SUBSTRATE CONTROL OF GLYCOGEN LEVELS IN ISOLATED HEPATOCYTES FROM FED RATS *

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Summary: Isolated parenchymal cells from fed rat liver rapidly lose glycogen when incubated with glucose. The addition of glycerol or fructose but not insulin prevents much of the breakdown. When cells are incubated with glycerol and glucose, more glycogen is retained with the further addition of xylitol than of fructose or pyruvate. Oleate stimulates glycogen breakdown. The results indicate that glycerol may play an important physiological role in the control of glycogen synthesis in the liver, possibly by esterifying fatty acids. Furthermore, the results support the concept that the main effect of insulin on liver glycogen levels in vivo may be the result of diminished flow of free fatty acids to the liver.

Net synthesis of glycogen in the liver from fed rats has been difficult to demonstrate in vitro. This has been due partly to the tissue preparations employed. For instance, the breakdown of glycogen in the damaged cells at the periphery and in the anoxic innermost cells of liver slices (1) obscures any synthesis which might have occurred. Damaged or anoxic cells, however, are not the only reason for the rapid breakdown of glycogen. The nature of the hormones and nutrients supplied to the liver also contributes. Thus, hyperlipemic serum has been shown to be necessary for the maintenance of glycogen levels in the perfused liver from fed rats (2).

The advent of intact, isolated hepatocytes has provided a system whereby several variables can be studied with the same liver. In addition, oxygen, substrates and hormones have ready access to all cells. Therefore, it seemed appropriate to investigate which nutrients and hormones affect glycogen levels in the liver from fed rats in an effort to demonstrate net synthesis of glycogen. Our initial results presented herein show that glycerol is the

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most effective substrate for the maintenance of glycogen levels and that oleate promotes degradation.

Methods. Isolated liver parenchymal cells from fed male Sprague-Dawley rats (220-300 g) were obtained by the technique described by Howard et al. (3). Basically, a solution of 0.05% collagenase and 0.1% hyaluronidase dissolved in calcium-free Eagle's Minimum Essential Medium is injected into the liver, which is then sliced and incubated in the enzyme solution at 37°C for 45-55 minutes. $CaCl_2$ solution is added to the slices after 25-35 minutes. The dispersed slices are then filtered through meshes, collected by low-speed centrifugation and washed twice.

In the present work the final cell pellet was suspended in Krebs-Ringer phosphate solution, pH 7.4, so that there were approximately 1 million cells per ml. One ml aliquots of the cell suspension were added to 25 ml siliconized flasks. The flasks were then gassed with 100% oxygen and incubated at 37°C with occasional shaking. Over 90% of the cells were viable, as determined by exclusion of trypan blue, at the beginning of incubations.

Glycogen was precipitated and estimated by modification of two described methods (4,5). At the end of incubation, 4 ml of 40% KOH were added to the flasks. The contents were boiled for one hour, cooled and made up to 10 ml with water. 2 ml duplicate aliquots of each solution were added to 50 ml

Table I

Glycogen Content of Liver Cells Incubated in Krebs - Ringer Phosphate Solution

Glucose Conc. (mM)	Additional Substrates	Glycogen Content (μg/10^6 cells)[a]		
		Expt. 1	Expt. 2	Expt. 3
		Not incubated		
10	None	550 ± 25	655 ± 37	899 ± 30
		Incubated for 1 hr. at 37°C		
10	None	210 ± 15	275 ± 22	529 ± 42
10	Glycerol[b]	435 ± 22	500 ± 8	781 ± 25
10	Fructose[b]	400 ± 44	415 ± 15	752 ± 10
10	Dihydroxyacetone (5mM)	330 ± 36	295 ± 12	-
10	Pyruvate (5mM)	290 ± 13	-	-
10	Xylitol (5mM)	285 ± 14	305 ± 11	-
10	Alanine (5mM)	250 ± 8	-	-
30	None	-	-	608 ± 17

[a]Mean average ± SEM of 4-6 analyses.
[b]5mM in Expts. 1 and 2; 10mM in Expt. 3.

centrifuge tubes. The glycogen was precipitated by the addition of 4 ml of 95% ethanol and 100 µl saturated sodium sulphate. The tubes were centrifuged at 2400 rpm for 10 minutes. The pellets of glycogen were then recrystallized twice. The final precipitate was dissolved in 1 ml of water and cooled before addition of 5 ml of 0.05% anthrone dissolved in 72% H_2SO_4. The tubes were placed in a boiling water bath for 20 minutes and then immediately placed in ice. The color produced was measured in a model 300 Gilford microsample spectrophotometer at 620 mµ.

RESULTS

Table I shows the effect of various substrates on the glycogen content of isolated parenchymal cells obtained from fed rat livers during one hour of incubation in Krebs-Ringer phosphate solution containing 10 mM glucose. It can be seen that when 10 mM glucose is the sole substrate, glycogen levels fall rapidly. Much of this breakdown is prevented by the addition of glycerol or fructose. Other substrates shown in Table I are not as effective. Raising the glucose concentration to 30 mM prevents some of the breakdown of glycogen observed with 10 mM glucose but the addition of glycerol and fructose is still more effective. In fact, more glycogen is retained in cells incubated with 10mM glycerol than with 10mM glucose (Table II). Other experiments have shown

Table II

Effect of Glucose and Glycerol on Glycogen Content of Liver Cells

Substrates	Glycogen Content (µg/10^6 cells)[a]	
	Expt. 1	Expt. 2
	Not incubated	
None	655 ± 37	396 ± 28
	Incubated for 1 hr. at 37°C	
Glucose (10mM)	275 ± 22	97 ± 2
Glycerol (10mM)	455 ± 7	174 ± 17
Glycerol (10mM) + Glucose (10mM)	500 ± 8	203 ± 14

[a]Mean average ± SEM of 6 analyses.
Approximately 1 million cells were incubated in 1 ml of Krebs - Ringer phosphate solution.

that the cells retain less glycogen when incubated with 10mM lactate than with 10mM pyruvate. In addition, insulin did not prevent breakdown but glucagon enhanced degradation over one hour of incubation.

By increasing the supply of substrates to the liver cells, breakdown of glycogen can be halted further. This depends, to some extent, on the nature of the substrates added. Table III shows that when cells are incubated with 10 mM glucose and 10 mM glycerol, more glycogen is retained on further addition of xylitol than of fructose or pyruvate. Other experiments have confirmed the differential effects of these substrates. However, the complete retention of glycogen in cells incubated with glucose, glycerol, xylitol and L-α-glycerophosphate (Table III) has not been repeated. This may be the result of the loss of 5-15% viability of the cells during different incubations.

Table III does not clearly show that xylitol actually enhances glycogen retention in the presence of glucose and glycerol. This is, therefore,

Table III

Effect of Combined Substrates on Glycogen Content of Liver Cells

Additional Substrates	Glycogen Content ($\mu g/10^6$ cells)[a]
	Not incubated
None	666 ± 12
	Incubated for 1 hr. at 37°C
None	180 ± 4
10mM glycerol + 10mM xylitol	595 ± 19
10mM glycerol + 10mM fructose	539 ± 11
10mM glycerol + 20mM pyruvate	485 ± 15
10mM glycerol + 10mM xylitol + 5mM L-α-glycerophosphate	676 ± 4
20mM glycerol	596 ± 11

[a]Mean ± SEM of 4 analyses. Approximately one million cells were incubated in Krebs - Ringer phosphate solution containing 10mM glucose.

Table IV

Effect of Xylitol on Glycogen Content of Liver Cells

Additional Substrates	Glycogen Content ($\mu g/10^6$ cells)[a]	
	Expt. 1	Expt. 2
	Not incubated	
None	540 ± 22	899 ± 30
	Incubated for 1 hr. at 37°C	
None	100 ± 9	529 ± 42
5mM glycerol	217 ± 10	781 ± 25
5mM glycerol + 5mM xylitol	410 ± 28	883 ± 33

[a]Mean average ± SEM of 6 analyses.
Approximately 1 million cells were incubated in Krebs - Ringer phosphate solution containing 10mM glucose.

Table V

Effect of Fatty Acids on Glycogen Content of Liver Cells

Additional Substrates	Glycogen Content ($\mu g/10^6$ cells)[a]	
	Expt. 1[b]	Expt. 2[c]
	Not incubated	
None	540 ± 22	666 ± 12
	Incubated for 1 hr. at 37°C	
None	100 ± 9	180 ± 4
Glycerol	217 ± 10	485 ± 15
Glycerol + Palmitate	189 ± 9	471 ± 8
Glycerol + Caproate	223 ± 8	445 ± 11
Glycerol + Oleate	104 ± 15	158 ± 12

[a]Mean average ± SEM of 4 analyses.

[b]5mM glycerol, 1mM fatty acid.

[c]10mM glycerol, 2mM fatty acid.
Approximately 1 million cells were incubated in 1 ml of Krebs - Ringer phosphate solution containing 10mM glucose. Ten times the final concentration of fatty acid was homogenized with 2% albumin. 0.1 ml aliquots of this complex were added to the flasks.

demonstrated in Table IV. Again in one experiment glycogen breakdown was negligible.

The addition of some substrates diminishes the effect of glycerol. Both 10 mM alanine and 10 mM serine lowered the glycogen content of cells incubated with 10 mM glycerol and 10 mM glucose by 25%. However, the effect of these two amino acids is not as dramatic as that observed with oleate. This unsaturated fatty acid markedly stimulates glycogen breakdown (Table V). In contrast, palmitate and caproate have essentially no effect.

The results presented have been obtained with cells incubated in Krebs-Ringer phosphate solution. Work in progress in this laboratory is showing that essentially the same results are obtained when Krebs-Ringer bicarbonate solution is the medium of incubation.

DISCUSSION

By adding glycerol or fructose to the incubation medium we have been able to prevent much of the breakdown of glycogen which occurs in isolated parenchymal cells from fed rat liver. Similar observations have recently been described with the isolated perfused liver, although fructose was not added until after 90 minutes of incubation (6). Consequently, the results were not as dramatic as those described here. Since, in our experimental conditions, there is a loss of 5-15% of viable cells during incubation, our results with added glycerol may reflect glycogen synthesis in living cells and breakdown in dead cells.

We believe that the transportation of glycerol to the liver or its synthesis there from precursors such as fructose plays an important physiological role in the control of glycogen synthesis in this organ. In our experiments, glycerol was the most effective substrate at inhibiting glycogen breakdown. Furthermore, low rates of glycogen synthesis are observed when the perfused liver (7) or isolated hepatocytes (8) from starved rats are provided solely with glucose as the exogenous substrate. Higher rates are observed when glycerol (9) or fructose are added (10). The addition of

serine and pyruvate in combination with glycerol and glucose has also been utilized in studies involving glycogen synthesis (11).

We suggest that glycerol plays a dual role in glycogen metabolism. First, it provides carbon for glycogenesis, as has been demonstrated by Longmore et al. (9). Second, it esterifies endogenous fatty acids and so removes those, such as oleate, which stimulate glycogen breakdown. The latter role would appear to be particularly important, as demonstrated by our results. Thus, if we assume that the carbon from glycerol is incorporated into glycogen by gluconeogenesis via the Embden-Myerhof pathway, then we should have observed similar effects on the glycogen content of hepatocytes with added xylitol or dihydroxyacetone. Both of these substrates greatly enhance gluconeogenesis (12,13). If the presence of glycerol is required for glycogen formation, then we have to assume that the glycerol formed from fructose is derived mainly from D-glyceraldehyde obtained by the cleavage of fructose-1-phosphate. The small effect of dihydroxyacetone and the large effect of fructose on preventing breakdown in our studies are indicative of this.

Our results also show that xylitol is more effective than fructose or pyruvate in preventing glycogen breakdown when glycerol is also present (Table III). This implicates that activity of the pentose phosphate shunt may also be involved in glycogen synthesis.

Several mechanisms could explain the effect of oleate in stimulating the breakdown of glycogen in our studies. The removal of glycerol by esterification to oleate would not appear to be a satisfactory explanation since palmitate did not significantly affect glycogen levels. Therefore, oleate may either control an enzyme involved in glycogen breakdown or partially uncouple respiration and so reduce ATP levels in the cells.

We have been unable to demonstrate that insulin directly stimulates glycogen synthesis in the liver. In general, such effects are small and require manipulated conditions (8,11). In view of our finding that oleate

stimulates breakdown of glycogen, one has to consider that the main effect of insulin on glycogen synthesis in the liver in vivo is to inhibit the release of oleate and perhaps other unsaturated fatty acids from adipose tissue (14) and so reduce their flow to the liver. Consequently, glycogen breakdown would be halted and synthesis could then occur.

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