Biochem. J. (1979) **180**, 389–402
Printed in Great Britain

Glycogen Synthesis by Rat Hepatocytes

By JOSEPH KATZ, SYBIL GOLDEN and PETRONELLA A. WALS
Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Los Angeles, CA 90048, U.S.A.

(*Received* 11 *September* 1978)

1. Hepatocytes from starved rats or fed rats whose glycogen content was previously depleted by phlorrhizin or by glucagon injections, form glycogen at rapid rates when incubated with 10mM-glucose, gluconeogenic precursors (lactate, glycerol, fructose etc.) and glutamine. There is a net synthesis of glucose and glycogen. ^{14}C from all three types of substrate is incorporated into glycogen, but the incorporation from glucose represents exchange of carbon atoms, rather than net incorporation. ^{14}C incorporation does not serve to measure net glycogen synthesis from any one substrate. 2. With glucose as sole substrate net glucose uptake and glycogen deposition commences at concentrations of about 12–15mM. Glycogen synthesis increases with glucose concentrations attaining maximal values at 50–60mM, when it is similar to that obtained in the presence of 10mM-glucose and lactate plus glutamine. 3. The activities of the active (*a*) and total (*a*+*b*) forms of glycogen synthase and phosphorylase were monitored concomitant with glycogen synthesis. Total synthase was not constant during a 1h incubation period. Total and active synthase activity increased in parallel with glycogen synthesis. 4. Glycogen phosphorylase was assayed in two directions, by conversion of glucose 1-phosphate into glycogen and by the phosphorylation of glycogen. Total phosphorylase was assayed in the presence of AMP or after conversion into the phosphorylated form by phosphorylase kinase. Results obtained by the various methods were compared. Although the rates measured by the procedures differ, the pattern of change during incubation was much the same. Total phosphorylase was not constant. 5. The amounts of active and total phosphorylase were highest in the washed cell pellet. Incubation in an oxygenated medium, with or without substrates, caused a prompt and pronounced decline in the assayed amounts of active and total enzyme. There was no correlation between phosphorylase activity and glycogen synthesis from gluconeogenic substrates. With fructose, active and total phosphorylase activities increased during glycogen synthesis. 6. In glycogen synthesis from glucose as sole substrate there was a decline in phosphorylase activities with increased glucose concentration and increased rates of glycogen deposition. The decrease was marked in cells from fed rats. 7. To determine whether phosphorolysis and glycogen synthesis occur concurrently, glycogen was prelabelled with [2-^{3}H,1-^{14}C]-galactose. During subsequent glycogen deposition there was no loss of activity from glycogen in spite of high amounts of assayable active phosphorylase.

We have previously reported (Katz *et al.*, 1976) that hepatocytes form glycogen at rapid rates when incubated with glucose, gluconeogenic precursors and glutamine or several other amino acids. We have now measured the activities of glycogen synthase and phosphorylase in parallel with glycogen synthesis from gluconeogenic substrates or from glucose. In the present paper we describe the enzyme pattern occurring during glycogen synthesis in cells from starved and fed rats. Our results are at variance with generally held concepts of the activation of these enzymes.

Methods

Preparation and incubation

To deplete liver glycogen of fed animals, the rats were injected intraperitoneally with 1ml of carbonate buffer (containing 50mg of phlorrhizin/ml)/100g body wt. To prepare this buffered solution, the phlorrhizin was suspended in 0.1M-bicarbonate and 1M-NaOH was added until the pH of the solution was 8.5–9. The rats had access to water and urinated copiously with marked glycosuria. At 2h after phlorrhizin injection they were injected subcu-

taneously with 5μg of glucagon/100g body wt., and anaesthetized 1h later. Starved rats were deprived of food for 20–24h.

Hepatocyte preparation was as described by Katz *et al.* (1978). The cells, usually 0.12–0.2ml of packed cells (20–30mg of protein), were incubated in a final volume of 3ml (without albumin or gelatin) in 25ml siliconized-glass Erlenmeyer flasks (incubation in cylindrical vials or centrifuge tubes is unsatisfactory; no glycogen synthesis occurs, presumably due to inadequate oxygenation) gassed with O_2/CO_2 (19:1, v/v; Katz *et al.*, 1975). The incubation was terminated with $HClO_4$. The flasks were kept at 37°C for 1h to complete extraction of glycogen by 5% (v/v) $HClO_4$. For enzyme assay, at intervals from 2 to 60min, the flasks were chilled in an ethanol/ice bath, 1ml was withdrawn and delivered into $HClO_4$ and the rest was decanted into tubes kept on ice and rapidly centrifuged in the cold. The pellet was frozen in a solid-CO_2 bath and stored at −50°C.

Analysis

Glycogen in the $HClO_4$ extract was determined by a modification (Katz *et al.*, 1976) of the method of Lust *et al.* (1975). Substrates were analysed as described in the manual of Bergmeyer (1974).

Isotope methods

[2-^{3}H]Galactose was synthesized as described by Wals *et al.* (1976). Methods for the separation of radioactively labelled compounds and their assay were as described by Katz *et al.* (1976, 1978). When [U-^{14}C]glycerol was the substrate it was necessary to separate [^{14}C]glucose and substrate glycerol. The neutralized $HClO_4$ extract was passed through three columns in tandem, with, beginning at the top, Dowex 50 (H^+ form), Dowex-1 (acetate form), and finally Dowex-1 (borate form) as materials respectively in these columns (Katz *et al.*, 1976, 1978). ^3H_2O is recovered in the eluate. Amino acids are retained by the top column, acids by the middle column, and glucose and glycerol by the bottom column. Glycerol is eluted with 0.02M-$(NH_4)_2B_4O_7$ or 0.02M-$K_2B_4O_7$. Glucose was eluted with 0.2M-tetraborate or with 0.5M-acetic acid.

Phosphorylation of glucose was obtained from the yield of ^{3}HHO from [2-^{3}H]glucose, multiplied by a factor of 1.4 to correct for incomplete detritiation (Katz *et al.*, 1978).

Extraction of enzymes

The medium was 50mM-Tris/HCl, pH7.4, containing 10mM-EDTA, 50mM-NaF, 5mM-dithiothreitol, and 0.5% glycogen (oyster glycogen purified by passage through a mixed ion-exchange bed). The frozen pellet was suspended in 10vol. of cold medium and cells were disrupted with a Tekmar (Cincinnati, OH, U.S.A.), or with a Brinkman Polytron homogenizer. The extract was used for the assay of synthase and phosphorylase, performed within the same day.

Assay of synthase

This was by the method of Golden *et al.* (1977). Briefly, 25μl of the above (uncentrifuged) extract was added to 50μl of a solution of 50mM-Tris/HCl, pH7.8, containing 5mM-EDTA, 1% glycogen, 1.5mM-UDP-[^{14}C]glucose (0.12μCi/ml). The solution used for active synthase assay was also 15mM in Na_2SO_4, and that for total ($a+b$) synthase activity contained no sulphate but 3mM-glucose 6-phosphate. The incubation (at 37°C) and separation of the labelled glycogen by anion-exchange resin was as described by Golden *et al.* (1977). To increase radioactive isotope incorporation and sensitivity the concentration of UDP-glucose in our assay was chosen as 1mM (Golden *et al.*, 1977) compared with 5–10mM in most conventional assays. The enzyme activity determined in our assay is about half that obtained at approximately saturating concentrations (10mM) of UDP-glucose. However, the patterns for both active and total synthase measured at the two concentrations are much the same.

Assay of phosphorylase

This was assayed by two methods, in the direction of glycogen synthesis from glucose 1-phosphate and in the direction of glycogen phosphorolysis.

Synthesis of glycogen

This was essentially as described by Golden *et al.* (1977). A portion (25μl) of the cell extract was added to 50μl of a solution containing 200mM-NaF, 15mM-glucose 1-phosphate, pH6.2, and 1.5% glycogen, and 0.12μCi of [U-^{14}C]glucose 1-phosphate/ml. The final pH was about 6.5. The solution used for phosphorylase *a* was also 0.75mM in caffeine, and that for total ($a+b$) phosphorylase contained no caffeine but 7.5mM-AMP. Uncentrifuged homogenate was used, but it was found that prior centrifugation had no effect on the assay. The incubation (at 37°C) and separation of radioactively labelled glycogen by anion-exchange resin was as described above.

Glycogen phosphorylation

The cell extract was centrifuged. The procedure was essentially as described by Thurston *et al.* (1974). In a spectrophotometer cuvette (at 37°C) 10–20μl of the clear supernatant was added to 1ml of a solution containing 50mM-imidazole/HCl (pH7.0), 10mM-$MgCl_2$, 0.5mM-EDTA, 0.5mM-KH_2PO_4, 0.5mM-$NADP^+$ containing 1.5 units of phosphoglucomutase and 1.5 units of glucose 6-phosphate dehydrogenase. The mutase suspension was centrifuged at 12000rev./min to free the enzyme from $(NH_4)_2SO_4$ and the pellet dissolved in 20mM-Tris/HCl, pH7.0. For assay of total phosphorylase the solution was 1mM in AMP.

Higher AMP concentrations did not cause further stimulation, and 5mM was inhibitory. The reaction was started by the addition of 20μl of a solution of 4% glycogen. The reduction of $NADP^+$ was linear after the addition for about 5min.

Activation of phosphorylase

In later experiments total phosphorylase was assayed after activation with phosphorylase kinase essentially by the method of Cake & Oliver (1969). The homogenate was added to an equal volume of a solution containing 10mM-ATP (pH7.0) and 10mM-$MgCl_2$ and 15 units of phosphorylase kinase (Sigma Chemical Co., St. Louis, MO, U.S.A.)/ml and incubated for 15min at 37°C. (We found that higher concentrations of ATP and magnesium caused the formation of a precipitate and enzyme loss.) Activation was virtually complete in 10min. The solution was chilled and kept on ice for up to 2h before assay.

Expression of results

The defatted dry weight of the cells (Katz *et al.*, 1976) was determined in each experiment. This corresponds fairly closely to cell protein content. The formation or uptake of substrates are expressed either as μmol/100mg of protein, or as μmol/ml of packed cells. Enzyme activities are expressed as μmol/min per ml of packed cells. Since the protein content of liver is about 20%, the data may be approximately converted from per 100mg of protein into per g wet wt. by multiplying by a factor of 2. The protein content of 1ml of packed cells is 120–135mg, so that the results can be converted into per g wet weight by multiplying by 1.5. However these values refer strictly to hepatocytes rather than whole liver.

Results

Comparison of methods of phosphorylase assays

Phosphorylase activity may be assayed either in the direction of glycogen synthesis or in the direction of phosphorylation. There have been relatively few studies of the assay in the direction of phosphorolysis and the two assays have been rarely compared. It has been reported (Mezl & Knox, 1972) that the activities determined in the direction of phosphorolysis were 10 times those in the reverse direction. It is by no means certain that the two rates would be affected in the same way by various conditions.

The assay of total (a+b) phosphorylase offers many vexing problems. The K_m of the a form for glucose 1-phosphate is 1–2mM, but that of the b form about 250mM without AMP and about 50mM with AMP (Tan & Nuttal, 1975). The K_m can be further decreased by high concentrations of several anions, but optimal conditions of pH and salt appear to differ markedly between species (Stalmans & Hers, 1975). In most methods a concentration of the order of 50mM-

Table 1. *Assay of rat liver phosphorylase by several methods*

Hepatocytes were incubated without added substrate for 15 or 30min with or without glucagon (1μg/ml). Conversion was with phosphorylase kinase (see the text). Caffeine and AMP, when present, were 0.5 and 5mM respectively. For liver, animals were anaesthetized with barbital and livers were frozen with solid-CO_2-cooled aluminium tongs. Results for liver are averages for two separate livers.

	Incubation (min)	Glucagon	Phosphorylase activity (μmol/min per ml of packed cells)											
Method of assay ...			Glucose 1-phosphate → glycogen (isotopic)							Glycogen → glucose 1-phosphate (spectrophotometric)				
Conversion with phosphorylase kinase ...			No			Yes				No		Yes		
Addition of caffeine ...			−	+	−	−	+	+		−	−	−	−	
Addition of AMP ...			−	−	+	−	−	+		−	+	−	+	
Procedure ...			a	b	c	d	e	f	b/f (%)	g	h	i	j	g/j (%)
Hepatocytes of starved rat	0	−	4.4	4.4	6.3	8.2	7.2	9.2	48	1.8	2.3	2.3	2.8	65
	15	−	2.3	1.6	4.0	5.0	3.8	6.6	24	0.56	0.96	0.91	1.4	39
	15	+	5.1	5.0	5.8	7.7	6.7	8.9	56	1.8	2.2	2.1	2.5	83
Hepatocytes of fed rat	0	−	7.0	6.8	8.2	10.8	9.8	12.2	56	3.7	3.8	4.0	4.3	89
	15	−	4.8	4.7	6.9	8.9	8.0	9.5	49	1.9	2.5	2.5	3.0	64
	15	+	7.0	6.9	8.3	10.3	9.9	12.2	57	3.0	3.5	3.7	4.0	76
Hepatocytes of diabetic rat	0	−	—	5.4	10.7	—	10.5	10.7	50	2.6	2.9	2.7	2.8	93
	30	−	—	2.2	4.9	—	6.6	7.9	28	1.1	1.1	1.2	1.2	92
Liver of starved rats	−	−	4.0	3.2	7.7	8.3	—	9.7	32	2.6	3.3	3.4	9.2	62
Liver of fed rats	−	−	4.7	4.0	8.6	8.5	—	10.2	39	2.8	3.9	4.3	4.9	51

glucose 1-phosphate is used, but recent procedures used saturated solutions (270mM; Tan & Nuttal, 1975; Lederer & Stalmans, 1976) for the assay of the total enzyme. To increase sensitivity, we use a concentration of 10mM (Golden *et al.*, 1977). The optimal assay for total phosphorylase would be by conversion of the *b* form into the *a* form by phosphorylation, since in theory after conversion the ratio of the active and total enzyme should be the same irrespective of the kind of assay. We thus compared several procedures, and the effect of various conditions, in the phosphorylase assay (Table 1).

In the isotopic assay of total phosphorylase in the direction glucose 1-phosphate to glycogen, the activity determined after conversion with phosphorylase kinase was always higher than that measured with AMP. However, even after conversion, AMP was still stimulatory. Phosphorylase is markedly inhibited by a variety of nucleotides and phosphate esters and this inhibition is overcome by AMP (Maddiah & Madsen, 1966) and this most likely accounts for the stimulation. Stalmans & Hers (1975) recommended the use of 0.5mM-caffeine in the assay of phosphorylase *a* to inhibit phosphorylase *b*. The results in Table 1 indicate that there is also inhibition of the *a* form. We have used routinely 0.5mM-caffeine in our assays of the *a* form, although under most conditions the effect of caffeine addition is marginal.

The activities of either the *a* form or total phosphorylase, as measured by the phosphorylation of glycogen, were one-third to one-half those measured in the direction of synthesis. The ratio $a/(a+b)$ was always much higher than the corresponding ratio obtained in the direction of synthesis. In our spectrophotometric assay we found caffeine to be highly inhibitory to the *a* form, 0.5mM inhibiting by 40% and 2mM inhibiting by over 80%. Thus caffeine cannot be used in the assay in the direction of phosphorolysis.

The rate of phosphorolysis would appear to provide more rational values for the activity as it occurs in the cell. This method, however, has been seldom used. The $a/(a+b)$ ratio is much higher and was near unity in cells of diabetic rats, and it is possible that dephosphorylation of the *b* form is not blocked. The assay is more laborious and slower than measuring the incorporation of glucose 1-phosphate into glycogen. We have thus used the spectrophotometric procedure to a limited extent. However, as shown below, the pattern of change obtained by the two methods is much the same.

In theory and practice assay of total phosphorylase after conversion with phosphorylase kinase appears to be the method of choice. It avoids the problems associated with the use of the very high concentrations of glucose 1-phosphate or salts required in other procedures. The kinase is commercially available and the phosphorylation conversion is fairly rapid and simple. This method was, however, adopted by us only in the later course of our experiments.

Carbon and ^{14}C balance

We have observed repeatedly that the uptake of lactate was nearly equivalent or exceeded somewhat the formation of glucose plus glycogen. Also the uptake of glycerol, dihydroxyacetone and fructose was somewhat higher than the yield of carbohydrate and lactate. The molar ratio of glutamate uptake/(alanine+aspartate production) was also in a series of experiments close to 1 (see also Katz *et al.*, 1976). These findings suggested that the three carbon compounds provided the carbon for carbohydrate, and glutamate was mainly oxidized to alanine. However, a different pattern is shown by the ^{14}C incorporation. In Table 2 we present a typical example of an analytical and ^{14}C balance, with lactate or glycerol as gluconeogenic precursor. There was extensive net glucose synthesis, but also glucose phosphorylation, as measured with [2-^{3}H]glucose (Table 2*a*). With lactate the rate of glucose 6-phosphatase was twice that of glucose phosphorylation, and with glycerol nearly 3.5 times. ^{14}C was introduced into the glucose 6-phosphate pool by exchange with unlabelled carbon, and subsequently was introduced into glycogen. In Table 2(*b*) the distribution of ^{14}C from the three substrates in the different products are shown. Total synthesis of glycogen carbon, as calculated as the sum of the contribution of ^{14}C from the three substrates, was only slightly less than that estimated by chemical analysis. With lactate the apparent contribution to glycogen of carbon from glucose, lactate and glutamine was nearly equal (Table 2*c*). Glycerol contributed over half, and glucose about one-quarter, of the glycogen carbon. It is apparent from Table 2(*b*) that the carbon atoms of the different substrates exchange and intermix in the intermediates, so that the ^{14}C yields do not provide complete information on the role of substrates as precursors for any product.

It is well known that, although incorporation of ^{14}C from CO_2 or acetate into glucose may be extensive, they are not precursors for glucose. Obviously exchange reactions also occur with other substrates, but still ^{14}C uptake is commonly used to quantify the rate of precursor incorporation in hepatic metabolism. Many investigators have used, both *in vivo* and *in vitro*, the incorporation of ^{14}C from glucose to evaluate the contribution of glucose and endogenous substrates to glycogen synthesis. A recent example is the work of Geelen *et al.* (1977), who estimated that glucose is the source of some 70 to 80% of the glycogen formed by rat hepatocytes, although in their experiments there was net production of glucose. Obviously when there is no net uptake of glucose, it is not a precursor for glycogen or any other product.

Table 2. *Carbon and radioactive isotope balance in glycogen synthesis by rat hepatocytes*

Hepatocytes (25mg in 3ml volume) from one starved rat were incubated for 1h with glucose (10mM), either 16mM-lactate or 20mM-glycerol, and glutamine (8mM). Each substrate combination was present in six replicate vessels, with one of the three substrates labelled uniformly with ^{14}C. Glucose was also labelled with ^{3}H in position 2. Abbreviation: N.D., not determined.

(*a*) Substrate balance

	Concentration change or uptake of substrate or product (μmol/100mg of protein per h)	
Substrates ...	Glucose, lactate and glutamine	Glucose, glycerol and glutamine
Change in glucose	21	42
Change in glycogen	16	16
Change in carbohydrate	37	58
Change in lactate	−102	5.3
Change in glycerol	—	−111
Change in glutamate*	−53	−54
Change in alanine	46	N.D.
Change in aspartate	10	N.D.
Apparent uptake of [^{14}C]glucose	8.1	6.5
Glucose phosphorylation†	16	17
Glucose 6-phosphatase‡	37	59

* Includes glutamine and glutamic acid.
† From [2-^{3}H]glucose, corrected for incomplete detritiation.
‡ Glucose production plus glucose phosphorylation.

(*b*) Isotope balance

Labelled substrate	^{14}C recovered (% of the amount added)							Isotope recovery (%)
	Glucose	Glycogen	Lactate	Glycerol	Amino acids	CO_2	Others	
Glucose	95	4.4	0.71	—	0.41	0.47	0.30	101
Lactate	17	4.8	26	—	32	15	4.2	98
Glutamine	17	5.2	10	—	46	23	2.2	103
Glucose	96	2.9	0.5	—	0.42	0.47	0.25	101
Glycerol	66	15	1.5	3.4	7.7	4.1	2.4	100
Glutamine	13	3.2	2.3	—	56	21	2.3	103

(*c*) Contribution of ^{14}C from the various substrates to ^{14}C in glycogen

Total carbon by analysis (Table 1*a*)	^{14}C contribution (μg-atoms of C/100mg of protein per h)				
Substrate ...	Glucose	Lactate	Glycerol	Glutamine	Sum
Lactate 95	36	26	—	30	92
Glycerol 98	24	—	54	17	95
	^{14}C contribution (%)				
Lactate (100)	38	22	—	31	—
Glycerol (100)	24	—	55	17	—

The rate of ^{14}C incorporation will depend on the rates of inflow from various sources into glucose 6-phosphate, and the relative rates of cycling catalysed by glucokinase and glucose 6-phosphatase (Katz *et al.*, 1978). However, quantitative estimates of such rates require complex calculations.

Our findings indicate the need for great caution in the interpretation of ^{14}C data for the evaluation of rates in hepatic metabolism, especially as applied to glycogen synthesis. Much of the conclusions in the literature based on ^{14}C yields on the role of glucose in hepatic metabolism may require re-examination.

Glycogen synthesis, synthase and phosphorylase

To correlate changes in enzyme activities to glycogen synthesis, cells from a single starved animal

were incubated with several substrate combinations and assayed in parallel for substrate uptake, glucose and glycogen synthesis and the activities of the enzymes in the *a* and (*a*+*b*) form. In Figs. 1–3 the correlation of glycogen synthesis to synthase activities is shown.

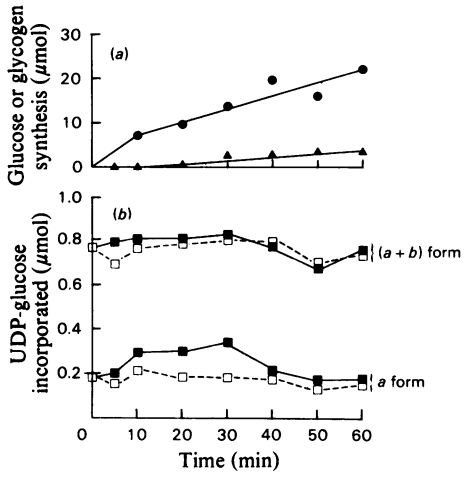

Fig. 1. *Glycogen synthesis and glycogen synthase activities in hepatocytes from one starved rat incubated with glucose with or without glutamine*

Results are expressed as μmol of glucose equiv. or μmol of UDP-glucose incorporated into glycogen/ml of packed cells. Cells were incubated with 10mM-glucose, and with 10mM-glutamine (——) or without glutamine (----). (*a*) Synthesis of glucose (●) and glycogen (▲) in the presence of glutamine (no glycogen was formed without glutamine); (*b*) activities of synthase *a* and (*a*+*b*) in the presence and absence of glutamine.

With 10mM-glucose there was no glycogen formation and no change in active and total synthase (Fig. 1). With glucose plus lactate (Fig. 2) there was negligible synthesis of glycogen, but this was greatly stimulated by glutamine. After a 10min lag period this was linear until 85–90% of the substrate was exhausted. Glutamine suppressed to some extent glucose synthesis, but the yield of total carbohydrate (glucose+glycogen) was increased. Glycogen synthesis from lactate (between 10 and 60min) ranged from 0.6–0.8 μmol of glucose equiv./h per ml of packed cells, equivalent to about 1 μmol/g of hepatocytes. Synthase *a* activity was doubled within 15min in the presence of glucose plus lactate, but then declined. The activity of the *a* form was increased over 3-fold in the presence of glutamine and remained high for most of the experimental period. Total synthase was also markedly increased.

With dihydroxyacetone as the gluconeogenic precursor (Fig. 3) there was some formation of glycogen and synthase activation even without glutamine, but both synthesis and synthase were greatly stimulated in its presence. Again total synthase was increased. After a 10–15 minute lag period, synthesis of glucose and glycogen proceeded at about equal rates of about 1.5 μmol/min per g wet wt. of liver. With fructose the pattern for synthase *a* and (*a*+*b*) was similar to that with dihydroxyacetone (results not shown). Total synthase was nearly doubled during glycogen synthesis. In the absence of substrates there was no change in the activities of active and total synthase.

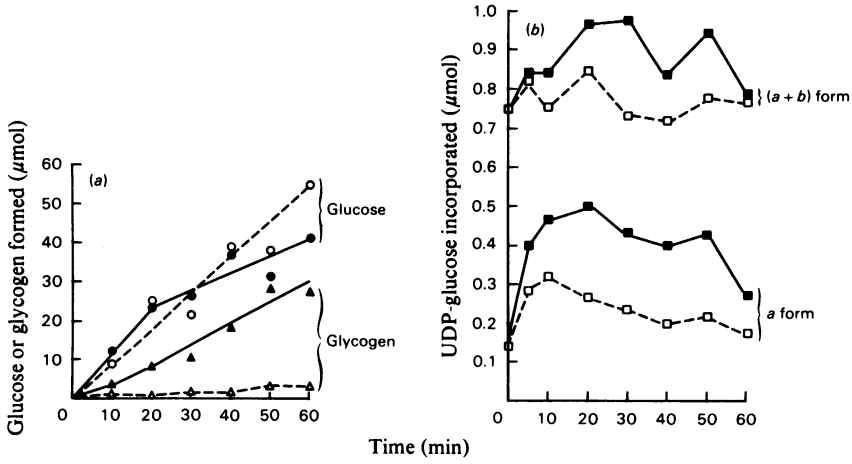

Fig. 2. *Synthesis of glucose and glycogen* (*a*) *and glycogen synthase* (*b*) *with glucose+lactate/pyruvate with and without glutamine*

Hepatocytes from one starved rat were incubated with 10mM-glucose, 15mM-lactate/15mM-pyruvate (9:1,v/v), and glutamine, when present, at 10mM. Explanation of units and lines is as in the legend to Fig. 1.

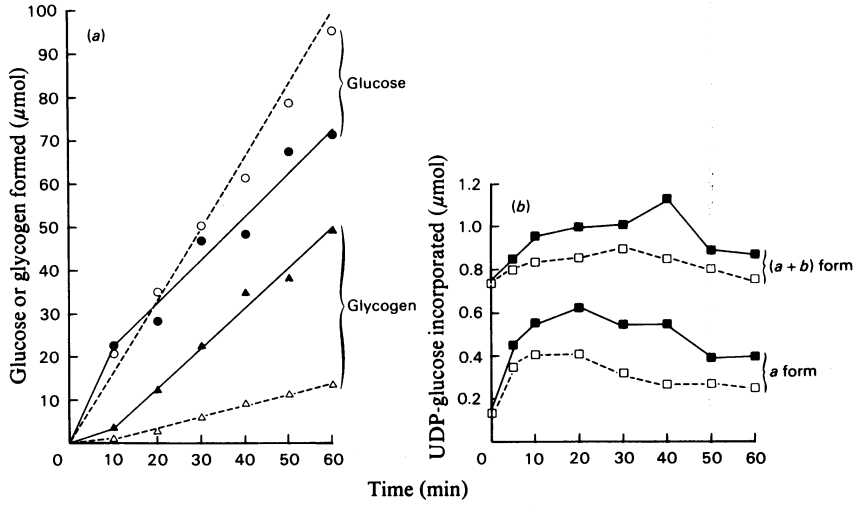

Fig. 3. *Synthesis of glucose and glycogen (a) and glycogen synthase (b) with glucose+dihydroxyacetone with and without glutamine*

Hepatocytes from one starved rat were incubated with 10mM-glucose, 20mM-dihydroxyacetone, and, when present, 10mM-glutamine. Explanation of units and lines is as in the legend to Fig. 1.

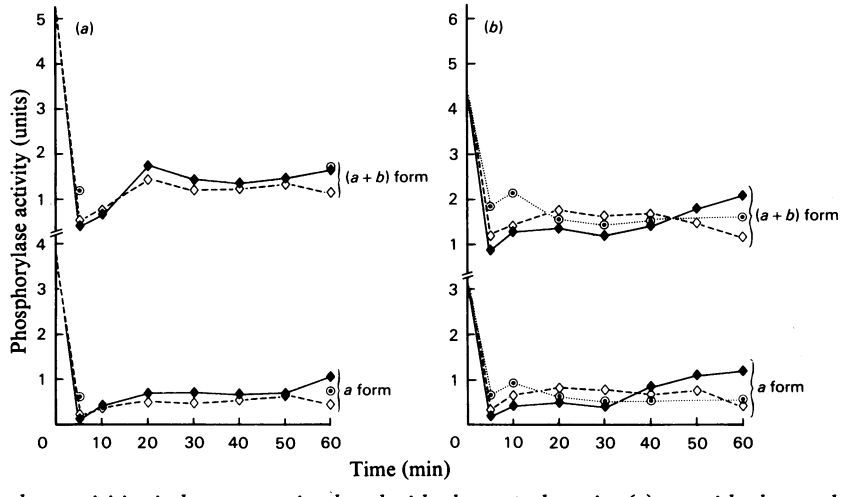

Fig. 4. *Phosphorylase activities in hepatocytes incubated with glucose±glutamine (a), or with glucose, lactate/pyruvate± glutamine (b)*

Results are for hepatocytes used for the results shown in Fig. 1. ——, Glutamine present; ----, glutamine absent; ····, no substrate. Phosphorylase was assayed by conversion of glycogen into glucose 1-phosphate. One unit of phosphorylase forms 1μmol of glucose 1-phosphate/ml of packed cells. Total phosphorylase (*a*+*b*) activity was measured in the presence of AMP.

In Figs. 4–6 the activities of phosphorylase for the above experiments are shown. There was always a very pronounced rapid drop of activity of both the *a* and total (*a*+*b*) form with the onset of incubation, whether substrate was present or not. This decrease occurred within 2min after incubation, and is likely to be caused by the transition from the relatively anoxic conditions in the cell pellet to the aerobic environment in the dilute suspension in the incubation vessels. The subsequent phosphorylase pattern dif-

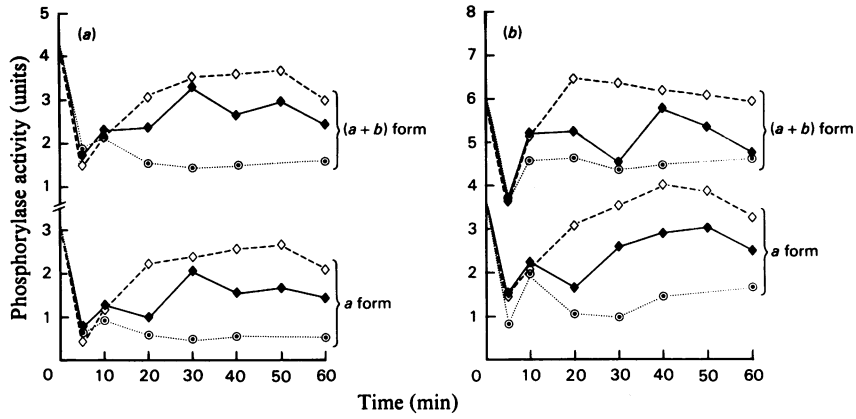

Fig. 5. *Phosphorylase activity of hepatocytes incubated with glucose+dihydroxyacetone ± glutamine*
Hepatocytes were those used for the results shown in Fig. 3. (*a*) Assay of phosphorylase by phosphorolysis; (*b*) assay of phosphorylase by glucose 1-phosphate conversion into glycogen. Explanation of units and lines is as in the legend to Fig. 4. Total (*a*+*b*) phosphorylase activity was measured in the presence of AMP.

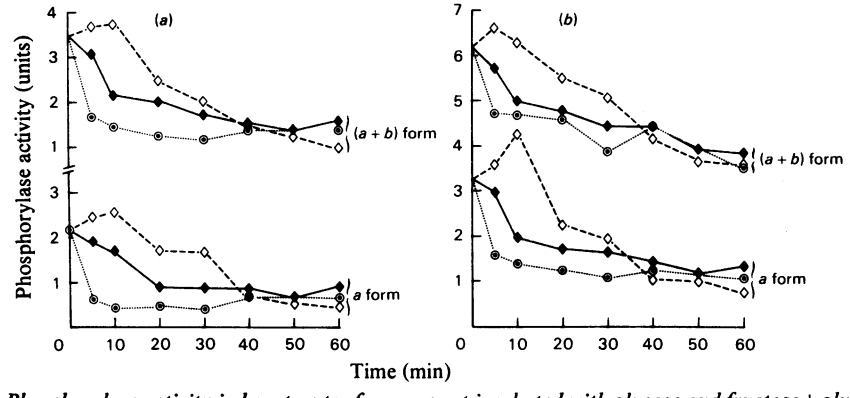

Fig. 6. *Phosphorylase activity in hepatocytes from one rat incubated with glucose and fructose ± glutamine*
Total (*a*+*b*) phosphorylase activity was measured in the presence of AMP. (*a*) Assay of phosphorylase by phosphorolysis; (*b*) assay of phosphorylase by glucose 1-phosphate conversion into glycogen. Explanation of units and lines is as in the legend to Fig. 4.

fered greatly with the type of substrate. The activity remained low in cells incubated without substrate, with glucose, or with glucose and lactate, and there was little effect of glutamine addition. On the other hand, with dihydroxyacetone the rapid decline was followed by a rise in active and total phosphorylase activities. Assay of phosphorylase in both directions is shown and it is apparent that the pattern of change with both methods parallel each other. Glutamine diminished the increase in phosphorylase activity, but it was still higher than in the absence of substrate. With fructose the initial decline in the active and total phosphorylase was less pronounced compared with cells incubated without substrate, and the decrease could be detected only by early sampling (1 to 2 min; results not shown). The decrease was followed by an increase in activity for about 15 min, which declined afterwards. Changes in total phosphorylase paralleled those of the active form. Similar results with fructose, showing a large transient increase in active and total phosphorylase followed by a decrease were reported with perfused liver by Walli *et al.* (1975).

Fig. 7 is representative of another series of experiments where hepatocytes from one starved rat were incubated with a number of substrates. Synthesis of glycogen and synthase *a* activity were maximal with fructose. The highest amounts of active phosphorylase were also seen with fructose. Whatever period was

examined, there was good correlation between active synthase amounts and glycogen synthesis, but there was no, or negative, correlation with assayable phosphorylase *a*.

These results present a pattern at variance with other findings in the literature and accepted notions of the behaviour of these enzymes. To sum up, in our system: (*a*) the activity of total synthase was not constant, but increased in parallel with the activity of the *a* form, and thus it is impossible to express activation as a fraction of the total enzyme; (*b*) the activity of total phosphorylase was also not constant, but varied in parallel with that of the *a* form; (*c*) there was a large and very rapid decrease in the activity of both forms of phosphorylase when cells were suspended in oxygenated buffer; and (*d*) there was no correlation between phosphorylase activity and glycogen synthesis. The activities of both forms of phosphorylase depend to a large extent on the nature of the substrates, rather than as the rate of glycogen synthesis.

Glycogen synthesis in cells from fed rats

We were not able to obtain consistent net glycogen synthesis with hepatocytes from fed rats unless the animals were pretreated to deplete the glycogen. With cells of fed rats, containing 100–150 μmol of glucose equivalents, there was first a period of glycogenolysis, which was followed after about 30 min by variable net glycogen deposition. However ^{14}C incorporation occurs both during glycogen breakdown and during deposition. There was glycogen synthesis when cells of fed rats were depleted of glycogen, at rates some 25 to 50% higher than cells of starved rats. A number

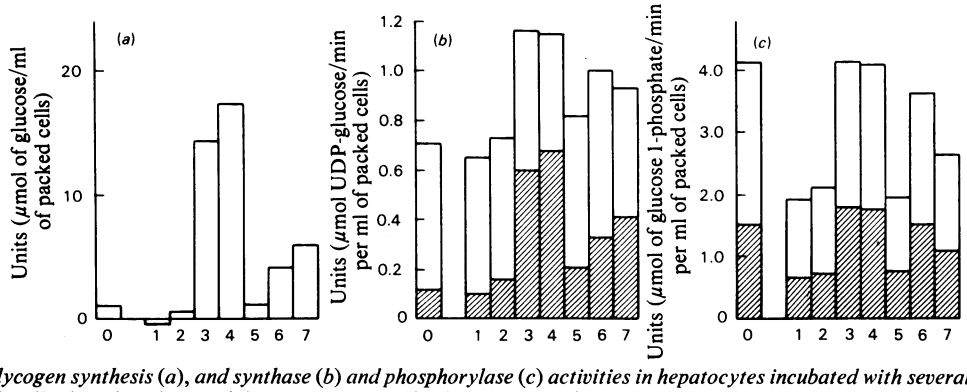

Fig. 7. *Glycogen synthesis* (*a*), *and synthase* (*b*) *and phosphorylase* (*c*) *activities in hepatocytes incubated with several substrates*
Total (*a*+*b*) phosphorylase activity was measured in the presence of AMP. Cells from one starved rat were incubated for 20 min with the following substrates: 0, initial value; 1, no substrate; 2, glucose; 3, glucose+fructose; 4, glucose+fructose+glutamine; 5, glucose+lactate; 6, glucose+lactate+NH_4Cl; 7, glucose+lactate+glutamine. Shaded area, synthase *a* or phosphorylase *a*; total height of bar, synthase (*a*+*b*) or phosphorylase (*a*+*b*). Enzyme units are defined in the Figure.

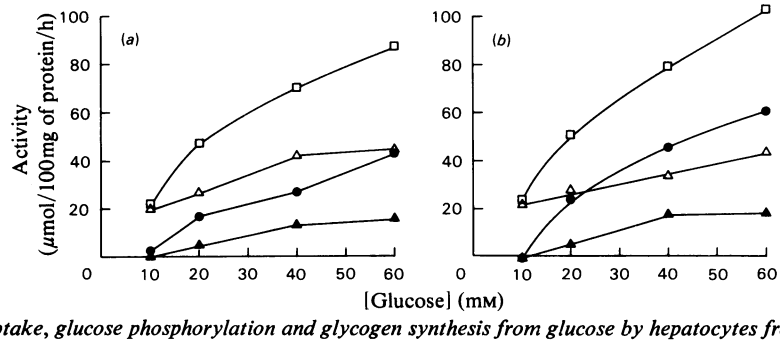

Fig. 8. *Glucose uptake, glucose phosphorylation and glycogen synthesis from glucose by hepatocytes from one starved rat* (*a*) *and one fed rat* (*b*)
The fed rat was treated with glucagon (see the text) to deplete liver glycogen. Cells were incubated for 1 h with [2-^{3}H]-glucose at various concentrations (no other substrate). □, Glucose phosphorylation as calculated from ^3HHO yields (see the text); ●, net glucose uptake (by analysis); △, glucose 6-phosphate dephosphorylation (phosphorylation minus uptake); ▲, glycogen synthesis. Results are expressed as μmol of glucose or glucose equivalents/100mg of cell protein.

of treatments, combining injections of phlorrhizin and glucagon and preincubation of cells without glucose, were employed to deplete the glycogen. The procedure that gave the most consistent results was that described in the Methods section. However, since rates may depend on the pretreatment, we do not present these results in detail. The enzyme patterns for synthase and phosphorylase were essentially those described for starved rats.

Glycogen synthesis from glucose

Seglen (1973) reported net glycogen synthesis from high concentrations of glucose by hepatocytes from starved rats. We confirm his findings. Glucose uptake commenced in various preparations between 10 and 20mM-glucose. Glycogen synthesis increased up to concentrations of 50–60mM. Glycogen synthesis was somewhat higher with cells of fed glycogen-depleted rats than with those from starved animals. In Figs. 8 and 9 we show typical results with hepatocytes from fed and starved rats.

Net glucose uptake, glycogen deposition, the rate of glucose phosphorylation and the calculated rate of glucose 6-phosphatase are shown. The rate of uptake was not saturated at 60mM. Uptake of glucose and glycogen synthesis by cells from fed glycogen-depleted rats was somewhat higher than that from starved animals. A comparison of our results with those for perfused liver is of interest. Probably the highest values were reported by Alvarez & Nordlie (1977), who perfused livers of starved and fed rats with glucose concentrations from 10 to 80mM. The rates of glucose uptake at 10mM were highly variable [0.27±0.19 and 0.40±0.35 (mean±S.D.) μmol/min per g of liver for starved and fed rats respectively]. At 20mM the uptake was 0.8μmol/min per g of liver for both conditions, and at a concentration of 40mM 1.6 and 2.2μmol/min per g of liver for starved and fed rats respectively. The rates for hepatocytes (Fig. 8) were 60–70% of the above values (at 20mM, 0.6 and 0.8, and at 40mM, 0.9 and 1.4μmol/min per g of liver for hepatocytes from starved and fed rats respectively). Alvarez & Nordlie (1977) reported a rate of glycogen synthesis with a liver of a starved rat perfused with 70mM-glucose to be 1.1μmol of glucosyl units/min per g of liver. Our rates at 40mM were about 0.6μmol/min per g of liver. The rate of glucose phosphorylation shown in Fig. 8 was calculated from the ^3HHO yield from [2-^{3}H]glucose, corrected for incomplete detritiation of glucose 6-phosphate (Katz

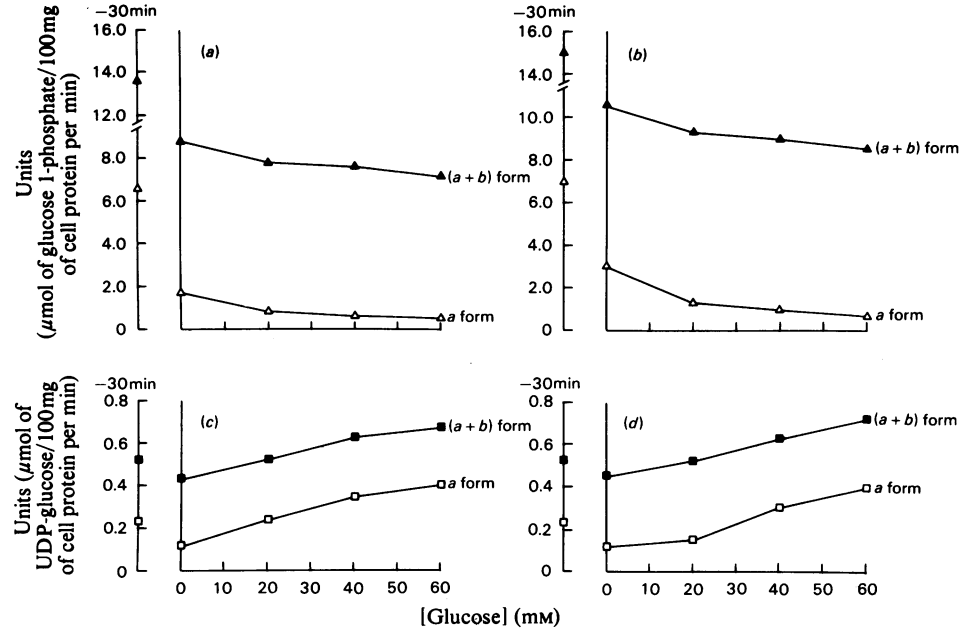

Fig. 9. *Glycogen phosphorylase (a and b) and glycogen synthase (c and d) by hepatocytes from one starved (a and c) and one fed rat (b and d)*

The cells were from the same animals as used for the results shown in Fig. 8. The cells were taken for enzyme assay at zero time, and after 30min incubation. Total phosphorylase (*a*+*b*) form activity was determined after converting into the *a* form with phosphorylase kinase, and phosphorylase was assayed by the incorporation of glucose 1-phosphate into glycogen.

et al., 1978). The net rate of glucose uptake represents the difference between phosphorylation and dephosphorylation of glucose 6-phosphate.

In Fig. 9 the activities of active and total synthase and phosphorylase as a function of glucose concentration in the medium are shown. There was a parallel increase in active and total synthase. Again the amounts of both forms of phosphorylase decreased markedly on incubation. However, in contrast with previous systems with gluconeogenic precursors, there was a decrease in the amount of both forms of phosphorylase with increasing glucose concentration and glycogen synthesis. The depression was most pronounced with cells of fed rats. This decrease in active phosphorylase confirms the findings of Hers and his co-workers, as reviewed by Hers (1976) and Stalmans (1976). It appears that the response of phosphorylase in glycogen synthesis from glucose differs from the response when glycogen is formed from gluconeogenic substrates.

Phosphorylase activity in intact cells

We have found a high activity of phosphorylase during glycogen formation from dihydroxyacetone or fructose. It appears possible that there is a 'futile' cycle between glycogen and hexose phosphates, catalysed by synthase and phosphorylase, with the rate of synthase in the cell exceeding that of phosphorylase.

To test for this possibility glycogen was prelabelled with galactose. Galactose utilization at concentrations of 5–10mM is rather low, but when added in trace amounts most of the tracer is taken up in 10–15min. In the absence of glutamine the major labelled product from [1-^{14}C]galactose is glucose, and from [2-^{3}H]galactose is water and smaller amounts of glucose. In the presence of glutamine the major product from both tracers is glycogen with a ^{3}H/^{14}C ratio from [1-^{14}C,2-^{3}H]galactose of approx. 1.0. The incorporation of galactose into glycogen is via galactose 1-phosphate, UDP-galactose and UDP-glucose and the overall reaction is irreversible. [2-^{3}H]Galactose was the radioactive tracer of choice, since there appeared little likelihood that any ^{3}H liberated on glycogen breakdown would be reincorporated, but when [^{14}C]glucose is the breakdown product, reincorporation of ^{14}C into glycogen would be possible. The glycogen labelled by galactose was isolated, hydrolysed by amyloglucosidase, and the hydrolysate phosphorylated with ATP and hexokinase. Nearly all the activity was adsorbed on an anion-exchange column, indicating that the activity in glycogen was indeed glucose.

Glycogen was prelabelled by incubation for about 20min with trace amounts of galactose, and then an excess of galactose and substrate (lactate or dihydroxyacetone) was added, and glycogen formation

Table 3. *Absence of glycogen recycling during synthesis*

Cells from starved rats (26 and 28mg of protein in Expts. 1 and 2 respectively) were incubated in 3ml containing 10mM-glucose, 10mM-glutamine and 20mM-lactate/20mM-pyruvate (9:1,v/v) or 20mM-dihydroxyacetone. After 10min a trace amount of [2-^{3}H]galactose was added, and 20min later 10μmol of galactose was added. After a further 10min (shown as zero time in the Table) sampling was begun and the synthesis of glycogen and ^{3}H remaining in the prelabelled glycogen and that liberated as water were monitored.

Expt. no.	Time (min)	Substrate	Distribution of ^{3}H (% of initial dose)		Glycogen content (μmol/flask)
			Glycogen	Water	
1	0	Dihydroxyacetone	38	8.4	4.1
	10		40	—	5.4
	20		40	9.1	6.1
	40		40	9.6	7.3
	60*		37	9.1	7.6
	60†		19	19	2.0
2	0	Lactate	47	—	5.8
	10		49	—	7.3
	30		48	—	8.4
	50*		41	15	7.2
	0	Dihydroxyacetone	49	—	9.4
	10		53	—	10.3
	30		50	—	12.3
	50*		43	9.5	11.0
	50†		51	—	2.0

* Substrate exhausted.

† Glucagon added 20min previously.

and the radioactivity in glycogen and water monitored (Table 3). There was continual glycogen synthesis and as long as substrate was present there was little if any radioactive isotope liberation from glycogen. These results indicate that although the amounts of assayable phosphorylase are high, there is very little glycogen phosphorolysis and no significant 'futile' cycle. Apparently phosphorylase *a*, although present in high amounts, is somehow inhibited in the cell. In these experiments with cells of starved rats the glycogen concentrations are low and in these conditions the phosphorylase in the intact cell may be completely inhibited. However, in cells with a high glycogen content it is possible that synthase and phosphorylase are both active and 'futile' cycling could possibly occur.

Discussion

Glycogen synthesis, synthase and phosphorylase

Our results establish the existence of two distinct systems of glycogen synthesis by hepatocytes. In the first type glycogen synthesis proceeds concurrently with glucose synthesis, and the glycogen is derived from gluconeogenic precursors. The requirements for efficient glycogen synthesis in hepatocytes are the presence of glucose at concentrations of about 10mM and a supply of gluconeogenic substrates and amino acids, most effectively glutamine (Katz *et al.*, 1976). Although in our experiments the initial concentrations of substrates and amino acids are high (above 10mM), synthesis proceeds at linear rates until 80–90% of the substrate is used up, down to concentrations of about 2mM.

A second system for glycogen synthesis from glucose as sole substrate requires rather high concentrations (30–40mM) for significant rates. Glucose is taken up rather than produced in this system, and there is also net glycolysis with production of lactate and other compounds.

A marked increase in glycogen synthase activity (both *a* and *a*+*b* forms) occurs during glycogen synthesis in both systems. They differ, however, in the response of the phosphorylase. The activity is depressed with an increase in glucose concentration and glycogen synthesis from glucose, but it is not changed or increased in glycogen synthesis from gluconeogenic substrates.

It is generally held that changes in the activities of synthase and phosphorylase, whether induced by substrate or hormones, involves transformation by phosphorylation/dephosphorylation from a largely inactive (*b*) form, to the active (*a*) form, so that the activity in the cell is determined by the fraction of the enzymes in the *a* form. Our results with both types of glycogen synthesis show, however, that the potential total activity of both synthase and phosphorylase (*a*+*b*) in our assay is not constant, but changes markedly in parallel with the amount of the *a* form. The behaviour of phosphorylase was most unexpected. In the gluconeogenic system there was no correlation between its activity and glycogen synthesis. With dihydroxyacetone and fructose, which are excellent sources of glycogen, the assayable activity of the *a* form was actually increased over initial amounts.

Hers and his co-workers (for reviews see Hers, 1976; Stalmans, 1976) proposed a mechanism for induction of glycogen synthesis by glucose in which a decrease in active phosphorylase precedes the activation of synthase. Our results are consistent with this mechanism for glycogen synthesis from glucose, but are at complete variance with the Hers hypothesis when glycogen synthesis is from gluconeogenic precursors. Actually the paradoxical association under some conditions of high phosphorylase with high glycogen synthesis has been noted by Hers (1976). A most striking example is in the foetal liver before birth (Watts & Gain, 1976). In the rat there is a very rapid glycogen deposition between days 18 and 21 after conception, with glycogen attaining 7% wet wt. of liver. Simultaneously the activities of assayable phosphorylase *a* as well as synthase *a* are greatly increased. Watts & Gain (1976) suggested that this implies a 'futile' cycle between glycogen and hexose 6-phosphate and glucose. However, we find that in spite of high assayable activity there is little phosphorolysis, and apparently the phosphorylase is inhibited. It is noteworthy that in assay of phosphorylase high concentrations of glycogen are employed, whereas in our experiments the glycogen concentration in the cells was very low. It appears that the assayable amounts of phosphorylase have little physiological significance, and it is likely that when there is glycogen synthesis, at least in cells low in glycogen, the phosphorylase is completely inhibited.

An unusual finding is the very rapid and drastic decrease in phosphorylase activity on incubation. Some preliminary experiments suggest that this is related to the transition from an anoxic state in the cell pellet to an oxygenated state on dispersion of the cells in the incubation medium.

Our results show that the assayable activity of the *b* form of the enzymes is variable and expressing results for the *a* form as a fraction of the total enzyme has little meaning for hepatocytes. The accepted concept is that modulation of the activity of synthase and phosphorylase is by a simple phosphorylation/dephosphorylation mechanism. More recent results indicate that the mechanism is much more complex, involving multiple sites of phosphorylation of synthase (Soderling *et al.*, 1977) and that there is more than one kinase, and probably more than one phosphatase. Four kinetically discrete forms of glycogen synthase and their interconversion were described in adipose tissue (Eichner, 1976). Our

results could be rationalized if, in addition to the classical *a* and *b* forms, there are in liver other forms that vary in their activity.

Precursors of liver glycogen

Soskin and his co-workers demonstrated (1938) the cessation of glucose production and a large uptake of glucose after administration of a massive (1.7g/kg body wt.) glucose load to dogs. These results were repeatedly confirmed and substantial accumulation of glycogen was shown to occur (for review see Stalmans, 1976). From such studies the concept evolved that liver has a major role to play in the disposal of increased blood-glucose concentrations, and that liver glycogen was the major product. It is noteworthy, however, that the glucose loads given were invariably in great excess of the body-glucose content, and also that free glucose is a rather minor component of most foodstuffs, so that the physiological relevance of such experiments is questionable. On the other hand, the large majority of studies *in vitro* indicate a limited capacity of liver to take up glucose at physiological glucose concentrations and to form glycogen, and also fat (see below). In our experiments glycogen formation from 20mM-glucose, a concentration rarely attained in portal blood, was a small fraction of that seen under suitable conditions from low concentrations of gluconeogenic precursors. In perfused liver Hems *et al.* (1972) and Whitton & Hems (1977) found very low formation of glycogen from 30mM-glucose, but substantial synthesis on addition of gluconeogenic precursors. The question is thus posed as to the relative contribution of glucose and gluconeogenic precursors to glycogen under a physiological range of conditions. Obviously there is no contribution of glucose to glycogen unless net uptake of glucose occurs. The capacity for glucose utilization seems to a large extent to depend on the composition of the diet and dietary conditioning. In a recent study, Remesy *et al.* (1978) kept rats on a diet of either 80% starch or 40% starch and 40% protein. At 4h after the onset of feeding, after starvation overnight, the rats conditioned to an 80% starch diet had portal plasma-glucose concentrations of 14mM and 13% of the glucose in portal-arterial blood was taken up. With rats on the 40% starch diet, portal plasma-glucose concentration was 8.8mM, and there was no uptake, but actually a small production of glucose. In dogs Landau *et al.* (1961) obtained substantial hepatic glucose uptake after a glucose load in animals conditioned on a high-carbohydrate diet, but very little with dogs kept on a normal high-protein diet. It is likely that efficient glucose uptake and utilization of glucose for glycogen synthesis depends on the induction of glucokinase, with a decrease in glucose 6-phosphatase activity.

There have been numerous studies with [^{14}C]-glucose to estimate the relative contributions of glucose and endogenous sources to liver glycogen. As discussed above, the incorporation of ^{14}C may predominantly represent exchange. It serves only to determine the upper limit of the contribution of glucose, and this may be a rather large overestimate of the true rate. To cite some examples of such studies, Moriwaki & Landau (1963) calculated that in rats in the second hour after an oral dose of 0.6g of glucose/100g body wt., about half of the glycogen carbon atoms were from glucose and half from endogenous C_3 precursors, but in the third hour nearly all the carbon was contributed by glucose. Baker (1977) fed glucose to starved mice, so that entry from the gut was maintained at a rate of at least 5 times the output by liver. From specific radioactivities of glucose and glycogen he calculated that about half the glycogen carbon atoms were derived from glucose and half from other sources. These experiments show that even with large loads of free glucose there is rather substantial synthesis of glycogen from gluconeogenic precursors.

It appears that at least in the rats fed 'normal' diets the role of glucose as a precursor of glycogen has been overestimated and that glycogen synthesis is predominantly a gluconeogenic process. The situation is akin to the role of glucose as a precursor for hepatic lipogenesis. Results with hepatocytes (Clark *et al.*, 1974) established that free glucose is not an efficient precursor of fatty acids, but that the major sources were glycogen and lactate. These findings have been confirmed (Salmon *et al.*, 1974; Katz *et al.*, 1977; Bloxham *et al.*, 1977). Most significantly the relatively minor role of glucose for hepatic lipogenesis was clearly established *in vivo* by Hems *et al.* (1975) in the starved and refed rat. Thus it appears that in the starved and refed rat on a high-carbohydrate diet, glucose is the major substrate for adipose tissue lipogenesis, but it is not used extensively for hepatic lipogenesis, and since the capacity for glycogen storage in liver is limited, most of the glucose is disposed of by extrahepatic tissues.

This research was supported in part by grants from the National Institutes of Health (R01 AM 12604 and NSF BMS74-22815).

References

Alvarez, F. L. & Nordlie, R. C. (1977) *J. Biol. Chem.* **252**, 8404–8414

Baker, N. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 253–256

Bergmeyer, H. U. (ed.) (1974) *Methods of Enzymatic Analysis*, Academic Press, New York

Bloxham, D. P., Fitzsimmons, J. T. R. & York, D. A. (1977) *Horm. Metab. Res.* **9**, 253–346

Cake, M. H. & Oliver, I. T. (1969) *Eur. J. Biochem.* **11**, 576–581

Clark, D. G., Rognstad, R. & Katz, J. (1974) *J. Biol. Chem.* **249**, 2028–2036
Eichner, R. D. (1976) *J. Biol. Chem.* **251**, 2316–2322
Geelen, M. J. H., Pruden, E. L. & Gibson, D. M. (1977) *Life Sci.* **20**, 1027–1034
Golden, S., Wals, P. A. & Katz, J. (1977) *Anal. Biochem.* **77**, 436–445
Hems, D. A., Whitton, P. D. & Taylor, E. A. (1972) *Biochem. J.* **129**, 529–538
Hems, D. A., Roth, E. A. & Verrinder, T. R. (1975) *Biochem. J.* **150**, 167–173
Hers, H. G. (1976) *Annu. Rev. Biochem.* **45**, 167–189
Katz, J., Wals, P. A., Golden, S. & Rognstad, R. (1975) *Eur. J. Biochem.* **60**, 91–105
Katz, J., Golden, S. & Wals, P. A. (1976) *Proc. Natl. Adad. Sci. U.S.A.* **73**, 3433–3437
Katz, J., Wals, P. A., Golden, S., Goldman, J. K. & Bernardis, L. L. (1977) *Horm. Metab. Res.* **9**, 59–63
Katz, J., Wals, P. A. & Rognstad, R. (1978) *J. Biol. Chem.* **253**, 4530–4536
Landau, B. R., Leonards, J. R. & Barry, F. M. (1961) *Am. J. Physiol.* **201**, 41–46
Lederer, B. & Stalmans, W. (1976) *Biochem. J.* **159**, 689–695
Lust, W. P., Passoneau, J. V. & Crites, J. V. (1975) *Anal. Biochem.* **68**, 328–331
Maddiah, V. T. & Madsen, N. B. (1966) *J. Biol. Chem.* **241**, 3873–3881
Mezl, V. A. & Knox, W. E. (1972) *Enzyme* **13**, 197–202
Moriwaki, T. & Landau, B. (1963) *Endocrinology* **72**, 134–145
Remesy, C., Demignee, C. & Aufrere, J. (1978) *Biochem. J.* **170**, 321–329
Salmon, D. M. W., Bowen, N. L. & Hems, D. A. (1974) *Biochem. J.* **142**, 611–618
Seglen, P. O. (1973) *FEBS Lett.* **30**, 25–28
Soderling, T. R., Lett, M. F., Hutson, N. J. & Khatra, B. G. (1977) *Biochem. J.* **252**, 7517–7524
Soskin, S., Essex, H. E., Hernick, J. F. & Marin, F. L. (1938) *Am. J. Physiol.* **124**, 558–567
Stalmans, W. (1976) *Curr. Top. Cell. Regul.* **11**, 51–98
Stalmans, W. & Hers, H. G. (1975) *Eur. J. Biochem.* **54**, 341–350
Tan, A. W. H. & Nuttal, Q. (1975) *Biochim. Biophys. Acta* **410**, 45–60
Thurston, J. H., Jones, E. M. & Hauhart, R. E. (1974) *Diabetes* **23**, 597–604
Walli, A. K., Birkman, L. & Schimassek, H. (1975) *Biochem. Soc. Trans.* **3**, 1039–1042
Wals, P. A., Golden, S., Rognstad, R., Katz, J. & Ebner, K. E. (1976) *Anal. Biochem.* **73**, 247–251
Watts, C. & Gain, K. R. (1976) *Biochem. J.* **160**, 263–270
Whitton, P. D. & Hems, D. A. (1977) *FEBS Lett.* **74**, 195–200