate and inorganic phosphate can partially restore the ATP effect. Glucose-6-P reverses the inhibition in this case too.

Sedimentation of the Enzyme in the Presence of ATP. The purified enzyme is obtained by elution from retrograded amylose with a glycogen solution (Algranati and Cabib, 1962), and therefore it contains a large amount of the polysaccharide. Centrifugation at 100,000*g* during 3 hr sediments the glycogen, to which the enzyme remains adsorbed. In order to verify if ATP might cause a gross change in the affinity of the enzyme for glycogen, a centrifugation was carried out in the presence of 4 mM nucleotide. It was found, however, that the supernatant, when tested with added glucose-6-P, was devoid of enzymatic activity, as in the absence of ATP.

Discussion

By the same criteria used in the preceding paper (Rothman and Cabib, 1967), it appears that the inhibition by nucleotides at pH 5.9 is allosteric. Chloride which does not change its ionization state in the range of pH used, is equally effective as inhibitor at pH 5.9 as at 7.5. It would appear therefore that the change in inhibitory power with pH found with other substances is more related to the ionization of the inhibitor than to that of the enzyme. It is still possible, however, that the shift in pH from 7.5 to 5.9 produces a net increase in positive charge in some group of the protein, which does not interfere with the small chloride ions but interacts with a bulkier anion.

ATP and ADP inhibit strongly at concentrations similar to those found in yeast (Kopperschläger and Hofmann, 1964; Betz and Chance, 1965) and at the pH value (about 6) reported for the yeast cell (Caldwell, 1956; Eddy, 1958). At a low level of glucose-6-P, small changes in concentration produce large variations in the activity of the inhibited enzyme. It is, therefore, attractive to suppose that these effects are functional in the *in vivo* regulation of glycogen synthesis. At first sight, it may

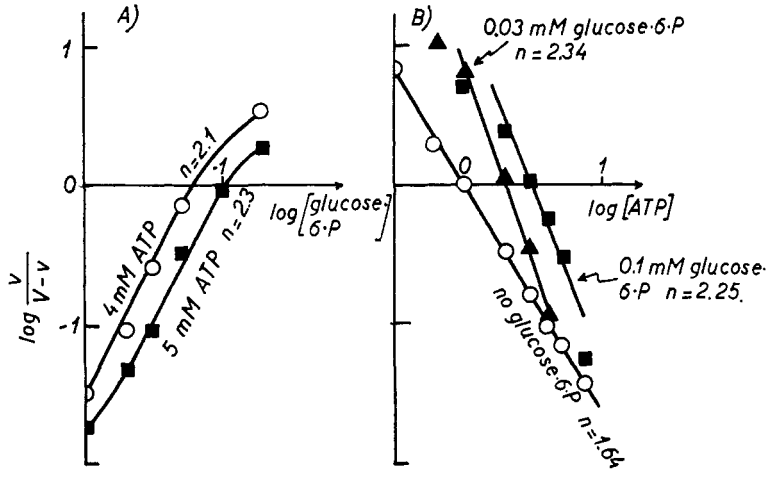

FIGURE 5: Hill plots (Atkinson, 1966) of the data shown in Figures 3 and 4.

TABLE IV: Effect of Magnesium Ions on ATP Inhibition at pH 5.9.[a]

Additions to Standard Reaction Mixture					
Mg^{2+} (mM)	Fructose-1,6-di-P (1 mM) + P_i (20 mM)	ATP (5 mM)	Glucose-6-P (5 mM)	Act.	Inhibn[b] (%)
–	–	–	–	100	
–	–	+	–	17	83
1	–	–	–	116	
	–	+	–	58	50
	+	–	–	103	
	+	+	–	23	76
	+	–	+	155	
	+	+	+	103	33
5	–	–	–	116	
	–	+	–	88	24
	+	–	–	80	
	+	+	–	43	46
	+	–	+	90	
	+	+	+	104	

[a] Conditions as in standard assay. [b] In each case the value obtained with the same reaction mixture minus ATP was taken as reference.

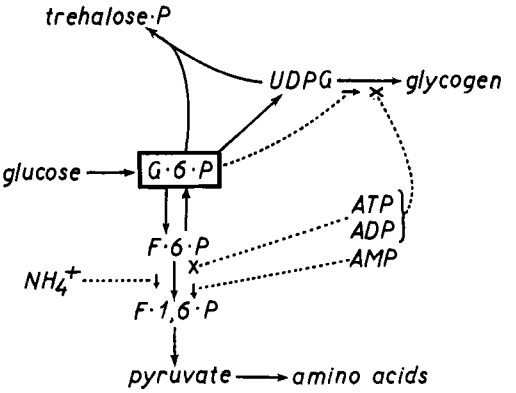

FIGURE 6: Tentative scheme of the regulation of glycogen synthesis in yeast. Stimulation of a reaction is indicated by a short arrow and inhibition by a cross, following Atkinson (1966). G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; F-1,6-P, fructose 1,6-diphosphate; UDPG, uridine diphosphate glucose.

seem paradoxical that ATP should be an inhibitor of glycogen synthetase, since one would expect that when plenty of ATP is available in the cell, it would be used as a source of energy for the formation of reserve carbohydrates. However, it should be borne in mind that ADP is almost as good an inhibitor as ATP. Thus, changes in the relative amounts of the two nucleotides probably do not affect *directly* the activity of the enzyme, and their combined concentration may be always sufficient to keep the enzyme in the inhibited state, were it not for the presence of glucose-6-P. The level of the phosphoric ester would then be the decisive factor in the regulation of glycogen synthetase activity. It is through the control of glucose-6-P concentration that the interconversion between adenine nucleotides may have an effect on the rate of glycogen synthesis. The diagram of Figure 6 illustrates how this regulation might work. Glucose-6-P is the first product of glucose metabolism; since yeast hexokinase is not inhibited by product, and assuming that glucose-6-P and fructose-6-P are in equilibrium, the level of glucose-6-P may be determined to a large extent by the phosphofructokinase reaction. The rate of this reaction depends, in yeast, on the ATP:AMP ratio (Ramaiah *et al.*, 1964); when this ratio is high, phosphofructokinase will be inhibited and glucose-6-P may accumulate, thus relieving the inhibition of glycogen synthetase by adenine nucleotides. The opposite effect would take place when AMP increases in relation to ATP.

This type of control would depend on the availability of energy in the cell. Some data of the literature suggest that a similar mechanism may be operative in connection with the requirements for growth. Trevelyan and Harrison (1956) have reported that yeast cells readily accumulate glycogen when incubated in a medium containing glucose and salts; as soon as ammonium ions are added, the formation of the polysaccharide ceases. Muntz has presented evidence indicating that ammonium activates the phosphofructokinase step in yeast (1947) as it does in animal tissues (Lowry and Passonneau, 1966). It is then possible that the presence of a nitrogen source in the form of ammonium ions gives rise to an inhibition of glycogen synthetase through a diminished level of glucose-6-P and helps to divert the flux of glucose carbon atoms toward the formation of the nitrogenous compounds needed for growth.

To be sure, these suppositions must be put to the test of experiment before they can be accepted. It will be necessary to measure the levels of the metabolites involved in these transformations under different conditions, and to correlate the results with the rate of glycogen synthesis. A further word of caution should be given, with regard to the fact that magnesium ions revert the inhibitory action of ATP. This point will be difficult to assess, until it is possible to determine the concentration of free Mg^{2+} in the cell. On the other hand, it is possible that other substances, in addition to the adenine nucleotides, inhibit glycogen synthetase *in vivo*. This would not invalidate the scheme of regulation presented above, as long as glucose-6-P can relieve the inhibition.

In the light of the results of this work it would seem pertinent to ascertain whether, in those cases in which glucose-6-P has been found to be inactive as a stimulator of glycogen synthetase, it may not still be functional as a

reactivator against inhibitors. Some data of Rosell-Pérez and Larner (1964) indicate that ATP, inorganic phosphate, and other substances can act as antagonists of glucose-6-P with the D form of dog muscle synthetase. A study of these effects in both the I and D forms of the enzyme may yield interesting results.

Acknowledgments

The authors wish to express their gratitude to Dr. Luis F. Leloir for his continued interest and support of this work. They are also indebted to their colleagues at the Instituto de Investigaciones Bioquímicas and to Drs. D. E. Koshland, J. M. Olavarría, and J.-P. Changeux for useful discussions and criticism. Special thanks are due to Dr. D. Sprinson and J. A. DeMoss for generous gifts of chorismate, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate and enolpyruvylshikimate 5-phosphate.

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