Regulation of Glycogen Synthesis in the Intact Yeast Cell*

Lucia B. Rothman and Enrico Cabib

ABSTRACT: Previous experiments *in vitro* indicated that yeast glycogen synthetase might be regulated *in vivo* through the antagonism between adenosine triphosphate and adenosine diphosphate as inhibitors and glucose 6-phosphate as activator. When nonrespiring yeast cells were incubated with glucose and salts, the sum of intracellular adenosine triphosphate and adenosine diphosphate remained relatively constant after an initial peak. The formation of glycogen proceeded steadily until, as glucose was being consumed, the concentration of glucose 6-phosphate fell under 0.4 mM; thereafter the rate of polysaccharide accumulation declined. When the glucose concentration of the medium was maintained constant, addition of ammonium ions brought the increase in glycogen to a sudden stop. At the same time glucose 6-phosphate decreased abruptly, while adenosine triphosphate and adenosine diphosphate showed only minor changes. Uridine diphosphate glucose was practically constant throughout the experiment; therefore regulation of glycogen synthetase by variations in substrate concentration is very unlikely. It was also shown that the effect of ammonium ions could not be explained by an increased degradation of glycogen or by changes in the properties or amount of glycogen synthetase. In other experiments yeast capable of respiration was incubated with glucose and salts. The rate of glycogen increase was rapid at first, but soon declined, despite a constant concentration of glucose in the medium. At the same time the concentration of glucose 6-phosphate in the cells fell continuously, while adenosine triphosphate, adenosine diphosphate, and uridine diphosphate glucose did not undergo pronounced variations. Thus, the results obtained are in general agreement with the hypothesis advanced previously. A modified method for determination of glycogen in yeast is described.

Previous work (Rothman and Cabib, 1967a,b) has indicated that the activity of yeast glycogen synthetase (UDP-glucose: glycogen α-4-glucosyltransferase, EC 2.4.1.11) is depressed by allosteric inhibitors, ATP and ADP being the most efficient among those tested. Glucose 6-phosphate specifically relieves the inhibition. A scheme was proposed (Rothman and Cabib, 1967b) for the *in vivo* regulation of the enzyme through the interplay of the above-mentioned effectors. In order to test further this hypothesis, it was deemed necessary to correlate the concentrations of the pertinent metabolites and their variations with the rate of glycogen synthesis *in vivo*. This was the object of the experiments to be described below.

Experimental Procedure

Materials

UDP-[^{14}C]glucose and [^{3}H]glucose were purchased from New England Nuclear Corp. The glucostat system, phosphoglucomutase, glucose 6-phosphate dehydrogenase, lactate dehydrogenase, myokinase, and pyruvate kinase were obtained from C. F. Boehringer und Soehne (Mannheim), while hexokinase, UDP-glucose dehydrogenase, and phosphorylase *b* were from Sigma Chemical Co.

The mixture of enzymes used for the determination of glycogen was obtained with the procedure described by Brown and Illingworth Brown (1967) for the preparation of the debranching system. The purification was only carried up to the ammonium sulfate fraction because this fraction contained phosphorylase and the debranching system, both needed for the assay. The 20–28% ammonium sulfate precipitate was resuspended in 5 mM Tris-HCl buffer (pH 7.2) containing 1 mM EDTA and 5 mM mercaptoethanol. After overnight dialysis against the same buffer, the opalescent solution was stored at $-10°$. Before use, the enzyme was diluted in the above-mentioned buffer.

Rabbit liver glycogen type III from Sigma Chemical Co. was used as a standard and shellfish glycogen from the same supplier for enzyme purification. All other chemicals were of reagent grade and were used without further purification.

Methods

Yeast Growth. Saccharomyces cerevisiae (strain No. 197, Cátedra de Microbiología, Facultad de Agronomía y Veterinaria, Buenos Aires, Argentina) was grown in two different media, depending on the experiment to be performed. Medium I contained 2% glucose, 0.3% Difco malt extract, 0.3% Difco yeast extract, and 0.5% Bacto peptone. Medium II contained 0.9% galactose, 1% Difco yeast extract, 0.5% oxoid casein hydrolysate, 0.9% KH_2PO_4, 0.03% $CaCl_2$, 0.5% $MgSO_4 \cdot 7H_2O$, and 0.6% $(NH_4)_2SO_4$.

Cells were allowed to grow at 30° in erlenmeyer flasks on a reciprocating shaker. They were harvested by centrifugation during the late logarithmic phase at a cell density of about 2 mg (dry wt)/ml and washed twice with cold water.

Cell concentration was estimated by measuring the absorbance at 660 mμ in a Coleman Junior spectrophotometer using 12 × 75 mm cuvets. A suspension containing 1 mg (dry wt)/ml gave an absorbance of 0.24.

Incubation of Resting Cells with Glucose and Salts. Yeast cells were suspended at a concentration of 1.2 g (wet wt)/100 ml, in a medium containing 50 mM succinate buffer at pH 5.5, 8 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, and 5 μM thiamine.

* From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received March 25, 1969.*

The experiment was started by the addition of glucose, from a 1.2 M solution, to yield a final concentration of 0.075 M. Incubation was carried out at 30° with shaking. When galactose-grown yeast was used, oxygen was bubbled through the suspension during incubation. The absorbancy of the cell suspension at 660 mμ was determined at intervals. There was an increase of approximately 10%/hr with cells grown in medium I and about 20%/hr with cells grown in medium II.

In order to maintain the glucose concentration constant in many of the experiments, it became necessary to monitor its changes during incubation with a very rapid method of determination. The titration procedure of Ionescu and Vargolici (1920)[1] was found to be appropriate for this purpose; results were obtainable in about 1 min. After each titration, glucose was added to the incubation mixture from a 3 M solution in order to restore the original concentration.

Periodically, samples were removed for the measurement of metabolites, as outlined below.

Determination of Metabolites. In order to calculate the concentration of different substances in the cell, it was assumed that the "free" water content of the yeast cells was 1.7 times the dry wt (Grylls, 1961).

Sampling was carried out by vacuum filtration of a 5-ml (for glycogen) or 10-ml (for all other metabolites) portion of the yeast suspension through a Millipore filter, pore size 0.45 μ. The filter was then treated as outlined below for the different determinations. The whole operation, consisting in measuring the sample, filtering, and transferring the filter to the appropriate solution, was performed in 20–30 sec.

In the enzymatic determination of glycogen and other metabolites changes in absorbance at 340 mμ were followed in a Gilford Model 2000 multiple-sample spectrophotometer.

Determination of Glycogen. The measurement of glycogen in yeast presents two main difficulties. In the first place, the cell wall hinders the extraction of the polysaccharide from the cell (Trevelyan, 1958). This problem was solved by disrupting the cells by sonic oscillations prior to alkaline digestion. Secondly, other polysaccharides present in yeast interfere in the determination, when general sugar reagents such as anthrone or phenol–sulfuric acid are used. Therefore, an assay method was chosen, which employs specific glycogen degrading enzymes (Bueding and Hawkins, 1964). The adopted procedure was as follows. After filtering the yeast suspension through Millipore, the filter was immediately placed in a 12-ml graduated conical tube containing 1.5 ml of water at 95–100°. After stirring with a Vortex mixer, the tube was placed in a boiling-water bath for 5 min and then cooled. The filter was removed, the volume was readjusted to 1.5 ml with water, and 1 ml of the yeast suspension was mixed with 0.11 ml of 60% potassium hydroxide in a stainless steel tube (International Equipment Co. No. 298). The suspension was treated for two 10-sec periods in a Branson Instruments Sonifier, using the 0.5-in. probe at an intensity of 8 A. By visual observation of samples in the microscope, it appeared that 80–90% of the cells was broken. An aliquot was heated for 30 min in a boiling-water bath, cooled, and centrifuged for 5 min at 2000*g*. The glycogen was precipitated from the supernatant fluid with ethanol (66% final concentration) and reprecipitated once more. The final pellet was resuspended in 0.4 ml of water to yield a turbid solution.[2]

TABLE I: Determination of "Glycogen" in Fractions Obtained According to Trevelyan and Harrison (1956).

Fractions[a]	Method of Determination: Anthrone[b] (mg/g dry wt)	Glucose Oxidase[c] (mg/g dry wt)	Phosphorylase Debranching[d] (mg/g dry wt)
Sodium carbonate (0.5 M)	15[e]	4.05	1.12
Acetic acid (2 N)	7.2	3.4	1.22
Perchloric acid (3%)	32.6[f]	28.5	0
Sum	54.8	35.95	2.34

[a] Lyophilized baker's yeast was used as assay material and extracted successively with the solutions listed below. [b] Trevelyan and Harrison (1952). [c] After hydrolysis in 1 N H_2SO_4 (Johnson *et al.*, 1963). [d] Bueding and Hawkins (1964). [e] After subtracting the mannan content, which was determined separately after Fehling precipitation (Trevelyan and Harrison, 1952). [f] After overnight dialysis against distilled water, 9.6 mg/g dry wt remained in the dialysis bag.

An aliquot of this suspension was incubated with the phosphorylase-debranching system, and after 30 min the released glucose 1-phosphate was measured enzymatically (Bueding and Hawkins, 1964). Recovery of an internal glycogen standard was 98%.

It was of interest to compare this procedure with that of Trevelyan and Harrison (1952) since the latter is probably the most widely used method for glycogen determination in yeast. After obtaining the different fractions according to the method of Trevelyan and Harrison, glycogen was measured in each fraction directly with anthrone (Sufter *et al.*, 1950), with glucose oxidase after hydrolysis (Johnson *et al.*, 1963), or with the method of Bueding and Hawkins. It can be observed in Table I that both anthrone and glucose oxidase resulted in very high values in the sodium carbonate and acetic acid fractions as compared with the enzymatic procedure. The latter method gave a value of zero with the perchloric acid fraction, probably because this consists mainly of glucose obtained by acid hydrolysis of polysaccharides. Nevertheless, 30% of the anthrone reacting material was not dialyzable. The same discrepancy between the methods was obtained after sonication and extraction with our procedure (see Table II). However, it should be noted that the results obtained with the enzymatic assay were very similar to those yielded by the iodine method (Krisman, 1962), which is also specific for glycogen. The results obtained here by the procedure of Trevelyan and Harrison were of the same order of magnitude as those in their original report (Trevelyan and Harrison, 1956).

Radioactive Glycogen Determination. The extraction proce-

[1] Cited in Sugar Analysis (1948), Browne, C. A., and Zerban, F. W., Ed., New York, N. Y., p 873.

[2] The turbidity appears to be due to a glucan fraction which is extracted by alkali after sonication and is insoluble at neutral pH.

TABLE II: Determination of "Glycogen" after Extraction According to the Present Method.[a]

Expt	Method of Determination: Anthrone[b] (mg/g dry wt)	Glucose Oxidase[c] (mg/g dry wt)	Iodine[d] (mg/g dry wt)	Phosphorylase Debranching[e] (mg/g dry wt)
1	38	20.5	5.46	5.1
2	40	19.5	5.0	4.8

[a] Lyophilized baker's yeast (same batch as in Table I) was used as assay material, sonicated, and extracted with alkali as described in text. [b] Trevelyan and Harrison (1952). [c] After hydrolysis in 1 N sulfuric acid (Johnson *et al.*, 1963). [d] Krisman (1962). [e] Bueding and Hawkins (1964).

TABLE III: Extraction of UDP-glucose by Different Procedures.[a]

Extraction Procedure	UDP-Glucose in Cell Water (mM)	Recov of Added Std (%)
Ethanol (66%)[b]	0.08	85
Perchloric acid (3 M)[c]	0.03	75
Methanol–chloroform[d]	0.14	90

[a] *S. cerevisiae* No. 197 was used as assay material. In all cases, UDP-glucose was measured enzymatically (Mills and Smith, 1962). [b] To 12 mg (dry wt) of yeast, 0.7 ml of water was added, and the tube was placed in a boiling-water bath for 5 min and cooled. Absolute ethanol (1.4 ml) was added, and the tube was shaken at room temperature for 15 min and centrifuged. The supernatant was evaporated to dryness under vacuum and redissolved in 1 ml of water. Aliquots were taken for the enzymatic determination. [c] As described in text for the determination of glucose-6-P and the adenine nucleotides. [d] As described under Methods.

dure and enzymatic treatment were as described in the preceding section. Subsequently, 1.5 volumes of ethanol was added, and the suspension was heated briefly to boiling, cooled, and centrifuged. An aliquot of the supernatant fluid was transferred to a scintillation vial and radioactivity was measured in a Packard scintillation counter, using the Triton-X-100 mixture recommended by Patterson and Greene (1965).

In order to determine the extent of labeling in the external branches, aliquots of radioactive glycogen were incubated under the conditions of Bueding and Hawkins (1964) but with phosphorylase *b* substituted for the phosphorylase-debranching system. Subsequent handling of the mixture and the measurement of radioactivity were carried out, as outlined in the preceding paragraph.

Determination of ATP, ADP, AMP, and Glucose-6-P. After filtration of the yeast suspension, the filter was placed in a 15-ml thick-walled Pyrex tube (International Equipment Co. No. 514), containing 2 ml of 3 M perchloric acid at −3°. The filter was removed after stirring on a Vortex mixer, the suspension was frozen in acetone–Dry Ice and thawed, and the freezing–thawing was repeated. The tubes were agitated on a rotary shaker for 5 min at 2 to 3° and centrifuged at 40,000*g* for 15 min. It is important to carry out the centrifugation at high speed and to obtain a clear supernatant fluid. When the supernatant remained turbid, it was found to be contaminated with nucleoside diphosphate kinase, which interferes with some of the assays. The supernatant was removed immediately, cooled to −2°, and neutralized with 10 N KOH. After 2 hr, the tubes were centrifuged as above and aliquots of the supernatant fluids were used for the determination of ATP, ADP, AMP, and glucose-6-P (Lowry *et al.*, 1964).

A second perchloric acid extraction of the yeast cells failed to yield measurable amounts of any of the metabolites tested. Addition of internal standards of the nucleotides and glucose 6-phosphate to the perchloric acid resulted in 90% recoveries.

UDP-glucose. The Millipore filter carrying the yeast sample was placed in a 15-ml thick-walled Pyrex centrifuge tube containing 0.5 ml of hot water and the tube was agitated briefly on a Vortex mixer. The filter was then removed and 1 ml of a 1:1 chloroform–methanol mixture was added. The tube was shaken mechanically for 15 min at room temperature and centrifuged at 12,000*g* for 10 min. After centrifugation, two phases were visible with the extracted yeast cells at the interphase. The presence of the interphase material did not allow removal of the upper layer without contamination from the lower one. Therefore both the upper and lower phases were separated from the cells by means of a Pasteur pipet and transferred to a 12-ml conical tube. After a brief centrifugation, the upper phase was transferred to a 13 × 100 mm test tube and evaporated to dryness under vacuum on a Rotary Evapo-Mix (Buchler Instruments). The residue was dissolved in 0.5 ml of water and an aliquot was used to measure UDP-glucose (Mills and Smith, 1962).

A second extraction of the cell residue with methanol–chloroform failed to yield any additional amount of UDP-glucose. Table III shows that of the three methods of extraction tested, the one outlined above gave the highest yield of both endogenous and added UDP-glucose. In addition, the results with perchloric acid were erratic, presumably because of partial hydrolysis of the labile glucose of the nucleotide. Lowering the concentration of perchloric acid to 0.6 M resulted in poorer yields.

Purification and Assay of Glycogen Synthetase. For enzymatic measurements, samples of the yeast suspension were centrifuged, the cells were washed twice with distilled water, and the residue was lyophilized. Purification was carried out according to Algranati and Cabib (1962), except that adsorption on retrograded amylose followed immediately after the dialysis of the crude extract, the ammonium sulfate step being omitted. Retrograded amylose was washed with 0.05 M glycylglycine at pH 7.5, containing 1 mM EDTA, and the same buffer was used to dissolve the glycogen employed for elution of the enzyme. On several occasions the activity was measured in the dialyzed crude extract (soluble enzyme) and in the residue from cell extraction (insoluble fraction), after resuspension

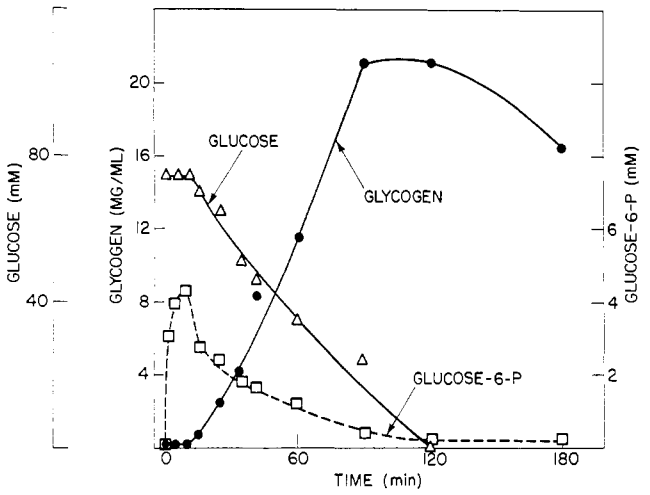

FIGURE 1: Changes in glycogen and glucose-6-P during incubation of yeast with glucose and salts. Conditions as explained under Methods. Glucose in the medium was not maintained constant.

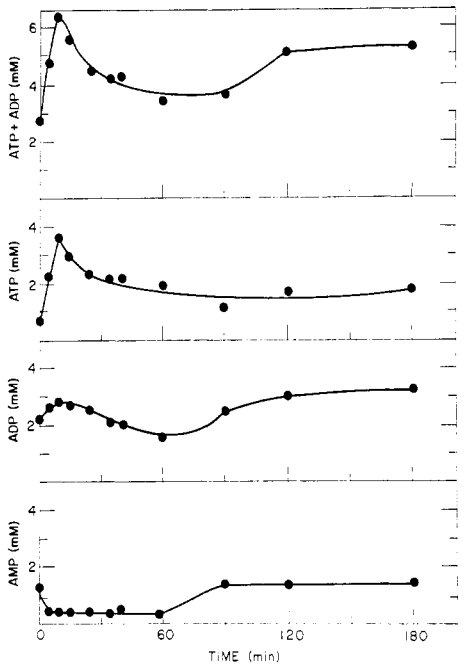

FIGURE 2: Changes in adenine nucleotides during incubation of yeast with glucose and salts. Same experiment as in Figure 1.

of the particulate material in 0.05 M glycylglycine at pH 7.5, containing 1 mM EDTA. As shown in Tables IV and VII, an important proportion of the activity remained in the insoluble material.

The activity of glycogen synthetase was measured as previously reported (Rothman and Cabib, 1967a,b).

Measurements of Oxygen Uptake. Respiration of intact cells was measured in 0.05 M phosphate buffer at pH 7 with a Clark oxygen electrode. Oxidation of DPNH was measured according to Bonner (1967) in the supernatant fluid of a yeast spheroplast lysate, obtained according to Behrens and Cabib (1968) and centrifuged at 1500*g*.

Results

Trevelyan and Harrison (1956) have shown that resting yeast cells accumulate glycogen when incubated in the presence of glucose and salts. Figures 1 and 2 show the results obtained in a simple experiment of this type in which the changes in several metabolites were followed well beyond the time at which the glucose was completely used. It can be observed that glycogen increased linearly after a short lag, and levelled off abruptly about 30 min before glucose disappeared completely from the medium. After a plateau, a decline in glycogen concentration ensued when glucose was exhausted. Glucose-6-P, which was present at very low levels in the yeast at the beginning of the experiment, showed a very sharp increase followed by a gradual decline which stabilized at a concentration of 0.2–0.3 mM. ATP and ADP, after an initial peak, did not undergo striking changes. AMP decreased initially and rose slightly, concomitant with the plateau in glycogen formation. It is rather surprising that the sum of the *three* nucleotides (not shown) more than doubled in the first 10 min. The source of the extra amount of adenosine compounds is unknown. The plot of the sum ATP + ADP *vs.* time indicates that the total inhibitory capacity toward glycogen synthetase did not vary greatly after the first 20 min. However, since glucose 6-phosphate declined steadily, it might be expected to reach a level where it would be unable to counteract nucleotide inhibition of the enzyme. This would happen at a concentration of about 0.4 mM, a point at which net glycogen synthesis had ceased.

Further information is provided by the experiment depicted in Figures 3 and 4. This experiment involved three stages, lasting, respectively, from 0 to 60 min, from 60 to 120 min, and from 120 to 300 min. During the first stage the concentration of glucose in the medium was maintained constant. At 60 min (second stage), the yeast suspension was split into two equal portions. To one of them sufficient 3.9 M ammonium sulfate was added to yield a final concentration of 50 mM. Intermittent additions of glucose to both incubation mixtures were continued to compensate for consumption of the sugar. At 120 min (third stage) the additions were discontinued, thus allowing the glucose concentration to fall.

As can be seen in Figure 3, the addition of ammonium ions brought the synthesis of glycogen to a sudden stop in agreement with the observations of Trevelyan and Harrison (1956). Simultaneously, the concentration of glucose 6-phosphate fell precipitously, in contrast to the slow decline in the absence of NH_4^+. It may be mentioned that the initial, unexplained oscillations in the glucose 6-phosphate level are reproducible in this type of experiment. When glucose was allowed to disappear, the results were similar to those outlined in Figure 1, *i.e.*, in the absence of NH_4^+ both glucose 6-phosphate and the rate of glycogen formation declined. When NH_4^+ was present, some disappearance of glycogen took place 30 min after glucose was exhausted. It is evident that the decrease in glucose 6-phosphate caused by ammonium sulfate cannot be attributed to a diminished uptake of glucose. On the contrary, the disappearance of glucose was actually stimulated by NH_4^+, as shown in Figure 3.

Changes in the adenine nucleotides (see Figure 4) are similar to those described in Figure 2, *i.e.*, a sudden increase of ATP and of the sum ATP + ADP, followed by a slow decline. It is noteworthy that the sum ATP + ADP did not decrease

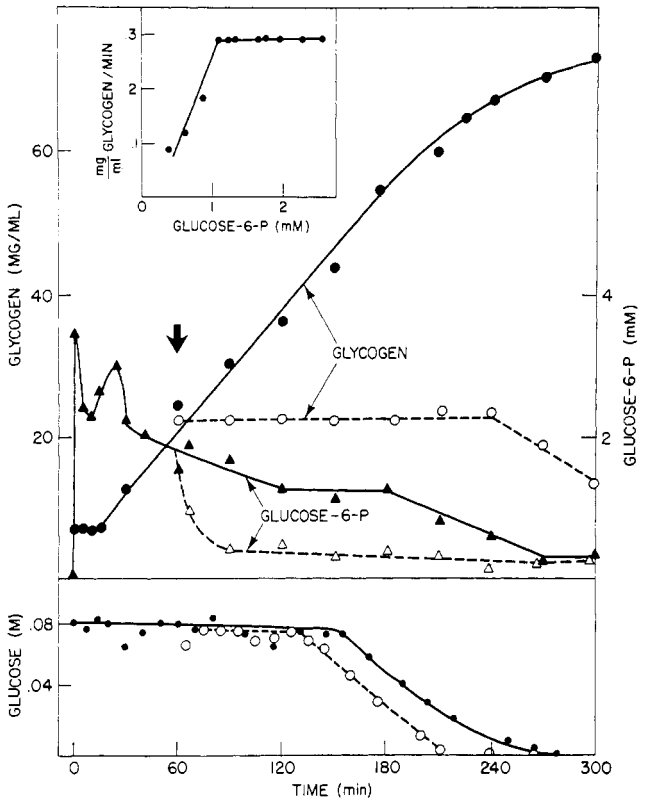

FIGURE 3: Effect of ammonium ions on glycogen and glucose-6-P concentration. Conditions as explained under Methods and in the text. The concentration of glucose in the medium was maintained constant until 120 min. Ammonium sulfate was added to one-half of the incubation mixture at 60 min (arrow). Full lines, without ammonium sulfate. Broken lines, with ammonium sulfate. Data for the insert were taken from the curves without ammonium sulfate. The bottom part of the figure shows the concentration of glucose in the medium.

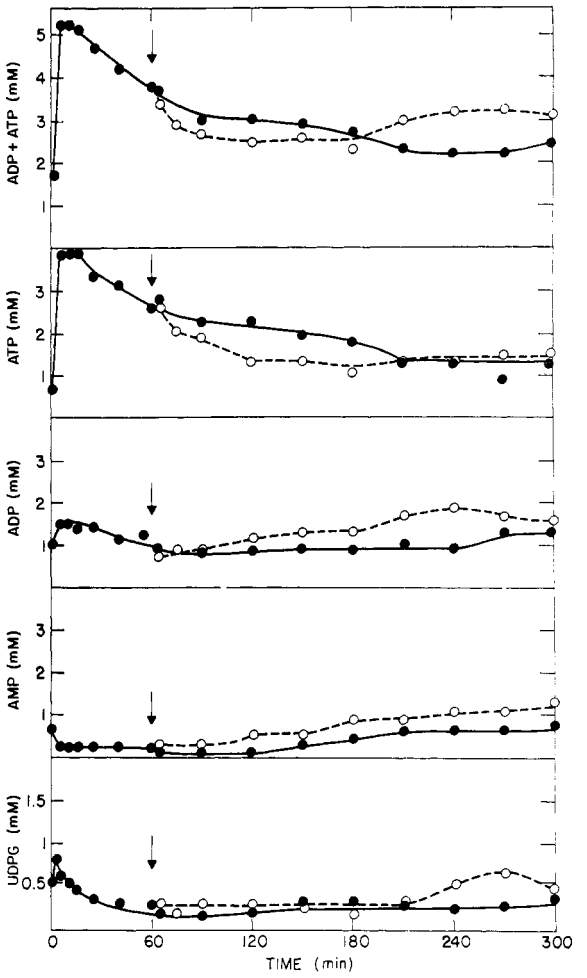

FIGURE 4: Effect of ammonium ions on the concentration of adenine nucleotides and UDP-glucose. Same experiment as in Figure 3. Ammonium ion was added to one-half of the incubation mixture at 60 min (arrow). Full lines, without ammonium sulfate. Broken lines, with ammonium sulfate.

significantly in the presence of NH_4^+. On the other hand, the ratio ATP/AMP showed a rapid decline upon addition of ammonium ion (from about 12, before the addition, to approximately 7, 30 min later).

It is remarkable that the concentration of UDP-glucose remained practically constant after the first 30 min, irrespective of the presence or absence of NH_4^+. Indeed, toward the end of the experiment an increase in UDP-glucose was observed in the incubation mixture containing NH_4^+. In other experiments in which glycogen synthesis had stopped, an increase in UDP-glucose was also noted in the yeast incubated without ammonium salts.

The constancy in glycogen content following the addition of ammonium ions may be explained in two ways: (a) the formation of glycogen is inhibited; (b) glycogen synthesis is proceeding at the same speed as before, but degradation is stimulated to an extent matching the rate of formation so as to result in a constant *steady-state* concentration. The latter possibility is made rather remote by the fact that in many experiments addition of ammonium ions always gave rise to a constant glycogen content. It seems unlikely that in all cases the rate of degradation would be exactly equal to that of synthesis. Nonetheless, an experiment was designed to test hypothesis b. Glycogen was labeled by including tritiated glucose in the medium and, after transferring the yeast to nonradioactive medium, the decrease of radioactivity in glycogen was followed in the presence and in the absence of ammonium ions. The experiment was carried out as follows. A suspension of yeast in a glucose and salt medium, made up as described under Methods in a total volume of 400 ml, was incubated at 30° with shaking. After 30 min, a 100-ml aliquot was removed and 60 μl (0.15 μCi) of uniformly labeled [^{3}H]glucose (3.4 mCi/μmole) was added. Incubation was continued at 30° for another 30 min. At that time the nonradioactive portion was divided into three equal parts and all aliquots, including the radioactive one, were centrifuged at room temperature for 1 min at 1500*g*. The supernatant fluids from two of the samples with unlabeled glucose were used to wash and to resuspend the radioactive yeast.[3] After centrifugation, the yeast from the third nonradioactive aliquot was resuspended in its own supernatant. Both this suspension and that of the washed labeled yeast were divided into two

[3] This procedure was necessary since a loss in glycogen content was observed when yeast was resuspended in fresh medium. For as yet unknown reasons, "used" medium did not produce this effect.

TABLE IV: Glycogen Synthetase Activity before and after Incubating Yeast Cells in Presence and Absence of Ammonium Ions.[a]

	Time at Which Yeast Samples Were Taken							
	0		45 min		110 min		110 min ($+NH_4^+$)	
	Total Act.[b]	Activ by G-6-P[c]	Total Act.[b]	Activ by G-6-P[c]	Total Act.[b]	Activ by G-6-P[c]	Total Act.[b]	Activ by G-6-P[c]
Crude dialysate[d]	100		48		36.4		22	
Insoluble fraction	80	1.05	44	2.75	55	2.3	40	1.8
Purified enzyme[e]	18	1.21	7.3	2.3	10	2.4	11.8	2.3

[a] Incubation of yeast cells in presence or absence of ammonium ions was carried out as in the experiment of Figure 3. Extraction, purification, and assay of the enzyme as outlined under Methods. The incubation mixture contained 5 mM UDP-glucose, 50 mM glycylglycine (pH 7.5), 1 mM EDTA, 3 mg of shellfish glycogen, and enzyme in a total volume of 50 μl. [b] Measured in the absence of glucose-6-P. The activity of the crude dialysate from the yeast extracted at zero time is taken as 100. [c] Activity with 10 mM glucose-6-P/activity without glucose-6-P. [d] The effect of glucose-6-P cannot be measured on this fraction, because of the interference by trehalose phosphate synthetase (Cabib and Leloir, 1958). [e] Combined eluates from retrograded amylose.

equal portions, to one of which 3.9 M ammonium sulfate was added to a final concentration of 50 mM. The four flasks were incubated at 30° for 2 hr.

During the entire course of the experiment, the glucose concentration was maintained constant, as explained in Methods. During the 30-min labeling period, tritiated rather than nonradioactive glucose was added, in order to maintain constant the specific activity of the sugar in the medium. Samples were taken periodically from the unlabeled suspensions in order to measure total glycogen, and from those containing prelabeled yeast, for determinations of glycogen radioactivity. The first sample in the latter series was taken immediately after resuspending the labeled yeast in the wash solution.

The results are shown in Figure 5. The lower part of the figure indicates that the formation of glycogen and its inhibition by ammonium ion occurred as expected. In the upper part of the figure it can be seen that the radioactivity in glycogen decreased only slightly during the experiment and, more important, that the decrease was about the same in the absence as in the presence of ammonium ions. If, in the latter case, the degradation of glycogen had been stimulated, one would expect a much greater turnover with consequent loss of radioactivity. The dotted line represents the theoretical curve, according to hypothesis b.

The percentage of radioactivity in the outer branches of glycogen was measured in the 80- and 140-min samples, from the flask where ammonium salts had not been added. These samples were incubated exhaustively with phosphorylase, and the amount of radioactivity which became soluble in 60% ethanol was determined (see Methods). Approximately the same result was obtained in both time intervals: 48% at 80 min and 45% at 140 min. The same values were obtained in a different experiment, in which the radioactive pulse lasted only 10 min.

Another possibility which had to be considered was that ammonium ions could cause a sudden change in the amount or in the regulatory properties of glycogen synthetase. The results of Table IV show that this is not the case. There was some decrease in the activity in the yeast incubated with ammonium sulfate, but the change is relatively small when the sums of the activities in the supernatant fluid and in the insoluble residue are compared. It is noteworthy that the effect of glucose 6-phosphate was greater on the two enzymes obtained from yeast incubated with glucose than on the glycogen synthetase from the cells collected directly from the growth medium. It is thus possible that the total activity, as mea-

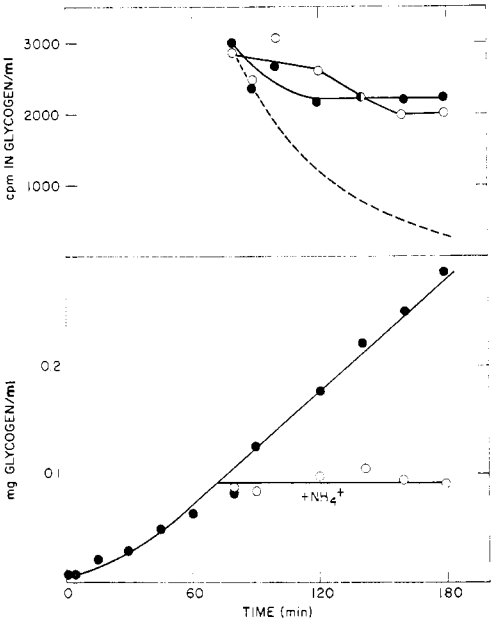

FIGURE 5: Pulse labeling of glycogen followed by incubation in nonradioactive glucose. Experimental details described in the text. The period during which the yeast was centrifuged and washed was counted as incubation time in the horizontal axis. The concentration of glycogen is given in milligrams per milliliter of yeast suspension. Full symbols, mixtures without ammonium sulfate; empty symbols, mixtures with ammonium sulfate. The dotted line shows the theoretical loss of radioactivity, if the rate of synthesis in the presence of ammonium ions were the same as in their absence, and degradation were taking place at an equal speed. The equation used is formally the same as for a convex chromatographic gradient (Bock and Ling, 1954).

TABLE V: Effect of ATP and Glucose-6-P on Glycogen Synthetase from Yeast Incubated in Presence or Absence of Ammonium Ions.[a]

Time at Which Yeast Sample Was Taken (min)	Insoluble Fraction			Purified Enzyme[b]		
		Inhibition by 5 mM ATP			Inhibition by 5 mM ATP	
	Activn by G-6-P[c]	−G-6-P (%)	+10 mM G-6-P (%)	Activn by G-6-P[c]	−G-6-P (%)	+10 mM G-6-P (%)
0	1.3	79	10	1.43	96	11.5
45	1.22	81	30	1.8	88	10.5
110	1.0	77	35	2.3	90	22
110 (+NH_4^+)	1.3	78	30	2.5	82	21

[a] Same enzymatic fractions as in Table IV. The conditions were identical, except that the assay mixture contained 0.4 mM UDP-glucose, 50 mM succinate–cacodylate buffer (pH 6), 1 mM EDTA, 3 mg of shellfish glycogen, and enzyme, in a total volume of 50 μl. [b] First retrograded amylose eluate. [c] Activity with 10 mM glucose-6-P/activity without glucose-6-P.

sured in the presence of glucose-6-P, did not vary greatly during incubation. All enzymes showed about the same inhibition with ATP and reversal with glucose-6-P (see Table V). The enzyme from the yeast strain used in this work differed from the baker's yeast enzyme which was previously used, in the range of glucose-6-P concentrations necessary for reversal of ATP inhibition. This range, which was between 0 and 0.15 mM for the baker's yeast enzyme at 0.4 mM UDP-glucose, was found to be from 0 to 0.5 mM for the synthetase of strain 197, at the same concentration of substrate. The sigmoid shape of the curve was unchanged.

In view of the relatively high concentration of glucose used in the growth medium, it was assumed that yeast obtained under these conditions would draw its energy needs mainly or exclusively from fermentation (Polakis *et al.*, 1964). It was considered of interest to investigate whether yeast cells capable of respiration would show a different pattern of glycogen deposition. Accordingly, *S. cerevisiae* was grown with galactose as the carbon source (Polakis *et al.*, 1965). The data in Table VI indicate that the yeast grown on medium I was indeed almost incapable of respiration, while that grown on galactose showed a rapid endogenous oxygen uptake which was further accelerated by the addition of glucose. The results were confirmed by measuring the oxidation of DPNH by a protoplast lysate (Table VI) and its inhibition by cyanide.

TABLE VI: Respiratory Capacity of Yeast Cells Grown in Different Media.

	Oxygen Uptake of Intact Cells[a,b]		DPNH Oxidn by Lysate Supernatants[a,c]	
	−Glucose	+12 mM Glucose	−KCN	+1 mM KCN
Yeast grown in medium I	15	15	0.16	0.055
Yeast grown in medium II	100	285	2	0.066

[a] Measured as outlined under Methods. [b] In microatoms of O_2 per minute per gram dry weight. [c] In micromoles of DPNH per minute per gram dry weight.

When the galactose-grown cells were incubated with glucose and salts (Figure 6) a different picture from that of Figure 3 was obtained. Glycogen increased immediately but the rate of synthesis began to decline before 30 min; the final level obtained was less than half that reached with fermenting cells. It can also be observed in Figure 6 that the change in level of glucose 6-phosphate bore an inverse relationship to the glycogen increase. This fall in glucose 6-phosphate was unexpected, since the concentration of external glucose was maintained constant. It is suggested that this effect is the result of a decreased penetration of the sugar into the cells. An approximate calculation of glucose consumption could be made from the amounts used to maintain the sugar concentration constant in the medium. In fact, it was found that the uptake of glucose was 0.37 mmole/min g yeast (dry wt) during the first 10 min and 0.06 mmole/min g yeast (dry wt) between 30 and 45 min.

In the experiments with fermenting yeast, the rate of glucose consumption did not decrease with time. A typical value was 0.17 mmole/min g yeast (dry wt) at 7 min after the addition of glucose, while the values for 30 and 70 min were 0.25 and 0.18, respectively.

During the experiment with galactose-grown cells the concentration of ATP plus ADP was remarkably constant after the usual initial peak and the same may be said for UDP-glucose (Figure 7).

In this case, too, enzyme activity or the susceptibility of the enzyme to effectors was not greatly different, whether the yeast was sampled during the ascent or at the plateau of the glycogen curve. On the other hand, the direct activation of the enzyme by glucose-6-P was very high in some cases.

In other experiments, continued beyond the 2-hr limit, an unexpected phenomenon was observed wherein a second burst of glycogen accumulation took place and was followed

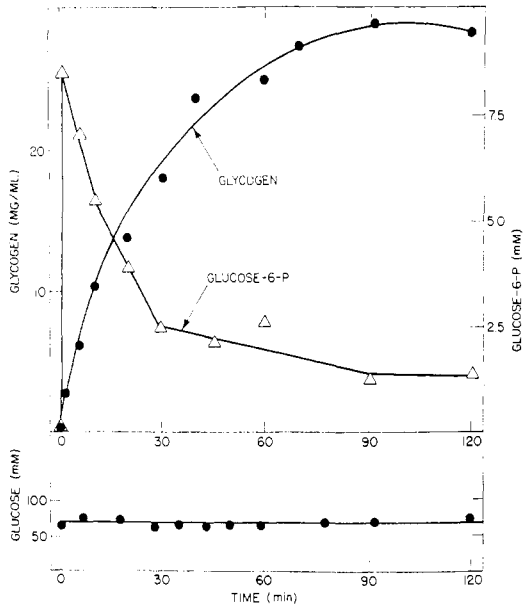

FIGURE 6: Changes in glycogen and glucose-6-P during incubation of galactose-grown yeast with glucose and salts. Conditions as described under Methods. The bottom part of the figure shows the concentration of glucose in the medium.

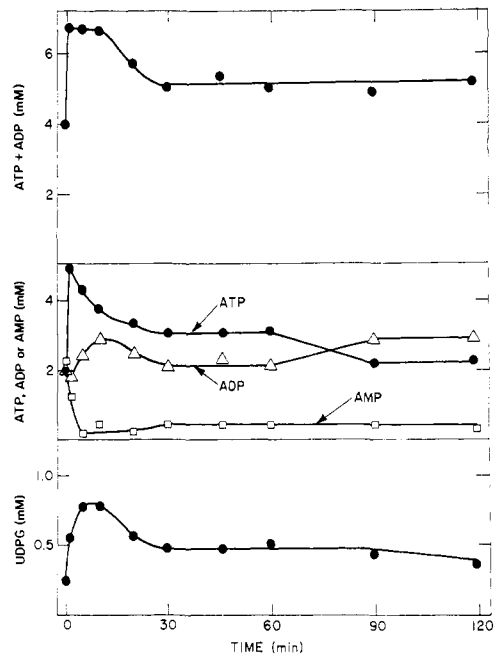

FIGURE 7: Changes in adenine nucleotides and UDP-glucose during incubation of galactose-grown yeast with glucose and salts. Same experiment as in Figure 6. Conditions as explained under Methods.

by a decrease. While the effect was qualitatively reproducible, the extent of the plateau and the amount of glycogen formed in the second increase varied in separate experiments. The maximal concentration of polysaccharide obtained after the last rise was usually about 110 mg/g of yeast (dry wt) or 64 mg/ml. This new increase in glycogen content could not be correlated with any change in metabolites or in glycogen synthetase activity. If there were a degree of turnover of glycogen in the previous stages, the second rise may be a manifestation of decreased degradation rather than of augmented synthesis.

Discussion

The type of results obtained in this study can only correlate different parameters, *e.g.*, the concentration of various metabolites; it cannot furnish proof that they are casually related. Furthermore, allowance could not be made in these experiments for the existence of different pools of the same substance at various locations in the cell. Yet, it is known that the yeast cell does contain several such compartments, *e.g.*, cytoplasm, nucleus, a large vacuole, mitochondria, and there is little doubt that the composition of the intracellular fluid is not uniform throughout. For this reason, it seems advisable to assign more importance to *changes* in metabolite concentrations than to their absolute values. Another reason for viewing these values with caution is the difficulty in determining individual substances in a mixture as complex as an unfractionated cell extract. Despite the care exercised in choosing specific enzymatic methods of analysis, it cannot be excluded, for instance, that the amounts obtained for some of the adenine nucleotides do not include other nucleotides. The risk of errors in these determinations is also illustrated by the fact that, in the measurement of glycogen, the use of widely employed methods yielded results more than 10-fold greater than those obtained with the specific procedure adopted for this work.

With these considerations in mind, the results may be evaluated. The experiments illustrated in Figures 1–4 confirm the report of Trevelyan and Harrison (1956) on the accumulation of glycogen in yeast cells incubated in the presence of glucose and salts. Glycogen accumulation can reach very high values. Thus, in an experiment in which the glucose in the medium was maintained constant for 7 hr, a linear increase in glycogen was observed for the entire period and a concentration of 182 mg/g dry wt was attained.

The total concentration of adenine nucleotides showed an initial rapid rise which remains unexplained; this was followed by a decline. There was no sign of an initial decrease of ATP as one would expect if some of the nucleotide present in the cell were used at early times for the phosphorylation of glucose. The sum ATP + ADP remained relatively constant during most of the experiments despite large changes in the rate of glycogen formation. It may be said, then, that the inhibitory capacity toward glycogen synthetase, as represented by the two nucleotides (Rothman and Cabib, 1967b), does not vary greatly with time. On the other hand, glucose-6-P displayed an initial abrupt peak (two under the conditions of Figure 3), which was followed by a fall. When external glucose was maintained constant, the level of glucose-6-P became stabilized; when glucose was allowed to fall, glucose-6-P also decreased. The rate of synthesis of glycogen varied, within limits, in accord with the observed changes in glucose-6-P, as can be seen by inspecting Figures 1 and 3, and the insert of the latter figure. These results may be interpreted in terms of a reversal of the ATP and ADP inhibition of glycogen synthetase, as a function of the concentration of the sugar ester.

Further support for this interpretation comes from the

striking effect of ammonium ions on the formation of glycogen. Upon addition of ammonium salts, the increase in polysaccharide content stopped abruptly, while a concomitant rapid decrease in glucose-6-P took place. Ammonium ions probably bring about this decrease by activating phosphofructokinase (Sols and Salas, 1966). The decrease in ATP concentration observed at the same time supports this hypothesis, although ATP would be expected to rise again, once the accelerated glycolysis results in an increased production of the nucleotide. That this did not occur suggests that ATP-consuming processes, such as the synthesis of glutamate (Holzer and Witt, 1958), are put in motion by the presence of ammonium salts. The consequent increase in the AMP/ATP ratio (see Results) would also contribute in the maintenance of a high phosphofructokinase activity.

Other interpretations of the effect of ammonium ions have been considered. The possibility of an increased degradation of glycogen is excluded by the finding that the turnover of prelabeled polysaccharide is the same whether or not ammonium salts are present. An interesting corollary of this experiment is that the percentage of radioactivity in the exterior chains of glycogen was found to be the same whether the cells were sampled immediately after labeling, or after further incubation in nonradioactive glucose for 1 hr. If, in this second period, the unlabeled glucose residues had coated the preexisting molecules of glycogen, the radioactive components should have been "buried" in the core of the structure by the action of the branching system. As a consequence, the percentage of radioactivity in external chains should have decreased. The fact that this was not the case may be interpreted to mean that practically all of the new glycogen was synthesized *de novo*. Taken together with the finding that a large portion of glycogen synthetase was found to be associated with particulate material,[4] this suggests the speculation that each individual molecule of glycogen is completed, possibly in one of the cell organelles, and released prior to initiation of synthesis of a new molecule (*cf.* Gahan and Conrad, 1968).

The results of Tables IV and V render very unlikely another possible explanation of the ammonium effect, namely, that sudden changes occur in the activity of the enzyme or in its sensitivity to effectors. An unexplained feature of these experiments is the increased sensitivity to glucose-6-P of the enzyme extracted from yeast after incubation with glucose, irrespective of the presence of ammonium ions. This result might be ascribed to a transformation of the enzyme to a different form, perhaps similar to the I to D transformation in muscle. The new form, however, would be less efficient, because of its increased dependence on glucose-6-P, than the previous one, despite being produced at a time of rapid glycogen synthesis. Another possibility is that the changed intracellular conditions at the time of extraction lead to the change in properties of the enzyme. Since the enzyme binds strongly to glycogen (Rothman and Cabib, 1967b) and the cells do contain glycogen after incubation, this may represent the explanation for the observed effects. Changes in the conditions of extraction have indeed been found to alter glycogen synthetase. For example, disruption of cells by sonic oscillation results in low yields of an enzyme preparation which is totally dependent upon glucose-6-P as well as being rather unstable.[5]

The relative constancy of the UDP-glucose concentration, despite large variations in the rate of glycogen synthesis, is striking. This finding suggests that the formation of the polysaccharide is not regulated by the concentration of substrate, and adds further support to the hypothesis that other effectors determine the rate. Indeed, in many cases, UDP-glucose tended to increase when glycogen synthesis stopped, as would be expected of an intermediate in a sequence of reactions, in which the next step was blocked. The situation in yeast would thus be different from that found in several bacteria. In the latter group of organisms the regulation of glycogen synthesis appears to be exerted at the level of the synthesis of the sugar nucleotide (Preiss *et al.*, 1966).

In the experiments performed with respiring cells the rate of glycogen synthesis decreased with time in spite of a constant concentration of external glucose. Concomitantly, the level of glucose-6-P fell continuously. The inverse relationship between the changes in glycogen and in the phosphoric ester are in agreement with our hypothesis since the sum of ATP plus ADP remained practically constant after an early peak. The level of glucose-6-P at which glycogen synthesis stopped was higher than that observed in the experiment of Figure 3. In that case, however, the combined concentration of ATP + ADP was smaller. The explanation for the decrease in glucose-6-P concentration is not clear, although it seems to be related to a fall in glucose uptake by the cells. In this respect it has been reported that glucose uptake by yeast is inhibited when the organism is shifted from fermentation to respiration (*cf.* Sols, 1968).

No changes were detectable in glycogen synthetase, whether extracted during the period of rapid glycogen synthesis or at the plateau phase.

Up to this point, only the changes in metabolite concentration, rather than their absolute levels, have been taken into account. For the reasons presented at the beginning of this discussion, it is felt that the absolute values should be assigned only limited importance. However, they will be briefly considered, for the purpose of comparison with the previous *in vitro* results. The sum of the ATP and ADP concentrations varied, in the different experiments between 3 and 6 mM, a range in which strong inhibition of glycogen synthetase would be expected in the absence of glucose 6-phosphate. The observed UDP-glucose level was between 0.2 and 0.6 mM, while the concentration used in the *in vitro* experiments was 0.4 mM (Rothman and Cabib, 1967b). On account of the observed sensitivity of the enzyme obtained from strain 197 to glucose 6-phosphate in the presence of ATP (see Results), one should expect effects on the rate of glycogen synthesis at concentration of the sugar phosphate up to 0.5 mM. The insert of Figure 3 shows a dependence on glucose 6-phosphate up to 1 mM, thus approximately in the expected range. Because of the sigmoid shape of the activity *vs.* glucose 6-phosphate curve (Rothman and Cabib, 1967b) very low or very high values of the sugar ester should fail to influence further the rate of polysaccharide synthesis. This would explain why, in the experiments of Figures 1 and 3, the rate of glycogen accumulation began to decrease only after glucose 6-phosphate

[4] See Table IV. A determination of the distribution of enzyme in a spheroplast lysate gave 35% of the total in the 20,000*g* pellet.

[5] L. B. Rothman, unpublished observations.

had fallen below a certain level. On the other hand, the concentration of the phosphoric ester in respiring cells (Figure 6) at the time when glycogen formation had ceased appears to be too high, even taking into account the higher nucleotide levels in this experiment. Some explanations might be offered for this anomaly, such as the presence of two or more pools of glucose 6-phosphate in these cells or a constant degradation of glycogen which would mask a continuing synthesis, but support for any of these hypotheses is lacking. Clearly, more work would be needed to elucidate this point.

The total yield of enzymatic activity recovered *in vitro* from nonrespiring cells and measured under optimal conditions of pH and glucose 6-phosphate and substrate concentration (Rothman and Cabib, 1967a,b) was enough to account for the synthesis of about 0.5 mg of glycogen per g (dry wt) of yeast per min, approximately the same value measured as maximal rate in the experiment of Figure 3. Nevertheless, when the assay was carried out at pH 6 and 0.4 mM UDP-glucose, conditions which presumably are nearer those prevailing *in vivo*, the activity was only about 10–20% of the above value. Aside from possible losses in the extraction procedure, this low value would reflect a lower efficiency of the synthetase in the *in vitro* environment. This result would be expected if, as above suggested, the formation of glycogen occurs in an organelle in which the operations of the synthetase and of the branching enzyme are somehow coordinated.

Within the limitations mentioned at the beginning of this discussion, it may be concluded that our observations are in agreement with the hypothesis that yeast glycogen synthetase is regulated *in vivo* through an antagonism between ATP and ADP (and possibly other substances) as inhibitors, and glucose-6-P as activator.[6] As previously discussed (Piras *et al.*, 1968) this scheme would fit into the general assumption that the biosynthesis of storage materials is regulated primarily by precursor activation rather than by end-product inhibition.

Acknowledgments

The authors are indebted to Dr. R. Hendler for the use of the oxygen electrode. We also thank Dr. G. Ashwell, Dr. W. B. Jakoby, Dr. F. A. Keller, and Dr. M. F. Singer for useful discussions and criticism. It is a pleasure to acknowledge the very valuable help of Mr. Kenneth Frank in several experiments.

[6] Piras and Staneloni (1969), working with rat muscle *in vivo*, have reached conclusions in agreement with ours.

References

Algranati, I. D., and Cabib, E. (1962), *J. Biol. Chem. 237*, 1007.
Behrens, N. H., and Cabib, E. (1968), *J. Biol. Chem. 243*, 502.
Bock, R. M., and Ling, N.-S. (1954), *Anal. Chem. 26*, 1543.
Bonner, W., Jr. (1967), *Methods Enzymol. 10*, 132.
Brown, D., and Illingworth Brown, B. (1967), *Methods Enzymol.* **8**, 515.
Bueding, E., and Hawkins, J. (1964), *Anal. Biochem. 7*, 26.
Cabib, E., and Leloir, L. F. (1958), *J. Biol. Chem. 231*, 259.
Gahan, L. C., and Conrad, H. E. (1968), *Biochemistry 7*, 3979.
Grylls, F. S. M. (1961), *in* Biochemist's Handbook, Long, C., Ed., London, E. & F.-N. Spon, Ltd., p 1050.
Holzer, H., and Witt, I. (1958), *Angew. Chem. 70*, 439.
Ionescu, A., and Vargolici, V. (1920), *Bull. Soc. Chim. Romania 2*, 38.
Johnson, J., Nash, J., and Fusaro, R. (1963), *Anal. Biochem. 5*, 379.
Krisman, C. R. (1962), *Anal. Biochem. 4*, 17.
Lowry, O. H., Passonneau, J. V., Hasselberger, F. X., and Schule, D. (1964), *J. Biol. Chem. 239*, 18.
Mills, G. T., and Smith, E. E. B. (1962), *in* Methoden der Enzymatischen Analyse, Bergmeyer, H. U., Ed., Verlag Chemie, Weinheim/Bergstr., Germany, p 581.
Patterson, M. S., and Greene, R. C. (1965), *Anal. Chem. 37*, 854.
Piras, R., Rothman, L. B., and Cabib, E. (1968), *Biochemistry 7*, *56*.
Piras, R., and Staneloni, R. (1969), *Biochemistry 8*, 2153.
Polakis, E. S., Bartley, W., and Meek, G. A. (1964), *Biochem. J. 90*, 369.
Polakis, E. S., Bartley, W., and Meek, G. A. (1965), *Biochem. J. 97*, 298.
Preiss, J., Shen, L., Greenberg, E., and Gentner, N. (1966), *Biochemistry 5*, 1833.
Rothman, L. B., and Cabib, E. (1967a), *Biochemistry 6*, 2098.
Rothman, L. B., and Cabib, E. (1967b), *Biochemistry 6*, 2107.
Sols, A. (1968), *in* Aspects of Yeast Metabolism, Mills, A. K., Ed., Philadelphia, Pa., F. A. Davis, p 47.
Sols, A., and Salas, M. L. (1966), *Methods Enzymol. 9*, 436.
Sufter, S., Dayton, S., Novic, B., and Muntweyer, E. (1950), *Anal. Biochem. 25*, 191.
Trevelyan, W. E. (1958), *in* The Chemistry and Biology of Yeasts, Cook, A. H., Ed., New York, N. Y., Academic, p 369.
Trevelyan, W. E., and Harrison, J. S. (1952), *Biochem. J. 50*, 298.
Trevelyan, W. E., and Harrison, J. S. (1956), *Biochem. J. 63*, 23.