GLYCOGEN SYNTHESIS BY A STRAIN OF EHRLICH ASCITES TUMOR

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Summary

A strain of Ehrlich Ascites cells, H, contains glycogen and synthesizes glycogen in vitro from added glucose or fructose. Only a fraction of the cells contain glycogen, and it is distributed in cytoplasm and nucleus.

The metabolism of glucose and activities of enzymes of glycogen metabolism in the H cells, was compared to a strain (HL) which does not contain or form glycogen. The activities of glycogen phosphorylase, glycogen synthase, phosphoglucomutase and UDP glucose pyrophosphorylase were of similar magnitude in both strains. Glucogon, epinephrine, and dibutyryl CAMP had no effect on the metabolism of glucose and its incorporation into glycogen in H cells, and were also without effect on its catabolism.

The occurrence of glycogen in rodent tumors is quite rare (1). We have investigated the metabolism of glucose in 3 strains of mouse Ehrlich ascites cells, which have been used by us in a previous study (2). We observed that one strain, H, incorporated in vitro glucose rapidly into glycogen, whereas the other 2 strains did not form any at all. It was of interest to determine the reasons underlying this difference. In the present study we report our findings on the utilization of glucose and the enzymes of glycogen metabolism in two strains, H, which forms glycogen and HL, which does not. The synthesis and regulation of glycogen metabolism in the ascites cells seems to differ in important aspects from that in liver and muscle.

METHODS

Ascites Cells. Two hyperdiploid strains, H and HL, were obtained from Professor T. Terranova of the Catholic University in Rome, Italy. The cells were propagated by i.p. injection in mice and harvested 9 to 15 days after inoculation.

Methods of Incubation, Fractionation and Metabolite Assays. These were essentially as previously described (2). Briefly, 0.15 to 0.25 ml packed cells were incubated at 38° in 1.5 or 2 ml of Krebs-Henseleit bicarbonate buffer in an atmosphere of 95-02-5% CO_2 with ^{14}C labelled substrates. The incubation was terminated with perchloric acid. The $^{14}CO_2$ was collected, and the extract made to volume and in part was neutralized with KOH. This was used for assay of glucose and lactate and isolation of labelled products as previously described (2). Glycogen is completely extracted with perchloric acid. It was precipitated from a portion of the extract with 2 volumes of ethanol after standing overnight in the cold and centrifuged down. The precipitate was dissolved in 1N KOH, reprecipitated with 2 volumes of ethanol and the glycogen washed with 70% ethanol and dissolved in 1 ml of 0.5 M sodium acetate buffer pH 4.5 containing 1 mg/ml of amyloglucosidase (Sigma). After 1 hour at 45° the solution was assayed for glucose and for ^{14}C. Cells were killed with perchloric acid at 0 time for the determination of initial glycogen levels and initial concentration of glucose and lactate. The cell residue was washed with 50% ethanol and dried at 50° to obtain the dry weight. Our results are expressed per 100 mg dry weight. One ml of packed ascites cells is equivalent to 100 to 120 mg dry weight.

Microscopic Techniques. Washed cells were fixed in cold cacodylate buffered 3% glutaraldehyde. Cells for electron microscopic examination were post-fixed in 1% cacodylate buffered oxmium tetroxide, dehydrated in a graded series of acetone and embedded in Epon 812. Thin sections were stained with uranium acetate and lead citrate. The sections were stained with azure II and methylene blue. Selected plastic and paraffin embedded sections were stained with periodic-acid-Schiff (PAS) Reagent with diastase controls.

Enzyme Assays. The cells were washed and stored frozen. For enzyme assays the thawed cells were sonicated in 6 volumes of medium at the maximal energy of the Bronson sonicator, for 20 seconds followed by 1 minute of chilling, repeated 4 times. The extract was centrifuged in the cold for 15 minutes at 12,000 G and the supernatant used immediately for enzyme assays. For glycogen synthase the extraction buffer was 50 mM Tris, 5 mM EDTA, 250 mM sucrose and 6 mM mercaptoethanol, pH 7.8. For the other enzymes the extraction medium was 50 mM Tris, pH 7.8, 50 mM sodium fluoride, 6 mM mercaptoethanol and 0.2% desoxycholate. Glycogen synthase (E.C. 2.4.1.11) was assayed by incorporation of ^{14}C glucose from UDP glucose into glycogen according to Thomas et al (3). Phosphoglucomutase (E.C. 2.7.5.1), UDP glucose pyrophosphorylase (E.C. 2.7.7.9) and phosphorylase (E.C. 2.4.1.1) were assayed spectrophotometrically, by coupling with auxiliary enzymes, by the oxidation of glucose-6P with glucose-6P dehydrogenase according to Kuhn and Loewenstein (4), Villar-Palasi and Larner (5) and Thurston et al (6), respectively.

Results

Cytology. Washed H cells were found to contain from 3 to 8 mg

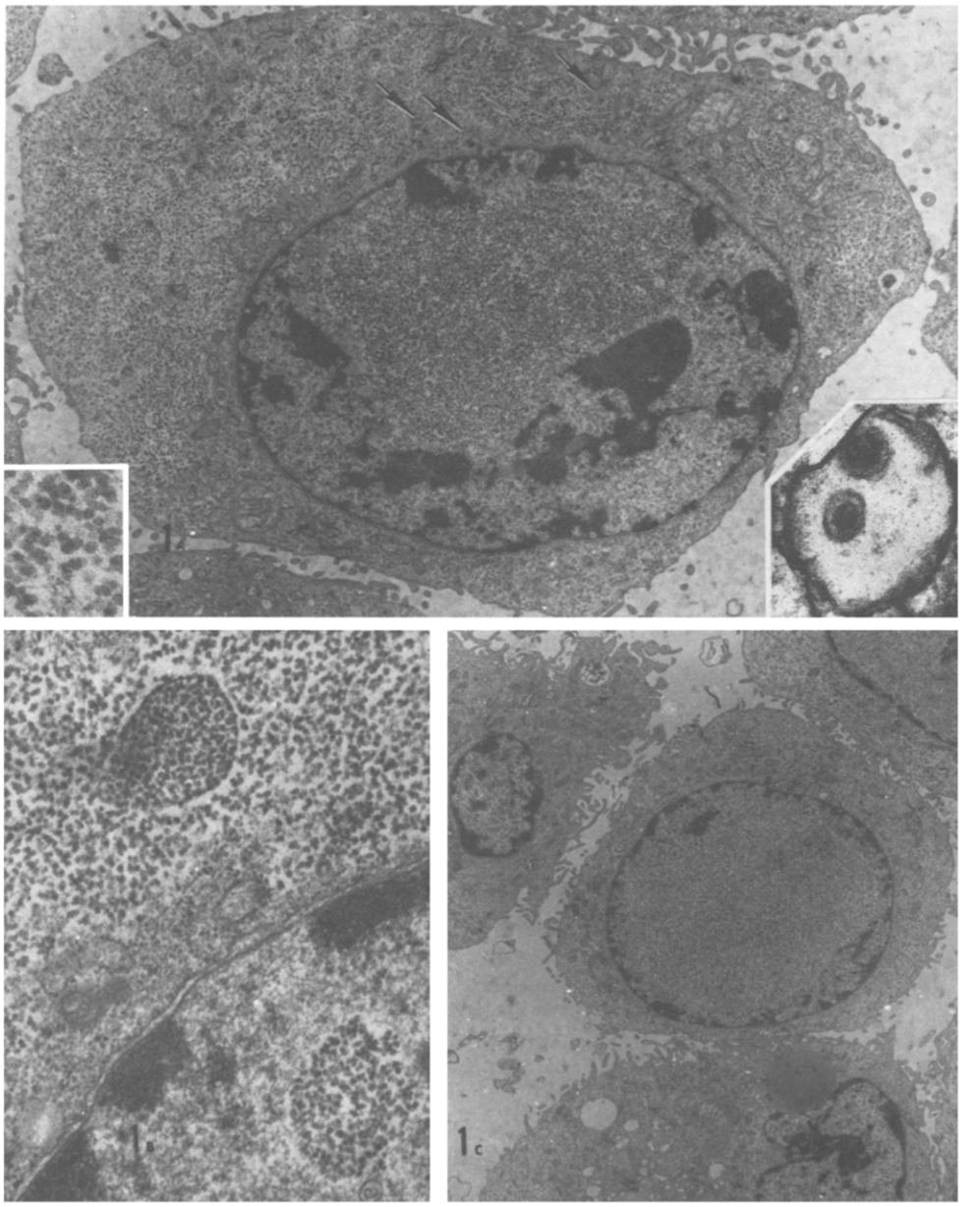

Figure 1

1A- Nuclear and cytoplasmic glycogen in H cells. In the nucleus the glycogen is confined to the central area and is surrounded by a band of karyoplasm of variable width. Arrows point to virus inclusions X 8750. Insert left, nuclear glycogen, X 80,000. Insert right, virus particles, X 100,000. 1B- Nuclear glycogen formation by small focus of glycogen particles. Note cytoplasmic glycogen particles surrounded by thin membrane. The significance of this structure is unknown X 40,000. 1C- Adjacent glycogen rich and glycogen free cells. Glycogen free cells are located at the top left and bottom of a tumor cell which shows a nucleus almost totally filled with glycogen particles X 5,000.

of glycogen per ml of packed cells. The presence of glycogen was confirmed histochemically with the periodic-acid-Schiff (PAS) stain. Diastase pretreated sections showed very little PAS positive material. The glycogen content of the cells differed greatly. From 15 to 25% contained heavy deposits uniformly distributed in cytoplasm and nucleus. Other cells contained little glycogen or the glycogen was localized predominantly in the nucleus. Over half of the cells appeared to be glycogen free. This is shown in electron micrographs of Figure 1. The glycogen was in the form of irregular granules 150-200 angstrom in diameter (Figure 1A). In some cells the glycogen deposit was in well defined dense small clusters, which was found in both nucleus and cytoplasm (Figure 1B). Cells that appeared otherwise very similar varied greatly in glycogen content (Figure 1C). Virus particles (Figure 1A) were present in cells with or without glycogen. Virus was also present in HL cells which contained no glycogen.

Glucose Metabolism. In early experiments we found close agreement between glucose uptake as determined from ^{14}C yields and by analysis in HL and W cells, but in H cells the ^{14}C recovered in the neutral fraction (eluted with water from the resins) exceeded considerably the calculated residual activity in glucose, as determined from the disappearance of glucose from the medium. A search for the extra ^{14}C revealed it to be glycogen.

In Table 1 we present typical carbon balances for the utilization of glucose by H and HL cells. Glucose uptake by H cells was somewhat higher than that of HL cells. H cells formed more acetate but less amino acids than HL cells. This pattern is also observed with lactate as substrate (2). The increase in cell glycogen in H cells was 2-4 mg/hr per ml packed cells. No synthesis whatsoever was seen in the other strains. Fructose served as well as glucose for glycogen synthesis.

TABLE I

Glucose Utilization by H and HL Strains of Ehrlich Ascites Tumor.

Experiment	1a	2a	1b	2b
Strain	H	H	HL	HL
	mg/100 mg*			
Glycogen**				
Initial	7.1	4.2	< 0.1	0
Final	8.9	7.8	< 0.1	0
	µatoms/100 mg*			
Glucose Uptake**	91	136	52	100
Lactate Formation**	122	124	78	127
	µatoms C/100 mg*			
^{14}C Recovery***				
CO_2	16	37	9	17
Acetate	11	23	6	13
Lactate	350	470	220	360
Pyruvate	46	42	27	23
Other Acids	6	7	8	4
Amino Acids	14	30	25	43
Glycogen	86	70	0	0
Residual		10		7
Total	525	689	295	467
Isotope Recovery	93	98	94	99

Cells, 24-30 mg dry weight incubated for one hour in 2 ml of buffer 15 mM in glucose, labelled uniformly with ^{14}C.

*Dry weight; ** determined by chemical analysis; *** calculated from ^{14}C recoveries in fractions.

Enzymes of Glycogen Metabolism. The absence of glycogen could be due to a deletion of a key enzyme by mutation of the HL strain. However enzyme assays (Table II) indicate that the activities of glycogen synthase, UDG pyrophosphorylase and phosphoglucomutase as well as phosphorylase are of the same order in both strains. The addition of AMP had little effect on phosphorylase activity, which appears to be in the phosphorylated active form. Glycogen synthase was however

TABLE II

Enzymes of Glycogen Metabolism in H and HL Strains of Ehrlich Ascites Tumor.*

		H	HL	Mouse Liver
		µmoles/min/ml packed cells		
Phosphorylase	-AMP	(11) 0.18 ± 0.04	(5) 0.44 ± 0.03	3.9
	+AMP	0.24 ± 0.03	0.46 ± 0.04	
P-Glucomutase		(8) 7.1 ± 1.3	(6) 4.1 ± 0.03	9.0
UDPG Pyrophosphorylase		(5) 4.0 ± 0.2	(4) 2.3 ± 0.2	21
Glycogen Synthase	-G6P	(6) 0.021 ± 0.004	(6) 0.017 ± 0.004	0.36
	+G6P	0.15 ± 0.005	0.094 ± 0.01	0.54

*( ) Number of animals, means and S.E.M.

predominantly in the dependent form, requiring glucose-6P for activity.

Effectors of Glycogen Catabolism and Synthesis. In liver and muscle glycogenolysis is stimulated by glucagon and epinephrine which act by increasing CAMP levels. In Table III the effect of these hormones and of dibutyryl CAMP on the breakdown of glycogen in H cells is shown.

The endogenous glycogen is utilized slowly when the cells are incubated in vitro without substrate, and no lactate accumulates. Apparently the rate of hydrolysis is less than the rate of lactate utilization. This contrasts sharply with the rapid glycolysis and lactate accumulation when glucose or fructose are the substrates. Addition of glucagon, epinephrine or dibutyryl cyclic AMP did not increase glycogen breakdown, and if anything decreased it. The addition of 2,4 dinitrophenol or of amytal stimulated however glycogen breakdown, and lactate accumulated.

TABLE III

Effect of Various Agents on Glycogen Breakdown.

Addition		Glycogen Breakdown	Lactate Formed*
	µg/ml	µmoles/100 mg	
None		8.1	+ 1.6
Glucagon	3	6.1	- 0.2
Epinephrine	20	6.8	- 0.3
Dibutyryl CAMP	50	7.5	+ 0.4
	mM		
2,4, DNP	0.2	11.4	18
Amytal	4	10.7	25

23 mg on cells (dry weight) incubated for 1.5 hours without substrate. Initial glycogen content, 41 µmoles/100 mg.

*(+) Indicates lactate formation; (-) uptake.

TABLE IV

Effect of Glucagon, Epinephrine, Dibutyryl Camp (DB CAMP) and 2,4 Dinitrophenol (2, 4 DNP) on Glycogen Synthesis.

Addition	Δ Glycogen	Δ Glucose	Δ Lactate	Glucose Carbon In: CO_2	Lactate	Other Cmpds	Glycogen	Total
	µmoles/100 mg*			µatoms C/100 mg*				
None	+24	-193	+300	14	860	115	130	1120
Glucagon	+24	-198	+305	14	880	110	132	1140
Epinephrine	+26	-197	+312	14	870	110	138	1120
DB CAMP	+25	-201	+310	13	890	105	135	1140
2,4, DNP	+ 4	-298	+550	40	1610	90	28	1770

7.8 mg of cells, (dry weight) incubated for one hour in 1.5 ml buffer, 15 mM in glucose labelled uniformly with ^{14}C. Concentration of additions as in Table III.

*Dry weight.

It appears that the glycogenolytic hormones which act via CAMP have no effect on glycogen catabolism in H cells. In liver and muscle they also block the synthesis of glycogen but in the H cells they were without any effect (Table IV).

DISCUSSION

Glycogen is found in human tumors where it is frequently localized in the nucleus (7), and it has also been observed in nuclei of mast tumors of dogs (8). It is rare however in rodent tumors (1). Thus only a few out of 70 strains of Yoshida rat ascites hepatoma can form glycogen (9). Nirenberg (10) could not detect neither glycogen nor glycogen synthase and phosphorylase in a number of tumors, including Ehrlich ascites, studied by him. Paweletz (11) found one out of five Ehrlich ascites strains to contain glycogen, which was present in nucleus and cytoplasm, and the occurrence of nuclear glycogen is a tetraploid strain of Ehrlich ascites and has been reported (12). We found no glycogen in a tetraploid strain, W, studied previously by us.

The variation in glycogen content in the cell population is of considerable interest. The content of some cells was probably as high as 3 to 5% of fresh weight, while the majority contained none. It appears reasonable that glycogen synthesis is associated with a certain transient stage in cell development of these rapidly dividing and growing cells. We could not however detect any obvious differences, apart from glycogen content. There is some indication that glycogen synthesis is initiated in special granules (See Figure 1B), found predominantly but not solely in the nucleus.

The strains of Ehrlich ascites are descendant from a mouse mammary adenocarcinoma cultured by Ehrlich since 1906 and in ascites form since 1932 (13). Letnanski (13) has compared the metabolism of over a dozen of these strains, including H, but did not examine them for their capacity to form glycogen.

The glycogen metabolism of ascites cells poses some interesting questions. It is not clear why only a few strains form glycogen, while others such as HL, which contain the necessary complement of

enzymes, do not. It is not clear why only a fraction of the cells accumulate glycogen and whether this is associated with cell development.

The enzymes of glycogen metabolism appear to differ from the "classical" system in normal tissues. The localization of the synthesis is at least in part nuclear and there is no hormonal regulation, mediated via CAMP, of synthesis and catabolism.

Further study of the properties of ascites tumor glycogen and glycogen forming enzymes should be of interest.

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