In Vivo Regulation of Rat Muscle Glycogen Synthetase Activity*

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ABSTRACT: *In vitro* and at low glucose 6-phosphate concentrations, the dependent form of rat muscle glycogen synthetase is inhibited more markedly by several metabolites (adenosine triphosphate, adenosine diphosphate, inorganic phosphate, etc.) than the independent form (Piras, R., Rothman, L. B., and Cabib, E. (1968), *Biochemistry 7*, 56). To test the significance of these findings the levels of the metabolites involved in glycogen metabolism and the forms of glycogen synthetase and phosphorylase have been simultaneously determined under different physiological situations. *In situ* electrical stimulation of the posterior muscles of the rat thigh produced a tetanic contraction with the resulting increased level of phosphorylase *a*, glucose 6-phosphate, pyruvate, lactate, and inorganic phosphate, and a decrease of glycogen and creatine phosphate. Within 10 min of the subsequent recovery period all the metabolites returned to the resting values. The rate of glycogen resynthesis *in vivo* was also studied during this period. Rates increased during the first 4-min recovery, reaching a maximum of 3.2 mM/min. The largest fraction of glycogen synthetase in the independent form was found 4–5 min after the end of a 10-sec stimulation. The levels of adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, and uridine diphosphate glucose did not vary significantly throughout the experiments. A comparison was carried out between the rates of glycogen resynthesis *in vivo* and those obtained *in vitro* under conditions which intended to simulate the concentrations of metabolites and the forms of glycogen synthetase found at rest, during contraction, and recovery. The *relative* rates of glycogen synthesis obtained when only glycogen synthetase was present in the assays, or the enzyme and glucose 6-phosphate, were different from those found *in vivo*. On the other hand, when the inhibitory metabolites were included in the assay, the relative rates were similar to those found *in vivo*, suggesting that these effectors play a role in the regulation of the enzyme. It is concluded that the regulation of glycogen synthetase activity under the conditions of muscular contraction and recovery can be best explained by the interconversion between the dependent and independent forms of the enzyme, and the differential inhibition and reversion by effectors of the two forms of glycogen synthetase. Both mechanisms act concomitantly, and have the effect of switching on and off gylcogen synthetase activity according to the physiological situation.

Muscle glycogen synthetase (UDP-glucose:α-1,4-glucan α-4-glucosyl transferase, EC 2.4.1.11) has been the subject of many investigations during the last years (Larner, 1966; Leloir, 1965, 1967); however, some aspects of its regulatory mechanism remained obscure. Thus, it was difficult to understand the physiological importance of the D and I interconversion of muscle GS,[1] since the concentration of glucose-6-P in the range found *in vivo* is sufficient to activate nearly completely both forms of the enzyme when assayed *in vitro* under "physiological" conditions of pH and substrate concentration. A possible solution to this problem was suggested by the finding that an antagonism exists between several metabolites (ATP, ADP, AMP, creatine phosphate and P_i) and glucose-6-P (Piras *et al.*, 1967, 1968). In fact, at concentrations of the phosphorylated sugar within the physiological range, D-GS is more strongly inhibited by the metabolites than the I form, and therefore, the interconversion would recover its meaning.

It became necessary to find out whether the regulatory mechanism based on these *in vitro* studies was compatible with the concentration of the relevant metabolites and enzymes present *in vivo* under different conditions. Unfortunately, the available data have been obtained under a variety of conditions and in different species (Danforth and Lyon, 1964; Danforth, 1965; Wilson *et al.*, 1967). Therefore, it was considered desirable to examine in a single set of experiments all the variables known so far to influence the activity of glycogen synthetase.

The results reported here suggest that, indeed, the regulatory mechanism proposed seems to operate *in vivo*. Glycogen synthetase activity is nearly absent at rest, becomes maximal 4–5 min after a tetanic

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[1] Abbreviations used: I-GS and D-GS, the glucose 6-phosphate (glucose-6-P) independent and dependent forms, respectively, of glycogen synthetase.

contraction, and decreases with a new stimulation.

Experimental Procedure

Stimulation of Muscles. Male rats fed *ad libitum*, weighing 150–250 g, were adrenalectomized 12–14 hr prior to the experiments. Sodium pentobarbital was administered intraperitoneally to obtain a deep anaesthesia throughout the experiment (5–7 mg/100-g body weight). The posterior muscles of the thigh were exposed bilaterally and freed of surrounding tissue, but leaving intact origin, insertion, and blood supply. The sciatic nerves were cut to prevent transmission of central impulses, the skin put in place, and the animals kept undisturbed in a warm environment for 30 min. The muscle from one leg was then removed (control) and immediately clamped in Wollemberger tongs precooled in liquid air. The muscles from the other leg were stimulated electrically *in situ* by an electrode applied to the sciatic nerve. The stimulation (5 V, 5 msec, and 40 stimulations/sec) was obtained by means of a Tektronix type 161–162 stimulator precalibrated with a Tektronix Model 502A oscilloscope. After the desired period of tetanus, the muscles were rapidly removed and clamped as before. In another series of experiments, the effect of a stimulation followed by various recovery periods was studied. In these instances, the sciatic nerves of both legs were stimulated simultaneously. The muscle from one leg was removed immediately (control) while the other was removed after the predetermined period of recovery. The time required to remove and freeze a muscle was approximately 1 sec. Muscles from rats with abnormal control values were discarded. Muscles were kept in liquid air until processed.

Extraction. A 400–600-mg portion of the frozen muscle was pulverized in a Nossal disintegrator (McDonald Co., Ohio) by a procedure similar to that described by Danforth *et al.* (1962). An 18-ml capsule precooled in liquid air was used, and the muscles were reduced to powder by a 15-sec shaking. Three volumes (v/w) of a solution containing 60% glycerol, 0.04 M glycerophosphate buffer (pH 6.8), 0.01 M EDTA, 0.02 M mercaptoethanol, and 0.04 M NaF were then added, and the capsule was cooled and shaken for 10 sec. Three more volumes of a similar solution to the one described, but without glycerol, were added and the shaking was resumed for 10 sec. At this stage the extracts were generally thawed. Cell debris were removed by centrifugation at 0° for 10 min at 15,000*g*. The clear supernatant was decanted and immediately assayed for glycogen phosphorylase and synthetase activities (see below). Occasionally, pulverization and extraction were carried out in a precooled mortar, with similar results.

A second portion of each frozen muscle (approximately 1 g) was pulverized as described and mixed thoroughly with 2 ml of cold 1 M perchloric acid–1.3 mM EDTA. The suspension was centrifuged at 0° for 10 min at 15,000*g*, and the supernatant was immediately neutralized with 30% KOH, allowed to stand at 0° for 20–30 min, and centrifuged as before. The clear supernatant was used for the determinations of metabolites (see below).

Glycogen was extracted from another aliquot (200–300 mg) of pulverized muscle, which was added, with rapid mixing, to a tared tube containing 2 ml of 33% KOH. The suspension was heated for 20 min at 100°, with occasional stirring. Glycogen was precipitated with two volumes of ethanol, centrifuged, and dissolved in acidified water.

Assay Procedures. Glycogen phosphorylase was assayed by the method of Cori *et al.* (1943) in the presence and absence of 1 mM 5′-AMP. The results are expressed as percentage of phosphorylase *a* present. This is calculated from the ratio of activities obtained without (phosphorylase *a*) and with AMP (total phosphorylase). At the dilution of enzyme used (1:250), endogenous AMP does not interfere with the assay. Glycogen synthetase was determined by the radioactivity incorporated into glycogen upon incubation with UDP-[^{14}C]glucose (Piras *et al.*, 1968). The standard reaction mixture contained 0.1 M cacodylate buffer (pH 6.8), 12 mM EDTA, 1.5% glycogen, 5 mM NaF, 2.5 mM mercaptoethanol, 4 mM UDP-[^{14}C]-glucose (60,000 cpm/μmole), enzyme, and 10 mM glucose-6-P when needed, in a total volume of 0.05 ml. The enzyme preparation was freed of metabolites by filtration through Sephadex G-25 (Pharmacia, Uppsala) columns equilibrated with 20 mM Tris-HCl buffer (pH 7.3), 5 mM EDTA, 5 mM mercaptoethanol, 10 mM NaF, and 0.1% glycogen. Alternatively, the extracts were diluted directly in the above solution so as to obtain a concentration of glucose-6-P in the assay smaller than 5% the K_m value for this metabolite. Under these conditions, and by using a UDP-[^{14}C] glucose of higher specific activity, there is good agreement between the two procedures.[2] Results are expressed as percentage of I form present. This is calculated from the ratio of activities obtained in the absence (I-GS) and presence (total GS) of 10 mM glucose-6-P.

Metabolite Determinations. Glucose-6-P, ATP, ADP, AMP, PCr, and pyruvate were assayed with the appropriate specific enzymes according to Lowry *et al.* (1964), following the changes in absorption at 340 mμ in a Gilford Model 2000 multiple-sample absorbance recorder. Similarly, UDP-glucose was determined by the reduction of DPN^+ in the presence of UDP-glucose dehydrogenase (Wilson, 1965). A final volume of 0.4 ml was used in all instances, and internal standards were run for each sample once the consumption of metabolites had ended.

P_i was determined by the procedure recommended by Lowry *et al.* (1964) and based on the method of Hohorst *et al.* (1959). Under the conditions used for the determination of P_i by the Fiske and Subbarow (1925) procedure, PCr is hydrolyzed. Therefore, its content can be inferred from the difference between the

[2] Incubation of [^{14}C]glycogen with the muscle extracts under standard conditions for GS assay does not release radioactivity into 66% ethanol, indicating that, under these conditions, degradating enzymes do not interfere with the assay.

two procedures. This value agrees closely with that determined with creatine kinase.

Lactate was assayed following LePage's (1957) modification of the Barker and Summerson (1941) procedure, and glycogen by Krisman's (1962) method, which agreed with the values obtained by glucose determination (Somogyi, 1945; Nelson, 1944) after acid hydrolysis.

The recovery of metabolites was checked by adding them to the perchloric acid used in the extraction. The recovery was 80–90% in all instances. The values given are corrected for this small loss, and are expressed in millimolar (micromoles of metabolite per milliliter of tissue water). Water content of rat muscle was assumed to be 76% (Threlfall and Stoner, 1957). Glycogen recovery was quantitative, and the values are expressed as glucose equivalents.

Materials. All chemicals used were of reagent grade and were used without further purification. Glucose-6-P dehydrogenase, lactic dehydrogenase, peroxidase, and creatine kinase were purchased from Sigma Chemical Co. (St. Louis). Myokinase and twice-recrystallized pyruvate kinase were prepared from rabbit muscle, and kindly supplied by Dr. H. N. Torres. UDP-glucose dehydrogenase was prepared from beef liver according to Wilson (1965). Dog liver glycogen, purified by treatment with charcoal, was a gift from Dr. L. Maréchal. UDP-[^{14}C]glucose (uniformly labeled in the glucose moiety) was purchased from The Radiochemical Centre.

Results

The content of metabolites and enzymes related to glycogen synthesis and degradation has been investigated in rat skeletal muscle under different conditions: rest, tetanic contraction *in situ*, and recovery (Figures 1–4). The metabolites determined were the GS substrates and effectors (Piras *et al.*, 1968). In addition, pyruvate and lactate were measured to determine the metabolic state of the muscle under the different conditions used. The values found in resting muscles are similar to those recently reported for rat (Marquez-Julio and French, 1967).

Muscle contraction was obtained by electrical stimulation, a simple technique which has been widely used in similar studies and which is known to bring about a variation in the content of several metabolites (Danforth *et al.*, 1962; Danforth and Lyon, 1964; Danforth, 1965; Wilson *et al.*, 1967). In fact, a rapid decrease of PCr and a concomitant increase of P_i could be observed (Figure 1A). On the other hand, ATP remained constant at 7–8 mM level, which might be accounted for by the fact that the PCr pool was not depleted under the conditions employed (Hohorst *et al.*, 1962).

Total phosphorylase activity found in rat muscle was 68 μmoles of P_i formed/min per g of muscle. This activity is similar to that reported for frog sartorius and mice caudofemoralis (Danforth *et al.*, 1962; Danforth and Lyon, 1964). At rest, 22% of the phosphorylase was in the *a* form[3] (Figure 1B) and increased rapidly with stimulation. The maximum transformation (60%

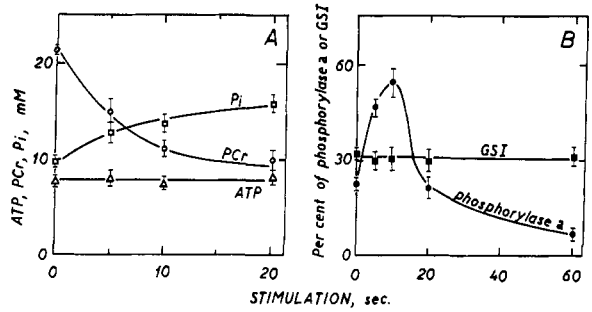

FIGURE 1: Effect of tetanic contraction on the levels of ATP (△), creatine phosphate (○), and P_i (□), and on the percentage of the glycogen phosphorylase *a* (●) and synthetase I (■) forms in rat muscle. Each point represents the average of five or more experiments, and the vertical bars the standard error of the mean.

phosphorylase *a*) was attained within 5–10 sec from the onset of the contraction, as previously described (Danforth *et al.*, 1962; Danforth and Lyon, 1964). Longer stimulations resulted in a decline in the percentage of the *a* form to values even lower than those present at rest. Danforth *et al.* (1962) and Danforth and Helmreich (1964) have reported sustained phosphorylase *a* values, under somewhat different conditions. On the other hand, Cori (1945) and Cori and Illingworth (1956) have observed that fatigued muscles have a low content of phosphorylase *a*, suggesting that, under the conditions here used, the muscles become fatigued after a 10-sec tetanus. Therefore, in the studies of recovery from contraction (see below) only a 10-sec tetanus was applied. Total phosphorylase activity was unchanged throughout the experiment.

The total GS activity found was 0.86 μmole of glucose transferred/min per g of muscle, a figure similar to those previously reported (Leloir *et al.*, 1959; Danforth, 1965; Williams and Mayer, 1966), providing that the values are reduced to equivalent assay conditions of temperature and pH. The latter has been recently determined in skeletal rat muscle to be 6.91 (Sanslone and Muntwyler, 1966), a value very similar to the one used in our assay. The resting value of the I form was 32% of the total, and it remained constant during the 60-sec period of stimulation employed (Figure 1B). In this instance, too, total activity was unchanged. The I form of GS is present at a 20% level in resting muscle of mice (Danforth, 1965). More recently, Goldberg *et al.* (1967) have reported a 10–15%

[3] This value is higher than that found in isolated muscle from frog and mice (Danforth *et al.*, 1962; Danforth and Lyon, 1964) but it is in the same range as that encountered in rat muscle *in vivo* (Posner *et al.*, 1965). This difference is not surprising, if it is considered that 20% phosphorylase *a* can be obtained by a 0.3- and 0.5-sec stimulation in frog and mice isolated muscle, respectively (Danforth *et al.*, 1962; Danforth and Lyon, 1964). In fact, the handling and time required to remove the muscle in the *in vivo* experiments may well be of the same order as the stimulation mentioned above. In addition, it should be pointed out that a very low content of phosphorylase *a* (2–6%) was produced by a prolonged tetanus (60 sec) or after a stimulation followed by recovery (Figure 3).

content in resting muscle from rat, a value lower than that obtained in the present study. This difference is probably due to the different assay conditions used. In fact, when the muscle extracts were assayed under the conditions described by Villar-Palasi *et al.* (1966) the percentage of I form found was of the same order, or even lower, than that reported by Goldberg *et al.* (1967).

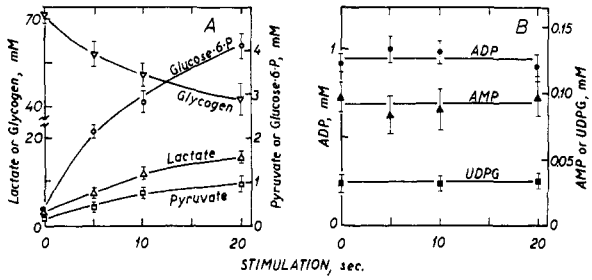

FIGURE 2: Effect of tetanic contraction on the levels of glycogen (▽), glucose-6-P (○), pyruvate (□), lactate (△), ADP (●), AMP (▲), and UDP-glucose (■) in rat muscle. Each point represents the average of five or more experiments, and the vertical bars the standard error of the mean.

The rapid activation of phosphorylase brings about the resulting glycogenolysis, and the increase in glycolytic intermediates (Figure 2A). Glycogen is diminished approximately one-third, while glycose-6-P is increased 12-fold over the resting value (0.3 mM). Glucose-1-P (not shown in the graphs) was always found at a concentration approximately 5% that of glucose-6-P. Other phosphoric esters were not measured, since they are not effective in reversing the inhibition of GS (Piras *et al.*, 1968). Pyruvate and lactate are also increased, though to a lesser extent, and the ratio of their content is nearly constant throughout the contraction. Approximately half of the glucose liberated from glycogen during the first 20 sec of contraction can be accounted for by the increase in pyruvate, lactate, and glucose-6-P. Other metabolites which might act upon GS, such as ADP and AMP (Piras *et al.*, 1968), did not vary (Figure 2B). UDP-glucose also remained constant at a 0.03 mM level.[4]

[4] This concentration is lower than that previously reported (Caputto *et al.*, 1950) but was obtained by the more specific and elaborate techniques now available (Wilson, 1965). It was found in muscle extracts assayed either directly or after purification by absorption on charcoal and chromatography (Leloir and Cabib, 1963). In this last instance an internal standard of UDP-[^{14}C]glucose was used to correct for losses incurred during the purification steps. Rabbit muscle also contains 0.03 mM UDP-glucose. Incomplete extraction, mechanical losses, decomposition or inhibition by the extracts of the reaction used for assay were ruled out as possible causes for the low value obtained. Furthermore, using the same procedure in other rat tissues, UDP-glucose was found at levels similar to those reported by other authors (Tarnowsky *et al.*, 1964; Williams and Mayer, 1966; Mersmann and Segal, 1967; Wong and Sourkes, 1967) as follows: 0.5 μmole/g of liver, 0.05 μmole/g of brain, 0.12 μmole/g of kidney, and 0.07 μmole/g of heart.

Danforth (1965) has shown that 3 min after a 20-sec tetanus, GS from mice muscle was mainly present in the I form. Preliminary experiments in rat muscle indicated that a 3-sec stimulation was not sufficient to induce a D to I transformation during the recovery period. On the other hand, 10-, 20-, or 40-sec stimulations all produced a similar transformation. Since stimulations longer than 10 sec produce muscular fatigue, as judged from the decline of phosphorylase *a* (Figure 1B), the shortest effective tetanic contraction (10 sec) was chosen as a standard condition to study the effect of different periods of recovery (Figure 3). At the end of the contraction 32% of the GS was present in the I form, and remained at this level for the first minute of recovery. However, 3–4 min later about 60% of the synthetase was in the I form. This percentage declined progressively over the next 5 min. These slow transformations contrast with the very rapid changes that take place in the phosphorylase system (Figures 1B and 3, and Danforth, 1965). While I-GS had nearly reached the percentage present at rest after 10-min recovery, phosphorylase *a* decreased sharply to very low values and remained constant even after 10-min recovery. UDP-glucose did not vary in this instance, either.

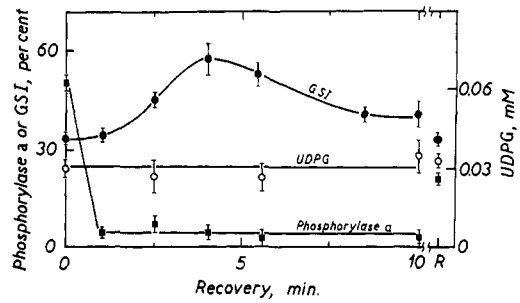

FIGURE 3: Effect of recovery, after a tetanic contraction, on the percentage of glycogen synthetase I (●) and phosphorylase *a* (■) forms, and the level of UDP-glucose (○). Muscles from both legs were stimulated simultaneously for 10 sec, the control (zero recovery time) immediately removed, while the other muscle was withdrawn at the times indicated. For reference, rest values (R) are also shown on the right side of the figure. Each point represents the average of five or more experiments, and the vertical bars the standard error of the mean.

The variations in metabolite content as a function of time of recovery are shown in Figure 4. Glucose-6-P, pyruvate, lactate, and P_i decreased while glycogen and PCr increased. ATP (as well as ADP and AMP, not shown) remained constant. In all instances the values achieved after a 10-min period were essentially those present at rest, indicating that the system is completely reversible under the conditions used.

The data obtained during the stimulation and recovery experiments were used to prepare mixtures with a composition corresponding to each of the situations studied. Thus, the activity of GS could be assayed *in*

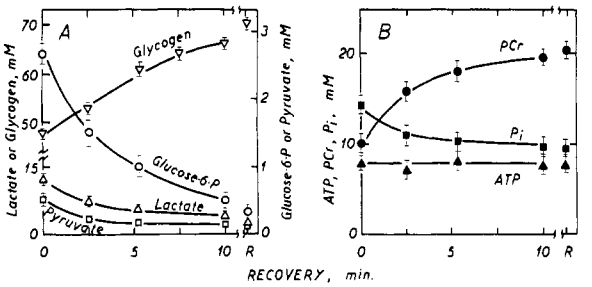

FIGURE 4: Effect of recovery, after a tetanic contraction, on the levels of glycogen (▽), glucose-6-P (○), pyruvate (□), lactate (△), PCr (●), P_i (■), and ATP (▲). Conditions were identical with those of Figure 3.

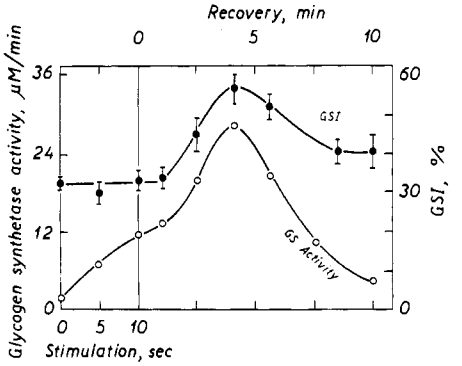

FIGURE 5: GS activity assayed *in vitro* under the conditions found *in vivo* during the tetanic contraction and recovery. The activity of GS was determined in the presence of 0.03 mM UDP-glucose, 6.5 mg/ml of glycogen, 8 mM ATP, 1 mM ADP, and 0.1 mM AMP. In addition, the concentrations of creatine phosphate, P_i, and glucose-6-P were varied at each point according to what has been found in Figures 1, 2, and 4. Also, GS used at each point has a percentage of I form which corresponds to the upper curve (data taken from Figures 1B and 3). GS of different percentages was obtained by mixing appropriate proportions of D- and I-GS. Assays were carried out at 30° and activities have been corrected for tissue water content (see Methods). Note the different time scales during stimulation and recovery.

vitro in the presence of mixtures which resembled the situations found *in vivo* (Figure 5). It is important to remark that both the metabolite composition of the mixture and the percentage of the I form of GS used vary with each point assayed according to the levels indicated in Figures 1–4. While glycogen concentration actually changed in muscle, it was nevertheless used at a constant level in the assays, since even the lowest value is sufficient to give maximal GS rates. The GS activity present at rest, when assayed in this way, was only 1% of that attainable at saturating concentration of glucose-6-P (10 mM) and in the absence of other metabolites. The *in vitro* activity increased 9-fold when the assay conditions were adjusted to represent the situation found *in vivo* at the end of a 10-sec tetanus. It increased further, reaching a maximum which coincided with that of the transformation curve, when the assay conditions corresponded to 4–5-min recovery from a 10-sec contraction. The total change in activity from rest to the maximum was a 20-fold increment. The *in vitro* activity corresponding to longer recovery periods declined slowly. Since the rate of glycogen synthesis might be influenced by the relative proportion of the forms of GS, as well as by the level of UDP-glucose and the presence or absence of glucose-6-P and/or inhibitory metabolites, the relative importance of each of these factors was studied separately. Thus, in addition to the data shown in Figure 5, which was obtained by using the average concentrations as found *in vivo*, curves were also obtained at saturating concentration of UDP-glucose (5 mM), or in the presence of the enzyme alone, or in the presence of the enzyme and glucose-6-P. Table I shows a summary of the rates, obtained from such curves, corresponding to the situation of rest and to three recovery periods (0–1, 2.5–4, and 7.5–10 min following a 10-sec tetanus). Also, for comparison, the rates of glycogen resynthesis *in vivo* are indicated. The *in vitro* rates are always larger at 5 mM UDP-glucose than at the concentration found *in vivo* of this metabolite (0.03 mM), as expected from the GS K_m for this substrate (0.34 mM; Piras *et al.*, 1968). Also, rates are maximal at the time when more I-GS is present. Large relative variations of the *in vitro* rates are observed only when the inhibitory metabolites are included in the assay.

In order to compare the GS activity profiles obtained *in vitro* (Figure 5 or Table I) with the actual situation prevailing *in vivo*, the rates of glycogen resynthesis after a tetanic contraction was carefully studied (Figure 6). It was found that the rate increased during the first 4-min recovery, then fell during the next 6 min. The total amount of glycogen synthesized during the 10-min recovery period was of the same order as the glycogen degraded during the preceding tetanic contraction.

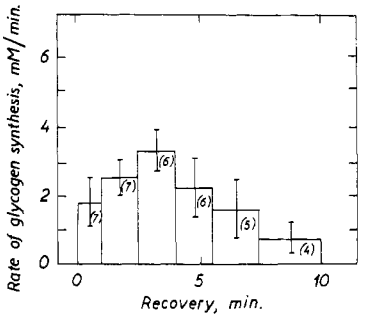

FIGURE 6: Rates of glycogen synthesis *in vivo* after a 10-sec tetanic contraction. Muscles were treated for glycogen determination as described under Experimental Procedures, except that two aliquots were independently processed for each muscle. The values obtained were paired with the corresponding controls, and the mean difference of glycogen synthesized in a given interval was calculated. Numbers in parentheses represent the animals used in each interval studied, and the height of the lines at each bar is the standard error of the mean rate of glycogen synthesis. The difference between the first and the third bar is statistically significant at a level of $0.05 > P > 0.02$.

TABLE I: Comparison of Rates of Glycogen Synthesis under Different Conditions.

	Condition			Rate of Glycogen Synthesis[a] (mmoles/min)				Ratios			
				R	A	B	C	B/R	B/A	B/C	A/C
1.	*In vivo*[b]				1.7	3.3	0.7		1.9	4.7	2.4
2.	*In vitro*[c]										
	UDP-glucose	Glucose-6-P	Inhibitor								
a.	F			0.05	0.05	0.1	0.07	2.0	2.0	1.4	0.7
b.	5			0.6	0.6	1.2	0.8	2.0	2.0	1.5	0.7
c.	F	F		0.15	0.2	0.2	0.16	1.3	1.0	1.2	1.2
d.	5	F		1.3	2.0	2.0	1.7	1.5	1.0	1.2	1.2
e.	F	F	F	0.002	0.02	0.04	0.01	20.0	2.0	4.0	2.0
f.	5	F	F	0.18	0.86	1.40	0.36	7.8	1.6	3.9	2.4

[a] The rates of glycogen synthesis correspond to rest (R), and to three periods of recovery after a 10-sec tetanic contraction: A (0–1 min), B (2.5–4 min), and C (7.5–10 min). [b] Data taken from Figure 6. [c] *In vitro* assays were carried out under conditions similar to those described in Figure 5, except that the metabolites included are those indicated in each instance. F Stands for the concentration of metabolite found for each particular situation (Figures 1–4), and 5 is the millimolar final UDP-glucose concentration present in the assay. To allow a direct comparison between the *in vivo* and *in vitro* data, the latter have been corrected for temperature (30–37°) and reduced to micromoles of glucose transferred per minute per milliliter of tissue water (see Methods).

Discussion

Knowledge, under different physiological situations, of the content of metabolites and enzymes related to glycogen metabolism in rat skeletal muscle has allowed the study of this system *in vitro* under conditions which intend to simulate some of the possible situations encountered in the cell. A comparison of the rates of glycogen synthesis determined from such studies can be made with the values actually observed *in vivo*. Even though this approach has obvious limitations, it might be useful to provide clues as to the actual situation prevailing in the vicinity of the enzyme *in vivo* and to the mechanisms involved in the regulation of its activity.

The maximal rate of glycogen synthesis *in vivo* is obtained 2.5–4 min after cessation of a 10-sec tetanus (Figure 6). This value is twice that observed during the first minute of recovery, and approximately five times larger than that found at 7.5–10-min recovery (Table I). While the absolute differences are small, they are statistically significant ($P < 0.05$ and 0.01, respectively). *In vivo* rates might be the result of the GS activity alone, or the net balance between the action of this activity and that of phosphorylase. However, since phosphorylase *a* reverts to the *b* form within 10 sec after the end of the stimulation, the rate of glycogen synthesis is probably not affected too seriously by this factor. In addition, the metabolites which might principally influence phosphorylase activity (Morgan and Parmeggiani, 1964) are constant, and therefore the general pattern of the rate of glycogen synthesis should not be affected. The maximal rate at which glycogen resynthesis proceeds *in vivo* is approximately 3% of the rate of glycogenolysis during tetanic contraction. A similar relationship is found by comparing the activity of GS with that of phosphorylase.[5]

Rates of glycogen synthesis of resting muscles could not be determined by the approach used in the present study. However, if it is considered that a rate of 0.76 mM/min is found at a time when glycogen has not yet reached the resting value (7.5–10-min recovery), it is evident that the rate for resting muscle should be considerably smaller. In fact, Stadie *et al.* (1953) have reported a value of 0.13 mM/min for rat hemidiaphragm. Thus, a large increase in the rate of glycogen synthesis (at least 5-fold, and more probably 10–30-fold) takes place *in vivo* from the rest condition to the maximal activity which follows a contraction. Stimulation of glycogenolysis by tetanic contraction is still larger by one to two orders of magnitude.

Studies of the rates of glycogen synthesis carried out *in vitro* on the basis of the determinations *in vivo* (Figures 1–4) are summarized in Table I. Three different situations were assumed in these *in vitro* experiments: (a–b) only the form of the enzyme is relevant, (c–d) both the form of the enzyme and the glucose-6-P concentration are important, and (e–f) the form of the enzyme, the concentration of glucose-6-P and those of the inhibitory metabolites are all important. In turn, each of the above alternatives was explored at the concentration of UDP-glucose found in muscle and at a saturating level. The first alternative

[5] The maximal rate of glycogenolysis during tetanic contraction in our preparation was 1.9 mM/sec, when obtained *directly* from the glycogen lost on stimulation. A lower value (0.8 mM/sec) can be calculated from the accumulation of metabolites (Figure 2A) in agreement with the data of Danforth and Lyon (1964). The phosphorylase activity used for the comparison with GS is that of the reaction going from glycogen to glucose-1-P, that is, approximately one-third of the value obtained in the assay.

implies that both glucose-6-P and the inhibitory metabolites are not available in the muscle to GS.

The time course of glycogen resynthesis (Figure 6) is similar to the time course of GS conversion into the I form (Figure 3 or 5) and might therefore suggest that the latter process is solely responsible for the *in vivo* synthesis of glycogen. However, closer inspection of the activities obtained from considering that only the relative proportions of GS forms are relevant (Table I, a–b) shows that this is not the case. In fact, comparison of the rates obtained at 2.5–4- and 0–1-min recovery with those found at 7.5–10 min shows that while the *in vivo* ratios are 4.7 and 2.4, respectively, the corresponding *in vitro* ratios are 1.5 and 0.7. In other words, the rate of glycogen synthesis anticipated from alternative a–b does not fall at the end of the recovery period as markedly as it is actually found *in vivo*. In addition, the increase of glycogen synthesis *in vitro* from rest to the maximum (B/R ratio, Table I) is only 2-fold, while *in vivo* this value is, at least, 5-fold (see above). Increasing the UDP-glucose concentration brings the actual rates closer to those found *in vivo*, but does not change the general pattern.

When glucose-6-P is included in the assays the rates are increased, but their ratios show that maximal changes in activities do not exceed 30–50%. In fact, glucose-6-P is present in most of the situations at a saturating concentration, and therefore the D into I conversion which takes place during the 1–4-min recovery period is not reflected in an increase in GS activity. It is evident that if these were the conditions prevailing within the cell the interconversion would be devoid of physiological significance.

Inclusion in the assays of the inhibitory metabolites alters substantially the pattern. The B/R ratio (Table I) is now increased 8–20-fold, depending upon the UDP-glucose concentration which is considered. Comparison of the maximal (2.5–4 min) and initial (0–1 min) rates of glycogen resynthesis with those found during the 7.5–10-min period of recovery shows a 4- and 2-fold increment, respectively. These ratios agree rather well with those obtained *in vivo*. Further support to the hypothesis that glucose-6-P reverts *in vivo* the inhibition by metabolites of the D form of GS is found in the fact that glycogen is resynthesized *in vivo* at an appreciable rate at a time when no conversion into the I form has yet taken place (0–1-min recovery, Figure 3 or 5 and 6). Thus, only the alternatives which consider the inhibitory metabolites as a part of the regulatory system gives *relative* rates in agreement with the *in vivo* data. The absolute rates obtained *in vitro*, by using all the concentrations at their face values, are substantially lower than those encountered *in vivo*. However, when UDP-glucose concentration is increased (which implies a certain degree of "compartmentation" for this sugar nucleotide), the *in vitro* rates become larger, and can account for approximately half of the *in vivo* values. This difference is not too surprising if it is considered that GS is a rate-limiting enzyme, and that the assays, even if carried out under "physiological" conditions, do not reflect the exact environment within the cell. Furthermore, a similar degree of relative efficiency can be found by comparing other systems when studied *in vitro* and *in vivo*. For instance, phosphorylase activity *in vitro* can account for the half-maximal rate of glycogenolysis during tetanic contraction,[5] and the rate of gluconeogenesis in the perfused liver is smaller than that in the intact rat (Exton and Park, 1967). It could be therefore concluded that comparison of the *in vivo* and *in vitro* data suggests (with the limitations discussed) that the regulation of glycogen synthesis, under the conditions studied, can be best explained by the alternative which takes into account the form of GS, the levels of glucose-6-P and inhibitory metabolites, as well as a higher concentration of UDP-glucose in the immediate environment of the enzyme.

Of the inhibitory metabolites used, ATP is, by far, the most important, since its concentration is seven to eight times higher than that of ADP. The ATP level does not vary under the conditions tested, consequently the inhibitory power of the mixture of metabolites will be nearly constant, regardless of the physiological situation it represents. Therefore, for a given form of GS, the main regulatory factor will be the variations in glucose-6-P concentration. Such a situation occurs during the tetanic contraction (Figures 1B and 2A). On the other hand, while glucose-6-P steadily decreases during the recovery period, GS activity continues to rise at the beginning of this period because of the D into I conversion. Therefore, of the two possible regulatory mechanisms (one by changes in concentration of the relevant metabolites, and the other by interconversion between the I and D forms) only the first is involved during the contraction and the first minute of recovery, but *both* of them play a role during the following period. Hence, metabolite regulation and interconversion would have the effect of switching on and off glycogen synthetase activity, as previously postulated from the *in vitro* studies (Piras *et al.*, 1967). An on and off mechanism has recently been proposed also for liver glycogen synthetase (Mersmann and Segal, 1967).

Finally, it should be pointed out that the conversions of muscle GS so far described are rather slow when compared with the phosphorylase system (Danforth, 1965; Figures 1B and 3). Moreover, electrical stimulation rapidly activates phosphorylase but does not decrease the level of I-GS present at rest (32%). This result might be considered surprising, given the widely accepted view that GS becomes activated when phosphorylase is inactivated, and *vice versa*. However, the GS activity (rather than I-form content) present at rest is very low (Figure 5), and therefore, there might be no special advantage in further diminishing the I content of GS in this situation.

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