Regulation of Muscle Glycogen Synthetase by Metabolites. Differential Effects on the I and D Forms*

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ABSTRACT: Both the glucose 6-phosphate independent (I) and the glucose 6-phosphate dependent (D) forms of rat muscle glycogen synthetase are inhibited by adenosine triphosphate (ATP), adenosine diphosphate (ADP), and P_i. The inhibition is, kinetically, of the competitive type with respect to uridine diphosphate glucose (UDP-glucose). Glucose 6-phosphate reverses this inhibition, without affecting, at pH 6.6, the K_m for UDP-glucose. UDP, one of the reaction products, is a strong inhibitor and its effect is not reversed by glucose 6-phosphate. Photooxidation of the I form in the presence of methylene blue causes a preferential loss of the inhibition by ATP, as compared to that by UDP. The sum of this evidence suggests that UDP-glucose and ATP bind to different sites. At low concentrations of glucose 6-phosphate, within the physiological range, the D form is much more strongly inhibited by ATP than the I form. Therefore, under most conditions, probably only the latter is significantly active. These results provide a physiological meaning for the interconversion of the two enzymatic forms. The metabolic control of the biosynthesis of reserve material is discussed in the light of these findings.

The regulation of the turnover of reserve polysaccharides has paramount importance for the economy of the cell. In recent years, fundamental advances have been made in this field, especially with regard to the synthesis and degradation of glycogen (Leloir, 1965; Caputto *et al.*, 1967). Most of this work has been concerned with the interconversion between two enzymatic forms: *a* and *b* in the case of phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1c) and I and D for glycogen synthetase (UDP-glucose[1]:glycogen α-4-glucosyltransferase, EC 2.4.1.11). Phosphorylase *b* and synthetase D need an activator (5′-AMP and glucose-6-P, respectively) and are supposed to have little activity under the conditions usually prevailing *in vivo*. Thus, the interconversion would have the effect of switching on or off the enzymatic activity, and consequently the synthesis or degradation of the polysaccharide. It was difficult to understand, however, how phosphorylase *b* or glycogen synthetase D could be almost inactive,[2] when concentrations of 5′-AMP and glucose-6-P in the range found *in vivo* are sufficient to activate the enzymes *in vitro*. A path that may lead to a solution of this problem was opened when an antagonism was found between ATP and AMP, in the case of muscle phosphorylase (Morgan and Parmeggiani, 1964b), and between ATP or ADP and glucose-6-P, for yeast glycogen synthetase (Rothman and Cabib, 1967a,b). Thus, the enzymatic activity depends not only on the level of the activators, but also on the relative concentration of the inhibitors. The results found with yeast glycogen synthetase were quickly extended to the rat muscle enzyme (Piras *et al.*, 1967), and it was found that the I and D forms exhibited a differential response to the effect of metabolites. A more detailed study of this problem is reported here.

Experimental Procedure

Materials. UDP-[^{14}C]glucose (uniformly labeled in the glucose moiety) was purchased from the International Chemical and Nuclear Corp. or The Radiochemical Centre. It was diluted with cold UDP-glucose (Sigma Chemical Co.) to a specific activity of 350,000

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[1] Abbreviations used: I-MGS and D-MGS, the glucose 6-phosphate (glucose-6-P) independent and dependent forms of muscle glycogen synthetase; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates; UDP-glucose, uridine diphosphate glucose; RIG, ratio of independence of glycogen synthetase; DPNH, reduced diphosphopyridine nucleotide; GTP, guanosine triphosphate; fructose-6-P, fructose 6-phosphate; UMP, uridine monophosphate.

[2] In some cases, such as during anoxia, it would seem that phosphorylase *b* is functioning (Morgan and Parmeggiani, 1964a).

TABLE I: Effect of Different Metabolites on MGS Activity.[a]

Inhibitor	Concn (mM)	Inhibition (%)			
		I Form		D Form	
			+ 10 mM Glucose-6-P	+ 0.5 mM Glucose-6-P	+ 10 mM Glucose-6-P
ATP	6	91	19	88	15
ADP	6	79	19	84	38
AMP	6	36	0	30	0
GTP	0.5	27	0	0	3
	5	86	4	65	11
GDP	0.5	15	0	0	12
GMP	0.5	5	0	0	0
UTP	0.5	47	25	11	9
UDP	0.5	86	72	66	58
UMP	0.5	29	10	0	4
P_i[b]	10	18	10	49	0
	20	37	5	82	0

[a] Assays were carried out with the standard reaction mixture which contained, when indicated, glucose-6-P and the inhibitors at the concentrations shown. [b] The inhibition by P_i is not caused by phosphorylase degradation of the glycogen formed by the synthetase, as shown by the following results: (a) when ^{14}C-labeled glycogen was incubated with the I form under standard assay conditions, no radioactivity soluble in 66% ethanol was released, either in the absence or in the presence of 20 mM P_i; and (b) the same inhibition was determined either by the standard assay or by measuring the UDP liberated in the reaction.

cpm/μmole. All other chemicals employed in these experiments were of reagent grade, and were used without further purification.

Enzyme Preparations. The I form of glycogen synthetase was obtained as follows. Male rats fed *ad libitum*, 200–250 g in weight, were decapitated and bled. The muscle from the hind legs and back was quickly removed, chilled with crushed ice, cut in small pieces and trimmed of fat. Homogenization was carried out in a Waring Blendor for 1 min with 5 volumes of cold 0.05 M Tris-HCl buffer (pH 8)–0.005 M EDTA. The homogenate was centrifuged at 7000*g* for 10 min and the supernatant was filtered through glass wool. This solution was incubated with 50 mM β-mercaptoethanol, at 30° for 45 min. The slight cloudiness appearing on incubation, was removed by centrifugation at 25,000*g* for 15 min, and shell fish glycogen was added to a final concentration of 1.5 mg/ml. This solution was then centrifuged in a Spinco L preparative ultracentrifuge at 80,000–100,000*g* for 90 min. The pellet was resuspended in 0.01 M Tris-HCl buffer (pH 7.5)–0.001 M EDTA (1 ml/10 g of muscle) with the aid of a hand homogenizer. The suspension was left standing at room temperature for 15–30 min and centrifuged at 25,000*g* for 20 min. The pellet was discarded. The supernatant was kept frozen at −15° and remained stable for several weeks. This procedure yielded 30–40% of the activity present in the crude homogenate.

The D form of glycogen synthetase was prepared by a similar procedure, except that 0.1 M NaF was included in the first homogenization, and that the incubation with β-mercaptoethanol was omitted. Occasionally muscle glycogen synthetase was prepared by precipitation at pH 5.2 (Belocopitow *et al.*, 1965).

The ratio of the activities measured under standard conditions (see below), in the absence and in the presence of 10 mM glucose-6-P, is called ratio of independence of glycogen synthetase or RIG. The RIG values of the I and D forms, as prepared in the way described, were, respectively, 0.75–1 and 0.05–0.15.

Methods. Protein was determined by the procedure of Lowry *et al.* (1951). UDP was measured by coupling the phosphoenolpyruvate–lactic dehydrogenase reaction and determining DPNH by its fluorescence in an Aminco-Bowman spectrofluorometer (Lowry *et al.*, 1964).

Enzyme Assay. The standard reaction mixture for the glycogen synthetase assay contained succinate–cacodylate buffer, 0.1 M in each (pH 6.6), 10 mM EDTA, 1.5% shell fish glycogen, 0.4 mM UDP-[^{14}C]glucose (350,000 cpm/μmole), and enzyme, in a total volume of 0.05 ml. Incubations were carried out at 30°. The reaction was linear both with respect to enzyme concentration and time. These were varied to obtain, when possible, incorporations into glycogen of the order of 10^3 cpm. The reaction was stopped by adding 1.5 ml of 66% ethanol containing 0.1 M ammonium acetate. The tubes were heated in a boiling-water bath for 10–15 sec and cooled for 15 min in ice. The pre-

TABLE II: Effect of pH and UDP-glucose Concentration on the Inhibition of MGS by ATP.[a]

pH	UDP-glucose (mM)	Inhibition (%)			
		I Form		D Form	
			+ 10 mM Glucose-6-P	+ 0.6 mM Glucose-6-P	+ 10 mM Glucose-6-P
6.60	0.4	89	10	74	15
7.15	0.4	73	14	60	6
7.80	0.4	56	0	40	9
7.80	5.0	25	0	12	0

[a] The incubation mixture contained: Tris–succinate–cacodylate buffer, 0.1 M in each, adjusted with NaOH to the proper pH, 15 mg/ml of glycogen, 0.1 M EDTA, 6 mM ATP, enzyme, UDP-[^{14}C]glucose, and glucose-6-P at the concentration indicated. The final volume was 0.05 ml.

TABLE III: Effect of Different Metabolites on the Inhibition of MGS by ATP.[a]

Expt	Metabolite	Concn (mM)	I Form			D Form		
			Activity (%)		Actvn Ratio[b]	Activity (%)		Actvn Ratio[b]
			−ATP	+ATP		−ATP	+ATP	
1			100	5		100	7	
	Glucose-6-P	10	134	101	20.2	129	58	8.3
	Glucose-1-P	10	130	12	2.4	88	5	0.7
	Fructose-1,6-P_2	10	114	16	3.2	50	9	1.3
	α-Glycero-P	10	117	9	1.8	80	12	1.7
	3-P-Glycerate	10	122	13	2.6	66	14	2.0
	Creatine-P	10	103	8	1.6	88	12	1.7
	Glucosamine-6-P	10	133	34	6.8	100	10	1.4
2						100	10	
	Glucose-6-P	2.4				107	35	3.5
	Glucose-6-P	4.0				113	54	5.4
	Glucose-6-P + Fructose-6-P	2.4 + 1.6				112	33	3.3
3			100	7				
	Glucose-6-P	0.36	134	61	8.7			
	Glucose-6-P	0.60	134	92	13.1			
	Glucose-6-P + Fructose-6-P	0.36 + 0.24	134	70	10.0			

[a] Assays were carried out with the standard reaction mixture, which contained, where indicated, the phosphoric esters and 10 mM ATP. Activities are expressed as per cent of that obtained in the absence of added metabolites and ATP. D-MGS was always measured in the presence of 0.6 mM glucose-6-P. [b] Ratio of the activities obtained at 10 mM ATP in the presence of each phosphoric ester and in its absence.

cipitated glycogen was redissolved in water, reprecipitated with 66% ethanol, plated on stainless-steel planchets, and counted in a gas-flow counter (Frieseke). Occasionally, activities were determined directly after the first precipitation by filtering the glycogen through a Millipore filter (pore 0.6 μ) of 2.1 cm in diameter fitted to a filtration apparatus (Tracerlab E8B). The filter was washed with 66% ethanol, dried under an infrared lamp, placed in a vial with 10 ml of a toluene-scintillating solution, and counted in a Packard liquid

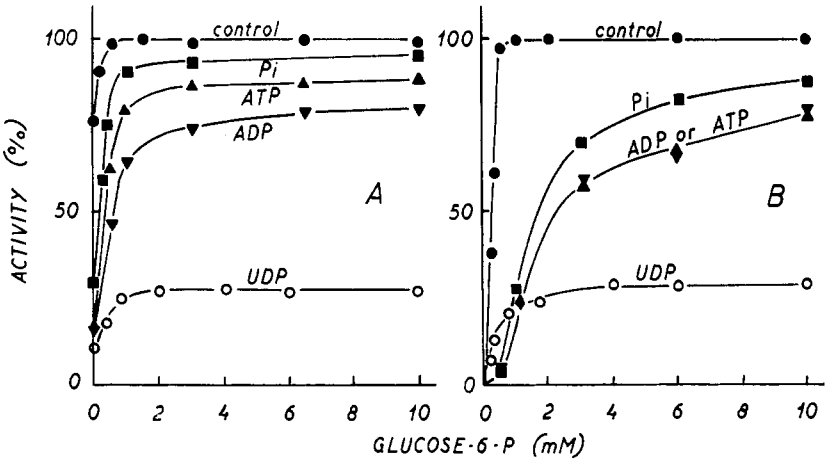

FIGURE 1: Effect of glucose-6-P concentration on reaction rates of I-MGS (A) and D-MGS (B) in the absence (●) and in the presence of 6 mM ATP (▲), 6 mM ADP (▼), 20 mM P_i (■), and 0.8 mM UDP (○). Assays were done under standard conditions. Since the D form contained 10% of the I form, the activities shown on part B of this figure were corrected using the data presented in A.

scintillation spectrometer. The efficiency is 20–30% higher than with the other method. This procedure is also faster, when few samples are processed.

Results

General Conditions for Inhibition and Reactivation of the I and D Forms. It has already been reported that ATP, ADP, and P_i inhibit both forms of glycogen synthetase and that glucose-6-P reverses this effect (Piras *et al.*, 1967). Table I shows the extension of these results to other nucleotides. It can be seen that GTP is a strong inhibitor at 5 mM, but this fact is probably without physiological significance, on account of the low concentration of guanosine nucleotides in muscle (Long, 1961). Among uridine compounds, UDP (a reaction product of glycogen synthetase) is by far the strongest inhibitor. It should be noted that this inhibition is not reversed appreciably by glucose-6-P.

The decrease in the activity of the I form, on addition of ATP in the absence of glucose-6-P, might have been caused by a transformation into the D form (Friedman and Larner, 1963). This possibility was excluded by determining the proportion of D form after incubation with ATP, under standard assay conditions, followed by passage through a Sephadex G-25 column. The percentage of D form was unchanged by the treatment.

As in the case of yeast, the inhibition by ATP was markedly dependent on pH, and was greater under more acidic conditions (see Table II). At a pH of 7.8 and at 5 mM UDP-glucose very little inhibition was found, in agreement with previous results of Rosell-Pérez and Larner (1964b).

Of the many compounds tried, only glucose-6-P (and glucosamine-6-P for the I enzyme) reversed the ATP inhibition efficiently, as shown in Table III. Marginal effects were observed with some other phosphoric esters. Fructose-6-P seems to be devoid of action, since an equilibrium mixture of this ester and glucose-6-P gave as much activity as glucose-6-P alone (Table III).

The fact that under our conditions (pH 6.6) glucose-6-P reverses the ATP inhibition without affecting the K_m for UDP-glucose (Piras *et al.*, 1967), suggested that the ATP effect might be allosteric, as it was demonstrated in the case of the yeast glycogen synthetase

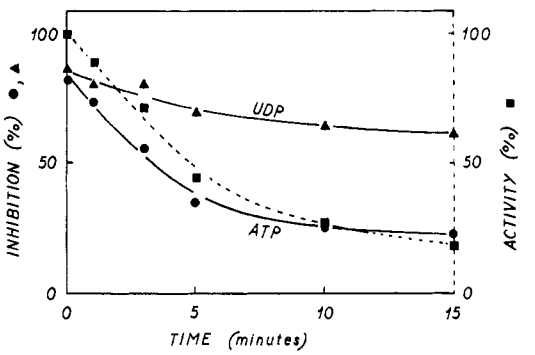

FIGURE 2: Effect of photooxidation with methylene blue on the total activity (■) of I-MGS, and on the inhibition by 5 mM ATP (●) and 0.6 mM UDP (▲). Photooxidation was carried out in the presence of 0.01% methylene blue and 0.03 M succinate–cacodylate buffer (pH 7.1), in a cell with a 3-mm light path illuminated by a GE 250-w lamp placed at 5 cm. The sample was kept at 4° by circulating iced water. Aliquots were removed at the time indicated and used directly, under dimmed lights, for enzymatic measurements.

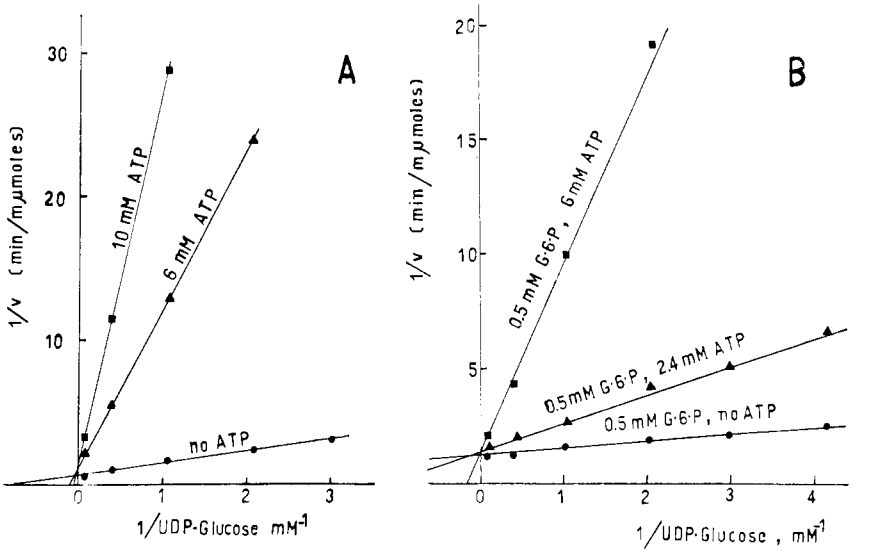

FIGURE 3: Effect of UDP-glucose concentration on reaction rates of I-MGS (A) and D-MGS (B) in the absence of (●) and in the presence (■, ▲) of ATP.

TABLE IV: Effect of D–I Interconversion on the Inhibition by ATP.[a]

Conversion	Treatment[b]	RIG	Inhibition (%)		
			Glucose-6-P (mM)		
				0.3	0.6	10
D → I	1. None	0.21	86		58	0
	SH–Mg^{2+}	0.68	76		6	0
I → D	2. None	0.87	84		16	2
	ATP–Mg^{2+}	0.36	86		57	15
	3. None	0.84	77	25		
	Trypsin	0.30	82	75		
	4. None	0.99	82	39		
	Ca^{2+}	0.42	72	64		

[a] Assays were carried out with the standard reaction mixture, and the inhibition was measured in the presence of 6 mM ATP. [b] The D → I conversion was obtained by treating at pH 7.2 D-MGS, prepared as described in Methods, with 50 mM β-mercaptoethanol and 10 mM $MgSO_4$ for 45 min at 30°. The reaction was stopped by filtration through Sephadex G-25. The I → D conversions were obtained as follows: for treatment 2, a 7000*g* supernatant from a crude homogenate was incubated for 45 min at 30° with 50 mM β-mercaptoethanol, and one aliquot was then treated at pH 7.4 with 50 mM NaF, 2 mM ATP, 10 mM $MgSO_3$, and 10^{-5} M cyclic 3′,5′-AMP for 15 min at 30°. More ATP and Mg^{2+} was added to bring their total concentration to 4 and 20 mM, respectively, and the incubation was continued for another 15 min. To both the untreated aliquot and that incubated with ATP–Mg^{2+}, glycogen was added to a final concentration of 1.5 mg/ml, and the enzyme was centrifuged at 105,000*g* for 90 min. The pellet was dissolved in 0.01 M Tris-HCl buffer (pH 7.2)–0.001 M EDTA and used for activity measurements. Treatments 3 and 4 were carried out as described by Belocopitow *et al.* (1967) on I-MGS, obtained as under Methods and on a pH 5.2 fraction, respectively (Belocopitow *et al.*, 1965).

(Rothman and Cabib, 1967b). A further confirmation of this hypothesis was afforded by the lack of reversal of UDP inhibition by glucose-6-P (see Table I and Figure 1). The limited stimulation observed in this case with increasing glucose-6-P concentration may signify that UDP binds to a small extent to the allosteric site.

In an attempt to obtain desensitization to inhibitors the enzyme was submitted to several treatments. A preferential loss of ATP inhibition was obtained when the I enzyme was subjected to photooxidation in the presence of methylene blue (Figure 2). Only one-fourth of the original inhibition remained, whereas

UDP still inhibited 70% of the value found with the intact enzyme. The RIG was unchanged during the photooxidation. The significance of this result is somewhat mitigated by the loss of enzymatic activity caused by the treatment (Figure 2). Attempts to protect the enzyme with UDP-glucose, glucose-6-P, or mercaptoethanol were unsuccessful.[3] Carrying out the photooxidation at a different pH value did not give better results.

Dinitrophenylation, heating at different temperatures, preincubation at several pH values, treatment with dioxane, urea, lithium bromide, *o*-phenanthroline, *p*-mercuribenzoate, and ultraviolet irradiation did not lead to a desensitization of the enzyme, under the conditions used.

Kinetic Studies with the I Form. The ATP inhibition is kinetically of the competitive type with respect to UDP-glucose, as shown in Figure 3A. The apparent K_m changes from 0.34 mM in the absence, to 5 mM in the presence, of 6 mM ATP. The addition of 10 mM glucose-6-P (not shown) restores the K_m to the original value (Piras *et al.*, 1967). UDP also showed competitive inhibition, with K_i = 0.03 mM. As above mentioned, this inhibition was not reversed by glucose-6-P.

The effect on the activity of increasing the glucose-6-P concentration at several different levels of ATP, is shown in Figure 4A. At variance with the results obtained with the yeast enzyme, where a cooperative effect was evident, here the curves appear to be hyperbolic. A high concentration of glucose-6-P reverses the inhibition almost completely, even at 10 mM ATP. In Figure 5A, the results of Figure 4A were replotted, to show the effect of ATP concentration on the enzymatic activity, at different glucose-6-P concentrations. Here, a sigmoid character of the curves is apparent, when glucose-6-P was present.

While a complete study with ADP was not carried out, Figure 6A shows that, in the absence of glucose-6-P, both ATP and ADP gave the same inhibition curve. The effect of P_i concentration is also shown, for comparison.

Kinetic Studies with the D Form. Similar experiments to those just described were performed with the D form, and the results can be observed in Figures 3B–6B. While the general pattern is similar to that obtained with the I form, several differences can be noted; the most striking is that the D form is much more strongly inhibited than the I form, within a certain range of glucose-6-P concentrations. The tenfold larger scale used for glucose-6-P in Figure 4B emphasizes this fact. At high ATP the reversal of inhibition for the D form is far from complete, even at 10 mM glucose-6-P.

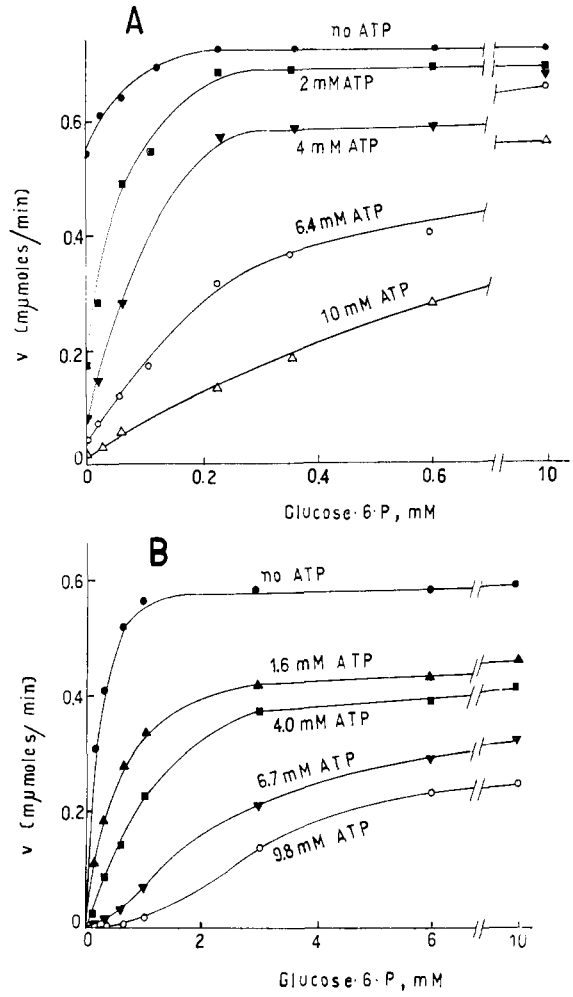

FIGURE 4: Effect of glucose-6-P concentration on reaction rates of I-MGS (A) and D-MGS (B) in the absence (●) and in the presence of several concentrations (○, ■, ▲, ▼, ▽) of ATP. Data for the D form were corrected as indicated under Figure 1.

Cooperative effects are here clearly observed above 5 mM ATP. On the other hand, curves of activity as a function of ATP concentration (Figure 5B) are not sigmoid. The inhibition curve is the same for ATP and ADP in this case too (Figure 6B). Figure 7 shows that a different type of D enzyme, that is, one obtained by treating the I form with trypsin (Appleman *et al.*, 1964), gives essentially similar results.

The visual appreciation of the sigmoid or hyperbolic character of the curves is confirmed by the Hill plots (Atkinson, 1966) of Figure 8. A slight cooperative effect (n = 1.1–1.3) may be present with the I form, when glucose-6-P was varied, or with the D form, when ATP was varied.

Effect of the I–D Interconversion on ATP Inhibition. In the experiments reported above, preparations of the I or D form were obtained independently, since in this way the contamination of one form with another could be minimized. Nevertheless, in order to obtain

[3] The enzyme is sensitive to *p*-mercuribenzoate (10^{-4} M inhibits 100%) but this inhibition can be reversed (70%) by incubation with SH compounds. Therefore, it was speculated that it might be possible to use *p*-mercuribenzoate to shield the SH groups of the enzyme during photooxidation, and then to recover the activity by treatment with mercaptoethanol. Unfortunately, under these conditions, the enzyme was inactivated to the same extent.

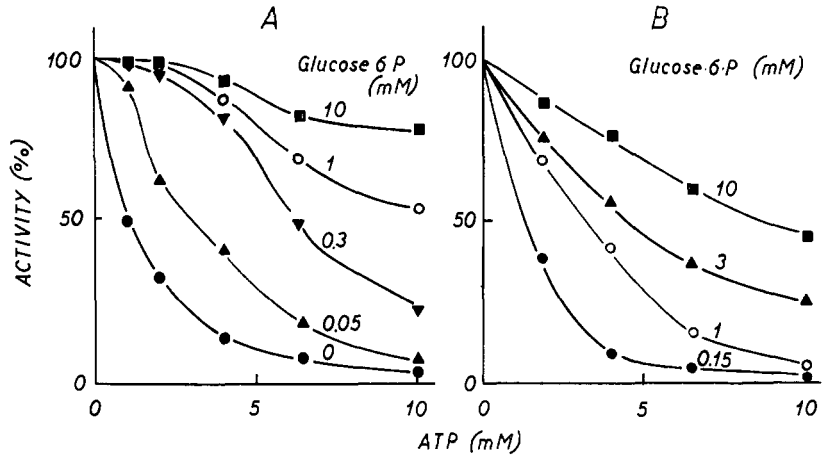

FIGURE 5: Effect of ATP concentration on reaction rates of I-MGS (A) and D-MGS (B) in the presence of several concentrations of glucose-6-P.

more telling evidence that the change in ATP inhibition was causally related to the I–D interconversion, and not to some other transformation, the experiment described in Table IV was performed. It can be seen that a transformation from the D to the I form produced a decrease, and one from I to D an increase in the ATP inhibition *at low glucose-6-P concentration.* Conversion of the I to three different D forms (with ATP–Mg^{2+}, trypsin, or Ca^{2+}, see Belocopitow *et al.*, 1967) always gave increased inhibition by ATP. In Figure 9, the results of several experiments were pooled to show the linear correlation between enzyme form and inhibition.

The Effect of Mg^{2+} and of a "Physiological Mixture" on Nucleotide Inhibition. Addition of Mg^{2+} greatly decreases the inhibition of yeast glycogen synthetase by ATP (Rothman and Cabib, 1967b). This also occurs, although to a more limited extent, with the muscle enzyme, as shown in Table V. In this experiment, ADP was used in place of ATP with the I form to avoid an I to D conversion, a possible event in the presence of both Mg^{2+} and ATP. It was also checked by incubation with ADP + Mg^{2+}, followed by passage through a Sephadex G-25 column, that under these conditions no interconversion took place.

In an effort to gain a better insight into the real importance of the Mg^{2+} effect under *in vivo* conditions, a "physiological mixture" was employed. The components of this mixture (ATP, ADP, AMP, P_i, phosphocreatine, and Mg^{2+}) were added in their probable average concentration *in vivo*, as assessed from several measurements reported in the literature (Long, 1961). The effect of glucose-6-P concentration on the activity, in the presence of such a mixture, is shown in Figure 10. It can be seen that in spite of the presence of 11 mM Mg^{2+}, the other components of the mixture restitute

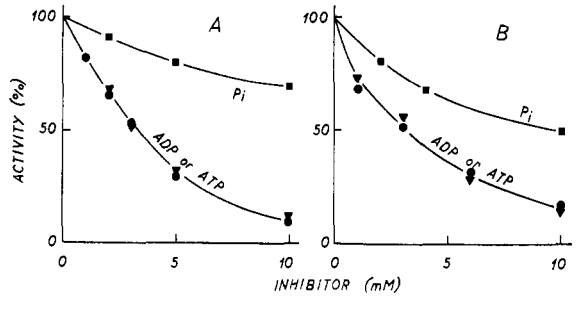

FIGURE 6: Effect of ATP (●), ADP (▼), and P_i (■) concentration on reaction rates of I-MGS (A) and D-MGS (B). The D enzyme was measured in the presence of 0.6 mM glucose-6-P.

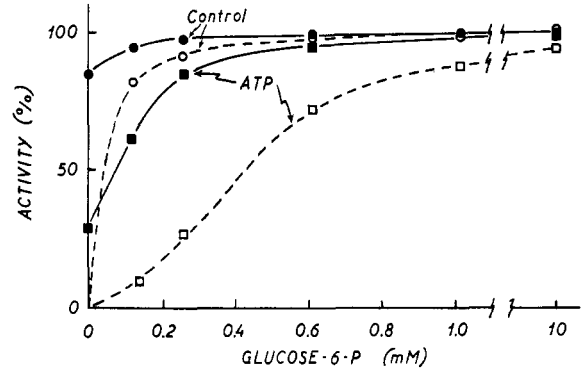

FIGURE 7: Effect of glucose-6-P concentration on reaction rates of I-MGS (——) and trypsin-MGS (– – –) in the absence (●, ○) and in the presence (■, □) of 8.4 mM ATP. I-MGS was obtained as indicated under Methods, and trypsin-MGS by treatment of the former by the procedure previously described (Belocopitow *et al.*, 1967). Data for the trypsin-MGS were corrected (as indicated under Figure 1) for residual I activity (20%).

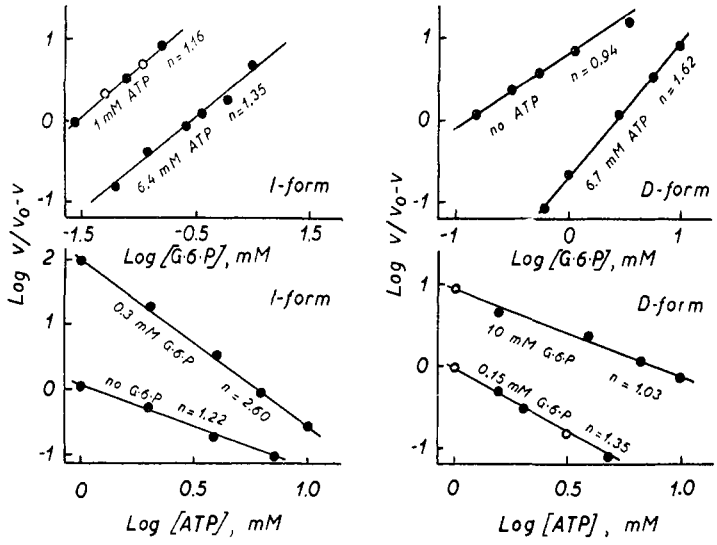

FIGURE 8: Hill plots (Atkinson, 1966) for some of the curves of Figures 4 and 5. Open circles represent points obtained by interpolation in the corresponding curves of Figures 4 and 5. v_0 represents the activity in the absence of effector, and v in its presence. G-6-P stands for glucose-6-P.

to a large extent the nucleotide inhibition. In fact, the curves are very similar to those obtained as a function of glucose-6-P concentration, in the presence of ATP alone (Piras *et al.*, 1967). Figure 10 also highlights the different behavior of the I and D form at low concentrations of glucose-6-P. Between 0.6 and 1 mM glucose-6-P, the D form is almost completely inhibited, while the I form is almost fully active.

Experiments with Rabbit Muscle Synthetase. Preliminary experiments were carried out with rabbit muscle synthetase (Rosell-Pérez and Larner, 1964a), partially purified by precipitation at pH 5.2, and containing 63% of the I form. Addition of 6 mM ATP gave 86% inhibition which was reduced to 38% by 0.6 mM glucose-6-P and to 2% by 10 mM glucose-6-P. These figures are almost identical with those obtained with a preparation of rat muscle synthetase of a similar RIG (Figure 9).

TABLE V: Effect of Mg^{2+} on the Metabolite Inhibition of MGS.[a]

		Inhibition (%)			
		I Form		D Form	
Inhibitor	Concn (mM)		Mg^{2+}		Mg^{2+}
ATP	5			83	56
ADP	5	82	68	86	64
AMP	5	55	30	42	38
UDP	0.5	87	73	79	63
P_i	10	39	24	65	60
	20	70	28	85	74

[a] The reaction was carried out under standard conditions. D-MGS was measured in the presence of 0.5 mM glucose-6-P. Mg^{2+} (10 mM) was used as the chloride. The presence of Mg^{2+} increased the activities of the controls in 40 and 20%, for the I and D forms, respectively. In each case inhibitions are expressed as percent of the value obtained in the absence of inhibitor.

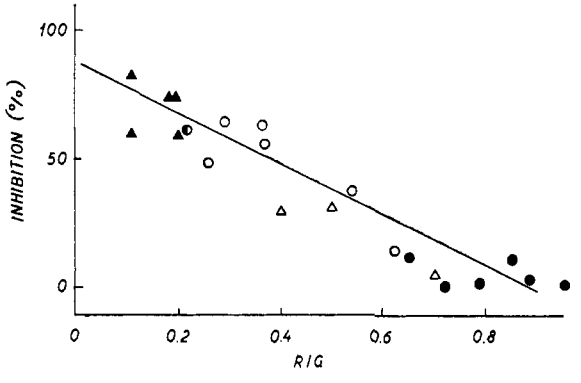

FIGURE 9: Graphical representation of RIG and ATP inhibitition of several MGS preparations and their conversion products. RIG was measured as indicated under Methods and the inhibition was determined with 6 mM ATP and 0.6 mM glucose-6-P. I-MGS (●) and D-MGS (▲) were obtained as described under Methods, and transformed, respectively, with ATP-Mg^{2+} and cyclic 3′,5′-AMP (○) or trypsin (◐), and with β-mercaptoethanol and Mg^{2+} (△), as indicated in Table V. The line was drawn by the least-squares method, and the correlation coefficient is 0.91 ($P < 0.001$).

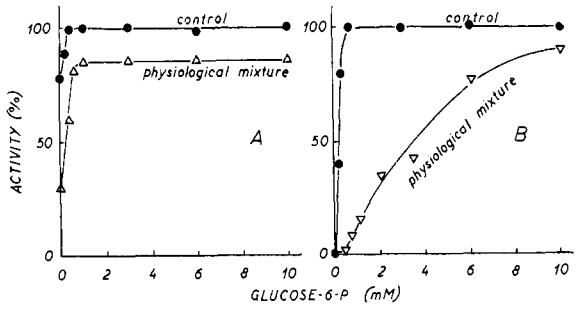

FIGURE 10: Effect of glucose-6-P concentration on reaction rates of I-MGS (A) and D-MGS (B) in the absence (●) and in the presence (△, ▽) of a "physiological mixture." This mixture contained 11 mM $MgCl_2$, 0.4 mM AMP, 10 mM P_i, 14 mM P-creatine, and 7.3 mM ADP, when used in A. Identical composition was used in B, except that ADP was 2.8 mM and that 4.5 mM ATP was also present. Data for the D form were corrected as indicated under Figure 1.

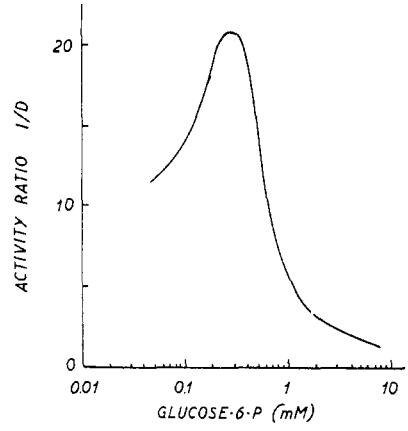

FIGURE 11: Effect of glucose-6-P concentration on the activity ratio of the I and D forms of MGS, when measured in the presence of 6 mM ATP. The curve is calculated from the data of Figures 4 and 5.

Discussion

Glycogen synthetases from yeast, as well as from rat and rabbit muscle, are inhibited by ATP, and this inhibition is reversed by glucose-6-P. It seems, therefore, that this is a general property of this enzymatic system that has been maintained through the evolutionary process. Since, in muscle, ATP, ADP, and, to a lesser extent, P_i inhibit at concentrations in the range found *in vivo* (Long, 1961), it seems probable that these effects may well have physiological importance. As found with the yeast enzyme, ATP and ADP are equally good inhibitors (see Figure 6). Therefore, a scheme of regulation similar to that proposed for yeast (Rothman and Cabib, 1967b) might be envisaged, according to which the combined concentrations of ATP, ADP, and in this case P_i, would be sufficient to keep the enzyme inhibited in the absence of glucose-6-P. The level of the latter would then determine, by competing with the inhibitors, the rate of glycogen synthesis.

Both muscle and yeast glycogen synthetases are more strongly inhibited by ATP as the pH decreases. The pH values found in muscle oscillate between 6.6 and 7.1 (Caldwell, 1956) and seem to depend on previous exercise. This variation in pH may contribute, together with the other variables discussed, to the regulation of activity. The pH used throughout this work was 6.6, in the lower physiological range, in order to show more clearly the inhibition and reactivation effects. For the same reason a small concentration of UDP-glucose (0.4 mM) was chosen, since the inhibition by ATP and related substances is formally of the competitive type and therefore more easily seen at low substrate level. In addition, the value employed is probably of the order of that found *in vivo* (Caputto *et al.*, 1950).

In spite of the seemingly competitive character of the ATP inhibition, its reversal by glucose-6-P, without changing the apparent K_m for UDP-glucose at pH 6.6, suggests that substrate and inhibitor bind to different sites. This hypothesis is reinforced by the finding that glucose-6-P has little effect on the inhibition by UDP, a substrate analog and probably a true competitive inhibitor. Some evidence in favor of an allosteric type of inhibition is also provided by the admittedly imperfect results of the desensitization experiments.[4] These arguments are strengthened by the similar behavior of the muscle and the yeast enzyme, since the allosteric nature of the latter is well supported by the experimental evidence (Rothman and Cabib, 1967a,b).

Cooperative effects (*i.e.*, sigmoid curves) were observed with glucose-6-P in the presence of ATP (D form) or with ATP in the presence of glucose-6-P (I form). Thus, more than one binding site would be indicated, both for the nucleotide and the phosphoric ester. It is not easily explained, however, why each of the two effectors show cooperativity with only one of the two enzymatic forms. An interpretation in line with the Monod–Wyman–Changeux (1965) theory would be that the equilibrium is displaced toward one of two possible conformations in the I form, and toward the other in the D form. Thus, in each case, only the effector that binds preferentially to the conformation present in smaller proportion would give rise to sigmoid curves. Nevertheless, the theory of Monod *et al.* (1965) conflicts, as in the case of yeast glycogen synthetase (Rothman and Cabib, 1967a), with other results, namely that UDP-glucose failed to show cooperative effects under any of the conditions tried.

[4] Brief treatment of the I form of the enzyme with papain led to an *increase* in ATP inhibition. Since, however, the K_m for UDP-glucose decreased at the same time, the significance of this result remained in doubt.

Probably of more consequence from the physiological standpoint is the finding that the D form requires a much larger concentration of glucose-6-P than the I-form, to counteract ATP inhibition. Transformations of the I into the D form, or *vice versa*, give rise to the expected change in sensitivity to modifiers. It is interesting that this occurs when the I form is converted into the D form, either by the kinase reaction, by trypsin, or by calcium ions plus the appropriate protein factor. The mechanism by which glucose-6-P dependence is attained in each of these cases is probably different (Belocopitow *et al.*, 1967), therefore this result indicates that the binding sites for glucose-6-P and ATP are closely interrelated. It cannot be decided whether both compounds actually bind to the same sites, but some evidence against this possibility is brought by the fact that with the D form, at high ATP concentration, glucose-6-P is unable to reverse the inhibition completely.

It can be inferred from Figure 4 that at low levels of glucose-6-P and in the presence of ATP, the difference in activity of the two forms is very large, but it gradually disappears as glucose-6-P increases. To bring out this point more clearly, the results of Figure 4 were replotted, in the manner shown in Figure 11. The curve has a maximum activity ratio of 20 at about 0.2 mM glucose-6-P. Not too much attention should be paid to these values, since they can vary with different preparations, perhaps owing to a limited desensitization of the enzyme. The important point to be made is that the maximal difference in activity between the two forms represents a severalfold activation and occurs within the range of the physiological concentration of glucose-6-P (Long, 1961; Danforth, 1965; Wilson *et al.*, 1967).

Another factor that modifies the effect of inhibitors and reactivators is the concentration of Mg^{2+} ions. It is difficult to assess its value *in vivo*, since an important proportion of the Mg^{2+} may be bound. Anyway, when Mg^{2+} is added to a mixture of several metabolites, approaching the composition reported for muscle, the behavior of the I and D forms was unchanged in its main features.

Thus, some uncertainty remains as to the activity of the enzyme *in vivo*, since it is at present impossible to know the exact concentration of the relevant metabolites under different conditions. Nevertheless we may conclude with some confidence that very probably the I and D forms will exhibit very different activity in the environment usually prevailing in muscle. This is due to the inhibition by ATP, ADP, and P_i. In the absence of this inhibition both forms would probably be equally active, in view of the small K_m of the D form for glucose-6-P at pH 6.6 (*ca.* 0.13 mM, see Figure 4B), and the interconversion would be devoid of physiological significance.

Muscle, then, is endowed with two mechanisms for the regulation of glycogen synthetase, I–D interconversion and metabolite effect, where yeast probably only possess the latter of the two. The enzymatic interconversions may have evolved as a result of the challenge posed to the differentiated tissue of a metazoan, which must integrate with other tissues and respond to the needs of the whole organism. In fact this system appears to respond to both hormonal (Belocopitow, 1961; Craig and Larner, 1964) and electrical (Danforth, 1965) stimulation. The once all-important metabolite regulation may, in the new environment, have been entrusted with the sole task of giving significance to the interconversion mechanism, as discussed above. It is, however, conceivable that in certain situations the metabolite level directly affects the enzymatic activity as, for instance, in the case reported by Okuno *et al.* (1966). They found that a genetically determined lack of phosphofructokinase was accompanied by a large increase in the glucose-6-P concentration and by an accumulation of glycogen. This may have been caused, at least in part, by the reversal of inhibition brought about by the phosphoric ester.

Finally, it might be asked whether some deeper meaning should be attributed to the fact that the control of the biosynthesis of a reserve material is assigned primarily to a precursor, as glucose-6-P, rather than to the end product, as is the common case. It is clear that the concentration of a biosynthetic building block must be strictly controlled, to avoid unbalanced growth and unnecessary expenditure of energy. On the other hand, the very purpose of a reserve material is to accumulate in times of plenty, so as to be available in case of need. It would be of doubtful utility for the cell to limit its production by the amount already formed, *at least within certain limits*.[5] Instead, continued synthesis should be ensured, as long as food is offered in excess of the amount needed for maintenance and growth: the concentration of glucose-6-P is a reflection of the nutrients' supply and consumption and seems therefore to be a sensible choice as a regulator of glycogen synthesis. It may also be stated that the rate of glycogen formation depends on the energy state or "energy charge" (Atkinson and Walton, 1967) of the cell, since the concentration of glucose-6-P is probably closely related through the phosphofructokinase step to the ATP:ADP or to the ATP:AMP ratio (Passonneau and Lowry, 1962).

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[5] It is obvious that a ceiling to the accumulation of a storage compound must be put somewhere. In fact Danforth (1965) has shown that the proportion of the (presumably inactive) D form in muscle is directly correlated with the glycogen level. According to Villar-Palasi and Larner (1966) the polysaccharide would act by inhibiting glycogen synthetase phosphatase.

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