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Glucose 6-Phosphate Dependent and Independent Forms of Yeast Glycogen Synthetase. Their Properties and Interconversions*

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ABSTRACT: Preparations of glycogen synthetase in the glucose 6-phosphate dependent (D) and independent (I) forms were obtained, respectively, from cells in the logarithmic and stationary phase of growth of a haploid strain of *Saccharomyces cerevisiae*. Some kinetic properties of the two forms were examined. The I form has a partial requirement for glucose 6-phosphate, but the corresponding K_m is 30 times smaller than that of the D form. The latter is much more strongly inhibited by adenosine triphosphate, at low concentrations of glucose 6-phosphate, than the I form. Inverse plots of the rate against uridine diphosphate glucose concentration yield two intersecting lines with both forms, and the two K_m values are similar in either case. The kinetic properties of glycogen synthetase from mutant GS 1-36, which does not accumulate glycogen during growth, appear to be very similar to, if not identical with, those of the D form from the parent strain. Storage of preparations of the D form at −20° resulted, in some cases, in a conversion into a glucose 6-phosphate independent (pseudo-I) form. The pseudo-I form was refractory to inhibition by ATP. The original properties of the D form were restored by incubation with high concentrations of β-mercaptoethanol. Incubation of glycogen synthetase in the I form with a cell-free extract from lyophilized yeast resulted in a decrease of the "ratio of independence" (activity without glucose 6-phosphate × 100/activity with 10 mM glucose 6-phosphate). ATP and Mg^{2+} were required for the conversion. After this treatment, glycogen synthetase showed the expected properties of the D form. Another extract, obtained from yeast spheroplasts, catalyzed the opposite effect, that is, an increase in the ratio of independence. The reaction was stimulated by Mg^{2+} and inhibited by fluoride. By allowing the increase in ratio of independence to progress to a certain extent and then adding ATP and fluoride, it was possible to reverse the effect in the spheroplast extract. The total activity of glycogen synthetase remained constant through all the changes in ratio of independence. It is proposed that the observed effects correspond to interconversions between the I and D forms of glycogen synthetase. Both interconverting systems are present in extracts from logarithmic phase and stationary phase cells, either of the wild-type strain or of the "glycogenless" mutant GS 1-36. Therefore, the factors which trigger unidirectional conversions between the I and D forms *in vivo* remain unknown.

The existence, in muscle and liver, of two forms of glycogen synthetase (UDP-glucose:glycogen α-4-glucosyltransferase, EC 2.4.1.11), which differ in their requirement for glucose-6-P,[1] was documented several years ago by Larner and his coworkers (Larner, 1966). Recently, the two forms were also detected in *Neurospora* (Téllez-Iñón *et al.*, 1969). In earlier studies of the yeast synthetase we found evidence for the presence by only one form, which showed a relatively small stimulation of glucose-6-P, in the absence of allosteric inhibitors (Rothman and Cabib, 1967a). However, recent results (Rothman-Denes and Cabib, 1970) indicate that the response of yeast glycogen synthetase to glucose-6-P depends on the stage of growth at which the cells are harvested. Thus, early and middle logarithmic phase cells yield enzyme in the glucose-6-P-dependent (D) form, whereas the glucose-6-P-independent (I) form predominates in early stationary phase cells. These results were obtained with a haploid strain of *Saccharomyces cerevisiae*, αS288C.

These findings opened the possibility of studying the

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[1] Abbreviations used are: glucose-6-P, glucose 6-phosphate; D, glucose 6-phosphate dependent form of glycogen synthetase; I, glucose 6-phosphate independent form of glycogen synthetase; UDP-glucose, uridine diphosphate glucose; *RI*, ratio of independence (for definition, see text).

properties of the D form, not previously obtained from yeast, and of comparing it to other preparations, such as the I form from the same strain, the enzyme from a "glycogenless" mutant (Rothman-Denes and Cabib, 1970) and the glycogen synthetases previously obtained from muscle and yeast. It was also feasible to determine the requirements for the interconversion of the two forms *in vitro*, thus laying the foundations for a search, at the enzymological level, of the factors which govern the transformations observed *in vivo*.

Experimental Section

Materials. The growth and harvesting of *S. cerevisiae*, strain αS288C and mutant GS 1–36, have already been described (Rothman-Denes and Cabib, 1970).

Phosphocreatine, creatine kinase, and shellfish glycogen were purchased from Sigma Chemical Co. UDP-[^{14}C]glucose was obtained from New England Nuclear Corp.

Methods. ENZYME DETERMINATIONS. Assays A and B were used in the presence or absence of 10 mM glucose-6-P, as previously described (Rothman-Denes and Cabib, 1970). The reaction was linear during the 10-min incubation. The specific activity of UDP-[^{14}C]glucose was varied between 0.06 and 2 μCi per μmole, depending on the substrate concentration and the activity of the enzyme preparation used, in order to obtain a minimum incorporation of about 500 cpm in glycogen. One unit of activity is defined as the amount of enzyme which catalyzes the incorporation of 1 μmole of glucose per min into glycogen, under the condition of assay A and with 10 mM glucose-6-P. The ratio of independence (*RI*) is defined as the activity without glucose-6-P divided by the activity with 10 mM glucose-6-P, the result being multiplied by 100. The total activity is that measured in the presence of 10 mM glucose-6-P.

PREPARATION OF GLYCOGEN SYNTHETASE IN THE D FORM. Yeast collected at an optical density of 0.1–0.12 at 660 mμ (logarithmic phase) was centrifuged, washed, lyophilized, and ground as previously reported (Carminatti and Cabib, 1965). The following operations were carried out at 0–5°. For each gram of yeast (dry weight), 4 ml of 0.05 M Tris-maleate buffer (pH 7.5), containing 1 mM EDTA and 50 mM KF,[2] was added. The suspension was homogenized for 2 min in a Lourdes Multi-mixer and centrifuged for 15 min at 20,000*g*. The pellet was washed with the same buffer, using 2 ml/g of yeast (dry weight), and the washing was combined with the first supernatant fluid. The solution was diluted twofold by adding the above buffer and centrifuged for 30 min at 150,000*g*. The pellet and a turbid layer above it were discarded. To the supernatant fluid, enough glycogen was added to obtain a final concentration of 12 mg/ml, and the mixture was again centrifuged for 90 min at 150,000*g*. The supernatant liquid was discarded and the pellet, which contained both glycogen and glycogen synthetase, was washed with 5 ml of 0.05 M glycylglycine (pH 7.5), containing 1 mM EDTA and 50 mM KF. The final pellet was resuspended in the same buffer to a final volume of 1 ml/g of yeast (dry weight), and dialyzed for 3 hr against two 1500-ml changes of 50 mM KF, containing 1 mM EDTA. The recovery of enzymatic activity, with respect to the 20,000*g* supernatant fluid was 30–55%, with a purification of 6- to 8-fold. The total activity obtained was on the average 0.6 unit/g of yeast (dry weight) as measured with assay A. Some preparations showed a relatively high *RI* (up to 35 with assay A), which could be decreased by a treatment with β-mercaptoethanol under the same conditions outlined below for the pseudoindependent form.

PREPARATION OF GLYCOGEN SYNTHETASE IN THE I FORM. The procedure was similar to that described in the preceding section for the D form, with the following modifications. (a) The cells were collected during the stationary phase 1 hr after growth had stopped; (b) the solutions used for extraction, washing, and dialysis contained no KF; (c) the 30-min centrifugation at 150,000*g* was omitted; since the stationary phase cells contained glycogen (Rothman-Denes and Cabib, 1970), a large amount of enzyme would have been lost to the sediment in this step; (d) the final pellet was resuspended in 2 ml of buffer/g (dry weight) of yeast used, before dialysis. The yield of glycogen synthetase activity, as referred to the 20,000*g* supernatant was 50 to 80% and the purification 5- to 6-fold. With extracts from stationary phase yeast, about half of the total activity sedimented with the 20,000*g* pellet (Rothman-Denes and Cabib, 1970), thus reducing the overall yield. The average amount obtained in several preparations was 2 units/g of yeast (dry weight).

The *RI* shown by the preparations of I form was variable, and sometimes as low as 40, as measured with assay A. Recently, it has been found that higher ratios could be consistently obtained if the 20,000*g* supernatant was dialyzed for 3 hr against 1 mM EDTA, before centrifuging at 150,000*g*. In three preparations, the ratio of independence was 82, 71, and 60, respectively, when measured with assay A, and 67, 64, and 58, respectively, with assay B.

Glycogen synthetase from stationary phase cells of mutant GS 1–36 was prepared as described for the I form of the parent strain.

PREPARATION OF *RI* DECREASING SYSTEM.[3] Yeast from the logarithmic phase of growth was harvested, washed, lyophilized, and ground as previously described (Carminatti and Cabib, 1965). The following operations were carried out at 0–5°. For each gram of yeast (dry weight), 4 ml of 0.05 M imidazole-acetate buffer (pH 7), containing 1 mM EDTA, was added. The suspension was homogenized for 2 min in a Lourdes Multi-mixer, and centrifuged for 15 min at 25,000*g*. To the supernatant fluid, enough glycogen was added to obtain a final concentration of 25 mg/ml and the mixture was centrifuged for 2 hr at 150,000*g*. The pellet was discarded, and 0.6 ml of the supernatant liquid was filtered through a Sephadex G-25 column (1 × 6 cm) which had been equilibrated with the above-mentioned buffer. After discarding the void volume, 1 ml of effluent was collected.

The preparation was devoid of glycogen synthetase activity, which is removed by the centrifugation in the presence of glycogen. The *RI* decreasing activity was lost after freezing and thawing. A fresh extract was obtained for each experiment.

PREPARATION OF EXTRACTS CONTAINING *RI* INCREASING AND *RI* DECREASING ACTIVITY. Extracts were obtained from spheroplasts, as already described for the interconversion experiment of Rothman-Denes and Cabib (1970). These preparations contained endogenous glycogen synthetase, which served as substrate in interconversion experiments. The centrifuged extracts, before Sephadex filtration, could be stored at −10 to −20° for at least a week without apparent

[2] KF was added to inhibit the D into I conversion (see below).

[3] The enzymatic systems which cause the observable changes in the ratio of independence of glycogen synthetase are named operationally, as *RI* increasing and *RI* decreasing system, respectively.

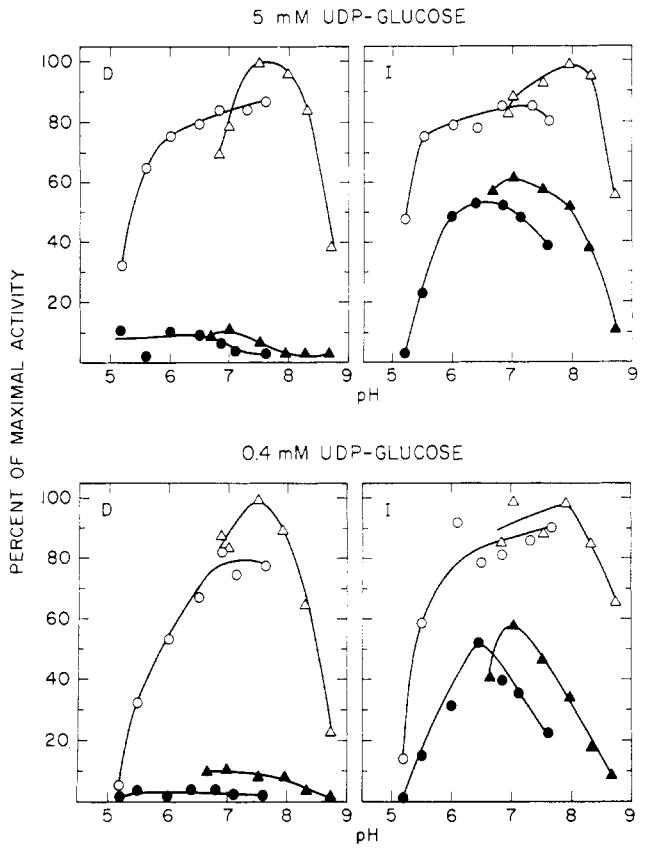

FIGURE 1: Effect of pH on the activity of the D and I forms at 5 or 0.4 mM UDP-glucose, as indicated. The amount of enzyme used in each assay was 0.74×10^{-3} unit for the D form and 0.95×10^{-3} unit for the I form. Other conditions were as for assay A, except that the buffer was either succinate–cacodylate (●, ○) or glycylglycine (▲, △). Filled symbols, no glucose-6-P. Empty symbols, 10 mM glucose-6-P.

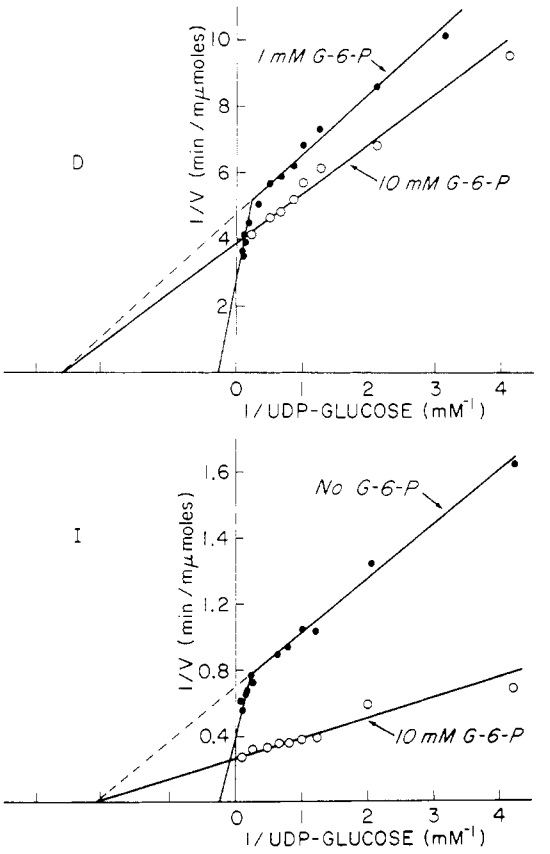

FIGURE 3: Effect of UDP-glucose on reaction rate with the D and I forms of glycogen synthetase. Conditions of assay B, except for the concentration of UDP-glucose. G-6-P, glucose 6-phosphate.

loss of activity of either glycogen synthetase or the interconverting systems. The ratio of independence of glycogen synthetase also remained unchanged. Prior to each experiment, samples were filtered through Sephadex G-25 columns, as for the *RI* decreasing preparation.

Results

Kinetic Properties of I and D Forms. In this study, many determinations of enzymatic activity were carried out at 0.4 mM UDP-glucose (assay B), a concentration close to that found in the yeast cell (Rothman and Cabib, 1969). However, in some cases the concentration of UDP-glucose was varied, and on several occasions it was desirable to determine the maximal measurable activity. For these reasons, the effect of pH on the activity was measured both at saturating (5 mM) and at 0.4 mM UDP-glucose (Figure 1). For each form, little difference was noted between the two concentrations of substrate, except for a steeper decrease in activity below pH 6.5 with the D form, when determined at 0.4 mM UDP-glucose. For this reason, most of the kinetic determinations were carried out at pH 6.5, rather than 6, as previously used (Rothman and Cabib, 1967b). From the degree of stimulation observed with glucose-6-P, it appears that the enzyme previously isolated from baker's yeast (Algranati and Cabib, 1962; Rothman and Cabib, 1967a,b) corresponds to the I form of strain αS288C. In fact, the curves in the absence of glucose-6-P are very similar (*cf.* Figure 1 and Rothman and Cabib, 1967b). In the presence of the sugar phosphate the activity at pH values below 6.5 was higher with the preparation from the present strain.

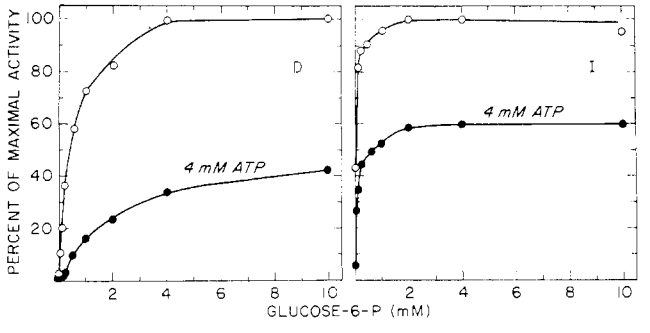

FIGURE 2: Effect of glucose-6-P on the activity of the D and I forms, in the absence and in the presence of ATP. Conditions of assay B. The amount of enzyme used in each assay was 1×10^{-3} unit for the D form and 0.5×10^{-3} unit for the I form.

Figure 2 shows the different response of the D and I enzymes to glucose-6-P. In the presence of ATP the two curves present strikingly dissimilar shapes, which are especially apparent at low concentrations of glucose-6-P (*cf.* Figure 5 of Rothman-Denes and Cabib, 1970). With both forms the effect of ATP was only partially reversed, even at very high concentrations of the sugar phosphate. Inverse plots of the activity as a function of glucose-6-P, in the absence of ATP, yielded straight lines. The K_m values were

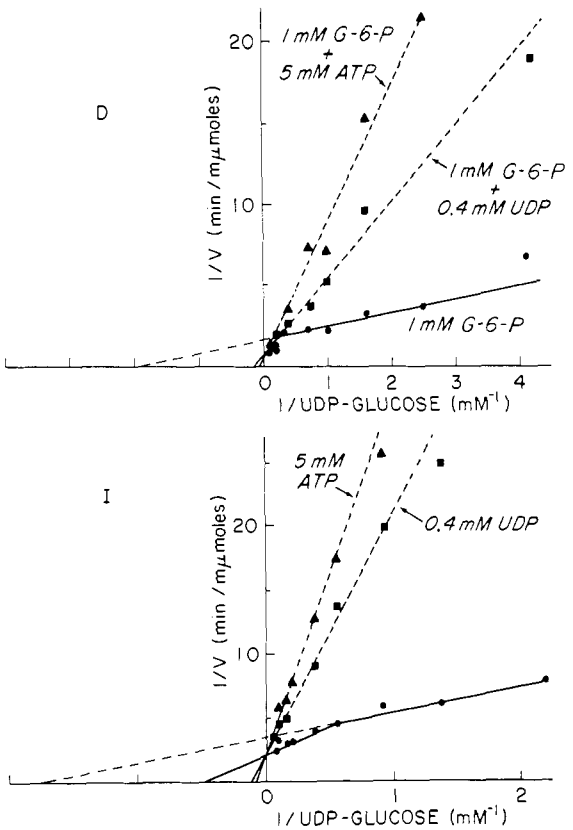

FIGURE 4: Inhibition of the I and D forms by UDP and ATP. Conditions of assay B, except for the concentration of UDP-glucose. G-6-P, glucose 6-phosphate.

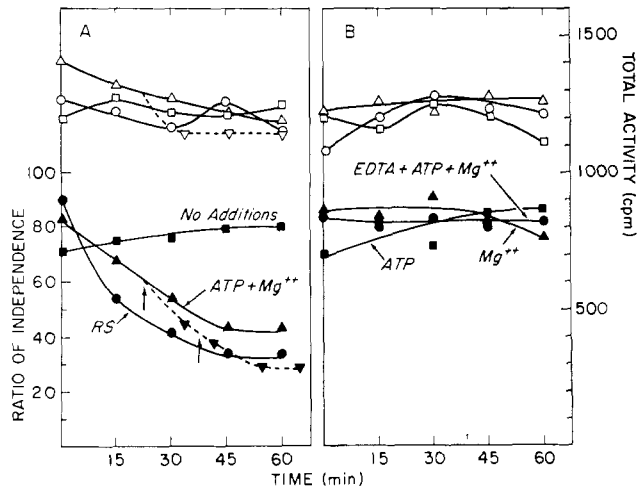

FIGURE 5: Requirements for the *RI* decreasing system. The incubation mixture contained 10 μl (22 munits) of glycogen synthetase in the I form, 40 μl of Sephadex-filtered preparation of *RI* decreasing system (see Methods) and the following additions, in a total volume of 60 μl: (A) (a) no additions (■, □); (b) 5 mM ATP and 5 mM magnesium acetate (▲, △); (c) same as in part b, but 3 μl of a solution containing 0.1 M ATP and 0.1 M magnesium acetate was added at the times indicated with arrows (broken line, ▼, ▽); (d) 5 mM ATP, 7 mM magnesium acetate, 30 mM phosphocreatine, and 33 mg/ml of creatine kinase (●, ○). (B) (a) 5 mM ATP, 5 mM magnesium acetate, and 20 mM EDTA (●, ○); (b) 5 mM ATP (■, □); (c) 5 mM magnesium acetate (▲, △). After incubation at 30° for different periods, 3-μl aliquots of each mixture were removed for measurement with assay A, of *RI* (filled symbols) or total activity (empty symbols). *RS*, ATP-regenerating system.

widely different, 0.5 mM for the D form and 0.017 mM for the I form. Similar graphs, from data obtained in the presence of ATP showed an upward concavity, as was expected from the curves of Figure 2, and of Figure 5 from Rothman-Denes and Cabib (1970).[4] From Hill plots (not shown) an n value of 1.5 was found for the D form and 2 for the I form.

The dependence of reaction rate on UDP-glucose concentration is illustrated in Figure 3. Unexpectedly, the points obtained in the absence of glucose-6-P (I form) or at low glucose-6-P (D form) yielded two straight lines with different slopes. At 10 mM glucose-6-P only the slope corresponding to a lower K_m can be seen. The two slopes of Figure 3 were reproducibly found with several preparations of both forms, although the numerical values of the K_m's varied to some extent. For the experiment of Figure 3, the K_m values for the D form were 0.42 and 4 mM, and those for the I form 0.48 and 4.5 mM. As shown in Figure 4, addition of either ATP or UDP yielded curves intersecting the ordinate axis at the same intercept obtained in their absence. This behavior is that expected from competitive inhibitors. However, the two lines observed in the absence of the inhibitors are no longer seen in their presence. The paucity and scatter of the points do not permit a definitive conclusion on this point. It should also be noted that the slopes of the two lines might be much closer to each other in the presence of the inhibitors, if the K_i values for each of the two hypothetical UDP-glucose sites are different.

Kinetic Properties of Glycogen Synthetase from Mutant GS 1-36. As reported in an earlier communication (Rothman-Denes and Cabib, 1970), the glycogen synthetase from the "glycogenless" mutant GS 1-36 appears to be in the dependent form, even when obtained from cells harvested during the stationary phase of growth. Nevertheless, a limited conversion into the I form was obtained *in vitro* (see below). It is not clear whether the defect of the mutant resides in the synthetase itself or in the interconverting systems. The dependence of the GS 1-36 enzyme on glucose-6-P, both in the absence and in the presence of ATP, and the pH-activity curve were very similar to those obtained with the D form of the parent. The K_m for glucose-6-P in the absence of ATP was 0.26 mM, as compared to 0.5 mM for the D form of αS288C. Determinations of the K_m for UDP-glucose at 0.1 mM glucose-6-P yielded two slopes in this case also, and the corresponding K_m values were 0.4 and 1.8 mM.

Pseudoindependent Form. When the D form of glycogen synthetase was stored at −10 to −20°, in some cases the ratio of independence increased up to a value of about 50. The new enzyme species obtained was a "pseudoindependent" rather than a "true independent" form, as shown by its resistance to ATP inhibition. Thus, in one case the inhibition by 4 mM ATP in the absence of glucose-6-P was 22% with the pseudo-I and 80% with the I form. Incubation of the pseudo-I enzyme with 0.1 M mercaptoethanol for 30 min at 30° restored the original properties of the D form. Lower concentrations of the SH compound were ineffective.

Interconversions. Incubation of glycogen synthetase with yeast extracts brought about changes in the *RI*, when certain additions were made. The following sections deal with the requirements for obtaining either a decrease or an increase in *RI*.

RI DECREASING SYSTEM. The I form of glycogen synthetase

[4] The curve of Figure 2 for the I form in the presence of ATP appears to be hyperbolic because of the compressed scale used.

TABLE I: Properties of Glycogen Synthetase before and after Incubation with ATP-Mg^{2+}.[a]

Treatment	Ratio of Independence		Inhibition by 4 mM ATP[b]		
	Assay A	Assay B	No Glucose-6-P (%)	1 mM Glucose-6-P (%)	10 mM Glucose-6-P (%)
None	76	72	64	28	20
Experiment A					
ATP-Mg^{2+}, Sephadex	40	7	63[c]	60	36
ATP-Mg^{2+}, Sephadex, SH	28.5	2.5	100[c]	68	35
Experiment B					
SH, Sephadex	73	61	70	35	25
SH, Sephadex, ATP-Mg^{2+}, Sephadex	30	4.5	80[c]	70	47

[a] In expt A, a sample (68 munits) of glycogen synthetase in the I form was incubated for 20 min at 30° with 5 mM ATP, 5 mM magnesium acetate, and 80 μl of *RI* decreasing extract, in a total volume of 0.1 ml. The incubated mixture was diluted 2-fold with 1 mM EDTA and filtered through a Sephadex G-25 column (0.5 × 6 cm), equilibrated with 1 mM EDTA (ATP-Mg^{2+}, Sephadex). A sample of the filtrate was incubated for 15 min in 0.1 M β-mercaptoethanol (ATP-Mg^{2+}, Sephadex, SH). In expt B, the I form was first treated with β-mercaptoethanol as above, and then filtered through Sephadex (SH, Sephadex). A portion of this filtrate was incubated with ATP and Mg^{2+} as above and again passed through Sephadex (SH, Sephadex, ATP-Mg^{2+}, Sephadex). [b] Measured with assay B. [c] Values of doubtful significance, due to the very low enzymatic activity in the absence of glucose-6-P.

was incubated with an extract from lyophilized yeast, prepared as described under Methods for the *RI* decreasing system.[5] As shown in Figure 5, the addition of ATP and Mg^{2+} resulted in a progressive decrease of the ratio of independence. When either ATP, or Mg^{2+}, or both were omitted, the *RI* remained essentially constant. It was found that after 15 min of incubation with ATP and Mg^{2+}, about 1.3 moles of P_i was liberated per mole of ATP present. Accordingly, repeated additions of ATP, or the presence of an ATP-regenerating system, led to a greater decrease in the *RI* (see Figure 5). Addition of 0.1 mM cyclic 3′,5′-AMP or 20 mM KF in the absence of the ATP-regenerating system did not influence the conversion. An extract obtained by boiling a preparation of the converting system which had not been filtered through Sephadex was also without effect. EDTA inhibited the conversion (see Figure 5), as would be expected from the Mg^{2+} requirement.

In all cases the "total" activity (measured at 10 mM glucose-6-P) changed little, as shown in Figure 5.

The purified preparation of I form appeared to be devoid of *RI* decreasing activity. Omission of the yeast extract in the ATP-Mg^{2+} system of Figure 5 resulted in *RI* values of 75, 82, and 85 after 0-, 30-, and 60-min incubation, respectively. Changes in total activity were less than 2%.

Glycogen synthetase is inhibited by several anions (Rothman and Cabib, 1967a,b). In the experiments described above, the enzyme was assayed at high UDP-glucose concentration, in order to minimize the inhibition by ATP or ADP, which is kinetically of the competitive type (see Figure 4). In the experiment shown in Table I, the converted enzyme was freed from small molecular weight substances by passage through Sephadex in order to confirm the change in properties. Furthermore, treatment with β-mercaptoethanol was included either before or after the incubation, in order to convert back into the D form any pseudo-I form that might be present. It may be seen that after incubation with ATP and Mg^{2+} and filtration through Sephadex, the enzyme showed a decreased *RI* and an increased inhibition by ATP, as corresponds to the D form.

RI INCREASING ACTIVITY. When an extract from spheroplasts (see Methods and Rothman-Denes and Cabib, 1970) was incubated at 30°, a slow increase in the *RI* of the endogenous glycogen synthetase was observed (see Figure 6). Addition of Mg^{2+} had a considerable stimulatory effect. The slow change observed without added Mg^{2+} took place despite the presence of 0.8 mM EDTA (contained in the Sephadex-filtered extract). It may also be seen in Figure 6 that fluoride inhibits the reaction. No effect was found by supplementation of the incubation mixture with 0.1 mM cyclic 3′,5′-AMP, 10 mM glucose, 5 mM ammonium sulfate, or 5 mM potassium phosphate. Glucose and phosphate caused some inhibition when added simultaneously. β-Mercaptoethanol, at 40 mM, was also inhibitory. Extracts from lyophilized cells were found to be devoid of *RI* increasing activity, either before or after passage through Sephadex. The experiment of Figure 6 also shows that changes in total activity were small.

Both the increase and the decrease in *RI* could be observed in the same extract, thus showing their reciprocal character. Such an experiment is shown in Figure 7. The addition of ATP after 60-min incubation with Mg^{2+} alone (curve A) caused a rapid decrease in the *RI*, which later began to rise again. At this time, all or most of the ATP had been hydrolyzed, as shown by determinations of P_i. The late rise in the *RI* can be greatly reduced by inhibiting the *RI* increasing activity with fluoride (curve B). The latter had only a slight inhibitory effect on the ATPases present in the extract. Replenishment of ATP by repeated additions led to an even lower *RI* (curve C).

Interconverting Systems at Different Phases of Growth and in Mutant GS 1-36. The rapid accumulation of glycogen at the end of the logarithmic phase of growth of the wild-type strain is accompanied by an increase in the *RI* of glycogen synthetase, whereas, in the "glycogenless" mutant GS 1-36

[5] In all interconversion experiments no buffer was added to the incubation mixture, because the Sephadex-filtered extracts already contained 0.05 M imidazole acetate at pH 7 (see Methods).

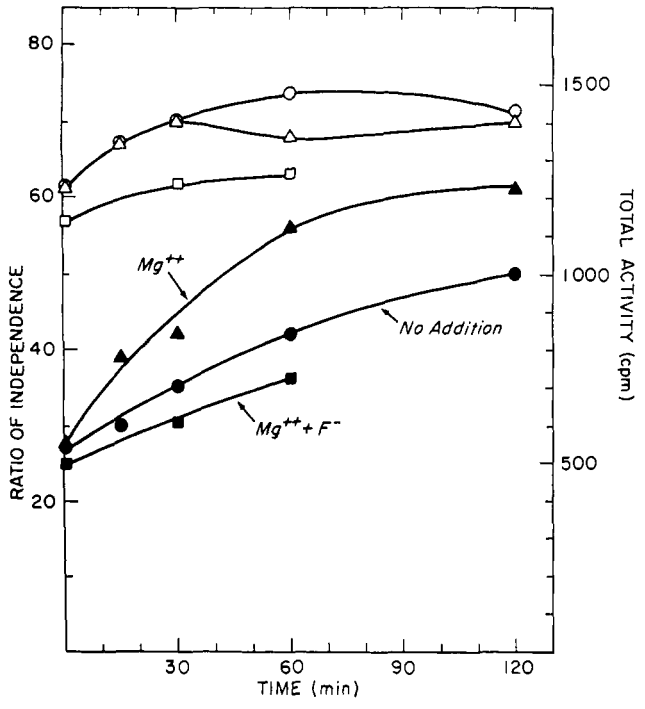

FIGURE 6: Conditions for *RI* increasing system. The reaction mixture contained, in a total volume of 60 μl, 50 μl of Sephadex-filtered spheroplast extract (see Methods and Rothman-Denes and Cabib, 1970), obtained from logarithmic phase yeast (A_{660} = 0.4). Magnesium acetate (4 mM) and KF (20 mM) were included where indicated. Incubation was carried out at 30°. Aliquots of 3 μl were removed at the times indicated, and the *RI* (filled symbols) and total activity (empty symbols) were determined with assay A.

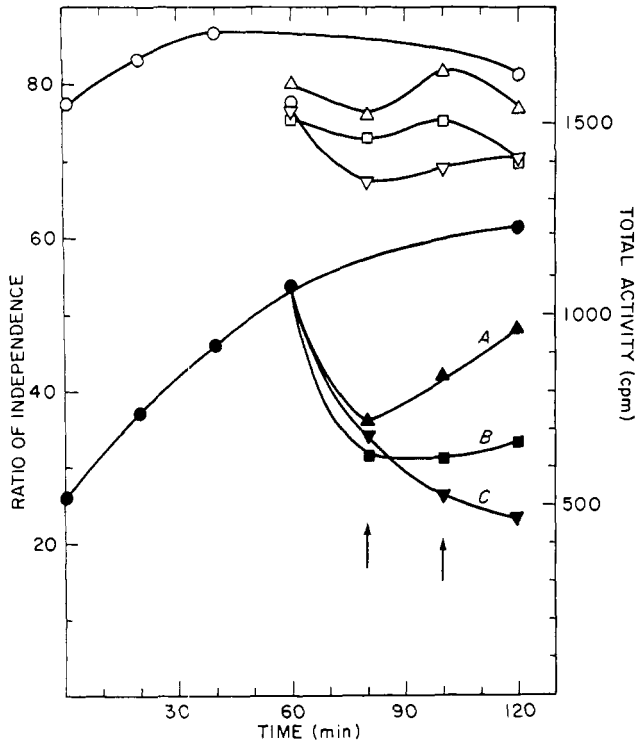

FIGURE 7: Reversibility of *RI* changes. A Sephadex-filtered spheroplast extract was obtained and incubated with 4 mM Mg^{2+} under the conditions of Figure 2 (●, ○); at 60 min, 0.1-ml aliquots were removed and supplemented with the following additions: curve A, 4 mM ATP (▲, △); curve B, 4 mM ATP and 20 mM KF (■, □); curve C, same as curve B except that 5 μl of a solution containing 0.1 M ATP and 0.1 M magnesium acetate were added at the times indicated with arrows (▼, ▽). Determinations of *RI* (filled symbols) and of total activity (empty symbols) were carried out as for Figure 6.

no conversion of the D into I form appears to take place *in vivo* (Rothman-Denes and Cabib, 1970). Therefore, it was of interest to examine the interconverting systems in both strains and at different stages of growth.

In experiments similar to that of Figure 7, it was found that variations in *RI* in both directions could be observed in all cases. With the wild-type yeast in the stationary phase the rise in *RI* was small, since the starting value was already high (Rothman-Denes and Cabib, 1970). With mutant GS 1-36 the overall changes in both directions were only about two-thirds of those observed with the parent strain.

Discussion

Properties of the I and D Forms. The experiments described in this report were carried out with enzymes obtained from lyophilized yeast. A certain variability in the ratio of independence was alluded to under Methods and in a previous communication (Rothman-Denes and Cabib, 1970). As mentioned above, some improvement could be obtained in the preparation of the D form with a β-mercaptoethanol treatment, and for the I enzyme with a dialysis of the crude extract. In the latter case, it is very unlikely that a conversion of D into I form was taking place, because the appropriate converting enzyme is not present in extracts from lyophilized cells, as mentioned under Results. Perhaps some of the difficulties were caused by enzymes or other substances present in the cell wall or in the periplasmic space, since more reproducible results were obtained with extracts from spheroplasts (Rothman-Denes and Cabib, 1970). The latter method, which was introduced more recently, may lead to better procedures for the preparation of the two forms, especially if the interconverting systems are used to transform all or most of the glycogen synthetase into the desired form. On the other hand, the use of lyophilized cells leads more readily to large-scale preparations, and the total activity obtained is only slightly inferior to that recovered from spheroplasts.

The enzymes studied were obtained from a 20,000*g* supernatant fluid. An important percentage of the activity remains in the pellet, especially when stationary phase cells are used (*cf.* Rothman-Denes and Cabib, 1970). It is not known whether the properties of this fraction differ from those of the supernate enzyme, except for the ratio of independence which tended to be somewhat higher in the sediment.

The purification obtained with the adopted procedure was very modest. Nevertheless, it was preferred, at this preliminary stage, to sacrifice purity to yield in order to obtain a representative sample of the total enzyme. This necessity is especially evident in the case described here, since at least two forms of the enzyme are present, and perhaps more, in view of the two K_m values obtained for UDP-glucose. Furthermore, purification of the D form with the method previously described (Algranati and Cabib, 1962) led to large losses in the amylose adsorption step.

As can be seen in Figure 2, the I form is not completely independent of glucose-6-P. At pH 6.5 and 0.4 mM UDP-glucose, a stimulation by glucose-6-P in excess of 2-fold was found, even in the absence of inhibitors. If this result were due to contamination with the D form, glucose-6-P

would be expected to yield a K_m of 0.5 mM. Since the K_m obtained was 30 times smaller, no D form appears to be present. Thus, the stimulation by glucose-6-P appears to be an intrinsic property of the I form. The situation seems to be very similar to that of the baker's yeast enzyme (Rothman and Cabib, 1967a), presumably also an I form. In that case it was shown that the two effects of glucose-6-P, *i.e.*, direct stimulation and antagonism against anionic inhibitors, were of different nature.

The curves describing the effect of glucose-6-P on the reaction rate of the I form, with or without ATP, are very similar to those previously determined with the baker's yeast enzyme (Rothman and Cabib, 1967b). The greater susceptibility of the D form to ATP inhibition is in agreement with the results obtained with the corresponding enzymes from muscle (Piras *et al.*, 1968). As previously discussed (Piras *et al.*, 1968) this behavior would ensure that only the I form is physiologically active, except under very special circumstances.

The two lines obtained in the graphs which relate the activity to the UDP-glucose concentration have not yet been explained.[6] Owing to the relatively crude state of the enzyme, they might perhaps result from some artifact. Nevertheless, the possibility that this behavior is due to the presence of an impurity is made unlikely by the fact that in the presence of glucose-6-P only one line can be seen.

The K_m values for the I and D forms are practically identical, as previously found with the muscle enzymes, when measured under the same conditions (Piras *et al.*, 1967). The value of the smaller K_m is also similar to that obtained with the muscle glycogen synthetases.

The properties of the enzyme from mutant GS 1-36 are very similar to those of the D form from the wild-type strain. Although some quantitative differences have been noticed, they are too small to decide whether a change in the enzyme has occurred as a result of the mutation.

The transformation of the D form of glycogen synthetase into the pseudo-I form, and the variability in the ratio of independence of the I form, as obtained from lyophilized stationary phase cells (see Methods), suggest that great caution is required in ascertaining the properties of each preparation under study.

Interconversions. The results presented above show that changes in the ratio of independence of glycogen synthetase, previously observed *in vivo* (Rothman-Denes and Cabib, 1970) can also be obtained *in vitro*, under controlled conditions. The following evidence supports the hypothesis that these changes correspond to interconversions between the I and D forms of glycogen synthetase: (a) the requirement of ATP and Mg^{2+} for the "*RI* decreasing" activity, similar to that found in the mammalian systems (Larner, 1966); (b) the reversibility of the changes, readily obtained by manipulating the composition of the incubation medium; (c) the constancy of the total activity throughout; (d) the changes in the kinetic properties of the enzyme after incubation with ATP and Mg^{2+}, which are as expected for a conversion of I into D form.

The requirement of ATP and Mg^{2+} for the I and D transformation suggests that a phosphorylation–dephosphorylation mechanism might be involved in the yeast system, as was found to be the case in muscle (Larner, 1966). Other interpretations are, however, possible.

The rapid increase in the ratio of independence of glycogen synthetase which takes place at the end of the logarithmic phase of growth (Rothman-Denes and Cabib, 1970), could not be correlated with a change in the interconverting systems, as measured *in vitro*. Both systems appear to be present at all times. It is, of course, possible that one or both of them are regulated by effectors, whose concentrations change before the onset of the stationary phase. From the results obtained in this work it does not appear that cyclic 3′,5′-AMP is such an effector, in contrast with the muscle system (Schlender *et al.*, 1969).

The experiments reported here do not explain the defect of mutant GS 1-36, which does not show the D to I conversion during growth, since both interconverting activities appear to be present in extracts of the mutant. Further studies on the regulation of the converting systems will be needed to solve this problem.

[6] The two lines were not observed in previous determinations with either yeast (Rothman and Cabib, 1967a) or muscle (Piras *et al.*, 1968) glycogen synthetase. However, it is possible that they escaped detection because of an insufficient number of points at high UDP-glucose concentrations.

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