Pathways of Glycogen Synthesis in Novikoff Ascites-Hepatoma Cells

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Affinity of glucose, fructose and mannose for tumour hexokinase and their rates of phosphorylation at saturation concentration have been correlated with rates of glycogen synthesis by intact tumour cells at different concentrations of the three substrates. Competition experiments with one sugar labelled and the other sugar unlabelled indicate inhibition of glycogen synthesis by the sugar with a low K_m for hexokinase. Glycogen synthesis from glucose 1-phosphate in aged cells and from nucleoside in freshly prepared cells is stimulated by fructose and inhibited by glucose. The decrease in glycogen formation from glucose 1-phosphate by oligomycin is partially overcome by increased fructose concentrations. These results are explained by an activation of α -glucan phosphorylase by fructose and an inhibition of this enzyme by glucose. It is suggested that differences in localization of glucose 6-phosphate, available to the intact cell in various ways, determine its transformation into glycogen by either the UDP-glucose- α -glucan glucosyl-transferase reaction or by the α -glucan phosphorylase reaction.

Recent studies from a number of laboratories including our own have shown some incompatibilities between the suggested pathway of glycogen synthesis (Leloir & Cardini, 1962) and certain experimental observations. For example, in liver slices (Figueroa & Pfeifer, 1964), in tumour cells (Nigam, 1967a) and in isolated rat diaphragm (Beloff-Chain *et al.* 1964; Landau & Sims, 1967), glucose 6-phosphate is a poorer precursor of glycogen than glucose, although its catabolism to carbon dioxide or lactate or both exceeds that of glucose. Glucose 6-phosphate also activates glycogen synthesis from [14 C]glucose in rat diaphragm (Beloff-Chain *et al.* 1964) and in tumour cells (Nigam, 1967a) despite its being an inhibitor of mammalian hexokinase (Crane, 1962) and a diluent of label in glucose 6-phosphate formed from [14 C]glucose. In other studies the rate of glycogen synthesis in the starved diabetic rat has been shown to be higher than the activity of hexokinase in the supernatant fraction of liver of these rats (Friedmann, Goodman & Weinhouse, 1967). Data on the specific radioactivity of glucose 6-phosphate and UDP-glucose in livers of rats given [14 C]glucose have been found to be inconsistent with the precursor-product relationship expected from the scheme: glucose $\rightarrow$ glucose 6-phosphate $\rightarrow$ glucose 1-phosphate $\rightarrow$ UDP-glucose (Threlfall, 1966).

Although these observations suggest that transformation of glucose into glucose 6-phosphate may not be an essential step in glycogen synthesis,

evidence is still lacking for the existence of a pathway not involving hexose phosphorylation. As an alternative explanation a number of authors (Muntz & Vanko, 1962; Nigam & Fridland, 1964; London, 1966; Nigam, 1967a; Landau & Sims, 1967; Threlfall & Heath, 1968) have suggested the possibility of compartmentation of glucose 6-phosphate during the metabolism of glucose in intact cells.

In this paper experiments designed to test exclusion or inclusion of hexokinase in glycogen synthesis in Novikoff ascites-hepatoma cells are described. Affinity of a sugar for tumour hexokinase has been correlated with the amount of glycogen formed at different concentrations of the sugar, and two sugars of different affinities for hexokinase (one labelled and the other unlabelled) have been incubated together with the tumour cells and the incorporation of label into glycogen compared in competition experiments. These results have lent support for hexokinase participation.

Further, it has been observed that fructose acts as an activator and glucose and mannose act as inhibitors of glycogen synthesis from glucose 1-phosphate in aged tumour cells, from glucose 6-phosphate formed from nucleoside, and from hexose, when oxidative ATP generation is inhibited. Since it has been shown by Holmes & Mansour (1968) that incubation of rat diaphragm with glucose results in a decreased activity of α -glucan phosphorylase, whereas fructose diphosphate yields

an opposite effect, it is proposed that, for tumour cells, activation of glycogen synthesis by fructose and inhibition by glucose and mannose in the above situations are a result of increased and decreased activities of tumour α -glucan phosphorylase respectively.

MATERIALS AND METHODS

Chemicals. Reagent-grade glucose (dextrose) was obtained from Baker and Co., Phillipsburg, N.J., U.S.A. Mannose and fructose were products of Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Sodium salts of glucose 6-phosphate and UDP-glucose and the dipotassium salt of glucose 1-phosphate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. All other chemicals were of reagent-grade purity available from commercial sources. [$1\text{-}^{14}\text{C}$]Glucose, [$1\text{-}^{14}\text{C}$]fructose, [$1\text{-}^{14}\text{C}$]mannose, [$1\text{-}^{14}\text{C}$]glucose 6-phosphate, [$1\text{-}^{14}\text{C}$]glucose 1-phosphate and UDP-[$1\text{-}^{14}\text{C}$]glucose were supplied by New England Nuclear Corp., Cambridge, Mass., U.S.A. [$1\text{-}^{14}\text{C}$]Adenosine and [$1\text{-}^{14}\text{C}$]uridine were obtained from The Radiochemical Centre, Amersham, Bucks.

Animals. Sprague-Dawley rats weighing 150–200 g. were obtained from the Canadian Breeding Laboratories, St Constant, Que., Canada. The rats were kept on a diet of Labena Chow supplied by Ralston Purina Co., St Louis, Mo., U.S.A. Food and water were available to the animals *ad libitum*.

Preparation of tumour cells. These were prepared as described by Nigam (1967b).

Isolation of glycogen and determination of its ^{14}C . Details of the procedure used for the isolation of glycogen and determination of its ^{14}C content were the same as described by Nigam (1967b).

Determination of lactic acid. Lactic acid was determined either by the enzymic procedure described by Hohorst (1963) or by the chemical procedure of Barker (1957).

Assay of hexokinase (EC 2.7.1.1) activity. This was measured by the procedure described by Viñuela, Salas & Sols (1963). For the determination of K_m of glucose for hexokinase different concentrations of hexose were employed. When different concentrations of fructose were employed for the determination of its K_m , 1 unit of phosphohexose isomerase was also added to the reaction mixture used for hexokinase assay. Mannose phosphorylation was observed by measuring the incorporation of [$1\text{-}^{14}\text{C}$]mannose into barium acetate-ethanol-insoluble material. The same reaction mixture as that used for the determination of the K_m of glucose for hexokinase was employed except that different concentrations of [$1\text{-}^{14}\text{C}$]mannose replaced different concentrations of glucose.

Rates of phosphorylation of glucose, fructose and mannose at saturation concentration were measured in a reaction mixture that contained ($\mu\text{moles/ml.}$): ATP, 15; MgCl_2 , 10; tris, 50; histidine, 50; EDTA, 2; hexose, 15 (approx. 100000 counts/ml.) and tumour homogenate equivalent to 0.012 ml. of packed cells contained in 0.1 ml. of 0.25 M-sucrose. Solutions of ATP, tris, histidine and EDTA were adjusted to pH 7.0 before addition. The reaction was carried out at room temperature (26–28°) for 5–10 min. It was stopped by the addition of 2 ml. of ethanol. Carrier 6-phosphates of glucose, fructose and mannose (5 μmoles each dissolved in 0.1 ml. of water) were added to

the appropriate digests, followed by the addition of barium acetate (50 μmoles dissolved in 0.1 ml. of water). The resulting precipitate was collected by centrifugation. The precipitate was washed twice with 3.0 ml. of 70% ethanol, dissolved in dilute HCl, treated with 0.05 ml. of saturated Na_2SO_4 soln., centrifuged, neutralized with NaOH soln. and the final volume of the solution was noted. A portion of the supernatant was transferred to a planchet for counting its ^{14}C . Control experiments were carried out with [$1\text{-}^{14}\text{C}$]glucose 6-phosphate of known c.p.m. and corrections were applied for self absorption due to salts. The same correction factor was employed for hexose 6-phosphate formed from the three sugars. The amounts of hexose 6-phosphate formed was given by multiplying the corrected c.p.m. in the isolated hexose monophosphate by 15 and dividing this value by the c.p.m. in the labelled hexose added to the reaction mixture.

Aging of tumour cells. Tumour cells were aged by keeping washed packed cells at 0–5° for 18–24 hr. (Nigam, 1967a). They were washed once before they were used in metabolic experiments.

Preparation of tumour-cell homogenates. Tumour cells were homogenized after exposure to osmotic shock as described by Hawtrey & Silk (1960) and Nigam (1967b). Packed tumour cells (1 vol.) were suspended in 5 vol. of one-quarter-strength cold tris-wash medium (Wu & Racker, 1959), and were shaken under ice for 10–15 min. The suspension was centrifuged and the swollen cells (now twice their original volume) were homogenized in a tight-fitting Potter-Elvehjem homogenizer for 2 min. at 0–5°, after the addition of an equal volume of cold water. To the homogenate an equal volume of cold 0.5 M-sucrose was added and the mixture was centrifuged at 2500g for 10 min. in an International refrigerated centrifuge. This resulted in the removal of unbroken cells and nuclei. Approx. 7 ml. of supernatant was recovered when 1 ml. of packed cells was used. Since 85–90% of the cells were broken during homogenization, 7 ml. of the supernatant contained the cytoplasmic content originally present in 0.85–0.90 ml. of packed cells.

Incubation procedure. Tumour-cell suspensions (0.9 ml. containing 0.2–0.5 ml. of packed cells, or as described in legends to tables and figures) were incubated with known amounts of labelled and unlabelled sugars dissolved in tris incubation medium (Wu & Racker, 1959). The total volume of the digest in each case was 1 ml. The reaction mixture was contained in 20 ml. beakers, which were placed in a Dubnoff metabolic shaker maintained at 37°, flushed with O_2 and shaken at the rate of 10–20 strokes/min. At intervals, the reaction was stopped by the addition of an equal volume of 60% KOH. Glycogen was isolated and its radioactivity was determined. The glucose equivalent of glycogen formed was obtained by multiplying the c.p.m. in isolated glycogen by the nmoles of sugar added and dividing this value by the c.p.m. in the labelled sugar added.

When determination of lactic acid was also required, identical duplicate reaction mixtures were used: one was used for the determination of glycogen and the other was transferred to a tube and heated at 100° for 2 min. After centrifugation, lactic acid was determined in a portion of the supernatant.

Although aging of the cell results in an increase in the tumour-cell volume, the results presented for aged cells are based on packed cell volume before aging.

Rate of glycogen synthesis during sequential addition of different amounts of ^{14}C -labelled hexose. These rates (Fig. 1) were measured as described by Nigam (1967b).

RESULTS

Relationship between hexose phosphorylation in homogenates and glycogen synthesis in intact tumour cells as a function of substrate concentration. Phosphorylation of glucose, fructose and mannose by tumour hexokinase was measured as a function of substrate concentration. From the Lineweaver-Burk plots of $1/v$ against $1/s$, the K_m values determined were $6.3 \pm 0.8 \times 10^{-5}\text{M}$, $3.2 \pm 0.3 \times 10^{-3}\text{M}$ and $3.8 \times 10^{-5}\text{M}$ for glucose, fructose and mannose respectively in three experiments. The K_m for mannose differed significantly ($P < 0.01$) from that for glucose. When phosphorylation of each sugar was measured at 15mM concentration, the rates of phosphorylation/min./0.1 ml. of homogenate were 0.02, 0.035 and 0.008 for glucose, fructose and mannose respectively. The K_m values are within the range observed for mammalian hexokinase I (Schimke & Grossbard, 1968) and particulate hexokinase of Krebs ascites-tumour cells (McComb & Yushok, 1959). The observed rates of phosphorylation are also in the same order as those obtained by McComb & Yushok (1959).

To study the effect of substrate concentration on glycogen synthesis by intact tumour cells it was necessary to add the substrate at short intervals to maintain its desired concentration approximately, since metabolism of the substrate by the tumour cells decreases its molarity in the medium. Thus experiments were carried out in which known and increasing amounts of ^{14}C -labelled hexose were initially added to cell suspensions. The concentrations of the substrate in each reaction mixture were maintained by supplementing with an appropriate amount of substrate at intervals of 1 min. The reaction was terminated after 4 min., glycogen was isolated and measured and an average value of glycogen synthesized/min. at different concentrations of the substrate could thus be obtained. Fig. 1 shows that increase in glycogen synthesis occurred as the amount of glucose and mannose added was increased from 0.1 to 0.2 $\mu\text{mole/min.}$

There was no increase when the amount of glucose and mannose added was raised to 0.5 $\mu\text{mole/min.}$ Fructose gave little glycogen synthesis at these low concentrations and therefore higher concentrations of fructose were employed. Glycogen synthesis from fructose increased as the amount of fructose added was raised from 1.0 to 5.0 $\mu\text{moles/min.}$ It is apparent that glycogen synthesis by intact tumour cells from glucose, fructose and mannose is related to changes in the substrate concentration, as is their affinity for hexokinase.

Glucose and mannose, which have low K_m values for hexokinase, attain maximal rates of glycogen synthesis at concentrations lower than those required for fructose, which has a high K_m for hexokinase.

Further, when a high concentration (15 mM) of the sugar was added to the tumour cells (see Table 1, values marked with an asterisk), the amount of glycogen synthesized by the tumour cells was in the order fructose > glucose > mannose, which is the same order as that observed for their rates of phosphorylation at 15 mM concentration.

Glycogen formation from a labelled sugar in the presence of an unlabelled sugar. The affinities of hexoses for tumour hexokinase predict that, if glycogen synthesis is dependent on hexose phosphorylation, a sugar with high affinity for hexokinase should inhibit glycogen synthesis from a sugar with a lower affinity for the enzyme. These

Fig. 1. Variations in the rates of glycogen synthesis by Novikoff ascites-hepatoma cells due to variations in the concentrations of the substrates (Δ , fructose; $\bullet$, glucose; $\blacksquare$, mannose). Tumour-cell suspensions (0.9 ml. containing approx. 0.18 ml. of packed cells) were added to nine 20 ml. beakers, which were later transferred to a Dubnoff metabolic shaker maintained at 37° . At zero time 0.02 ml. of labelled glucose and mannose solutions (0.1, 0.2 and 0.5 μmole ; 90000–100000 c.p.m.), or labelled fructose solution (1, 2 and 5 μmoles ; 90000 c.p.m.), were added to individual beakers to give three different initial concentrations of each sugar. These concentrations were maintained approximately constant by further addition of the same amounts of the labelled sugars at intervals of 1 min. to respective beakers to make up the fall in concentration due to utilization of the hexose. After 4 min., the digests were inactivated by the addition of 1 ml. of 60% KOH. The procedure of incubation and the isolation of glycogen and estimation of its ^{14}C are described in the Materials and Methods section. Rates of glycogen synthesized/min. were obtained by dividing the amount of glycogen formed by 4.

Table 1. *Formation of [^{14}C]glycogen from ^{14}C -labelled hexose in the presence of unlabelled sugar by Novikoff ascites-hepatoma cells*

The reaction mixture consisted of 0.9 ml. of tumour-cell suspension containing 0.18 ml. of packed cells, 0.05 ml. of ^{14}C -labelled hexose soln. (approx. 40 000 c.p.m.) and 0.05 ml. of unlabelled hexose soln. The sugars were added after dissolution in tris incubation medium. Experimental conditions are described in the Materials and Methods section. Results are expressed as means $\pm$ S.D. of those obtained in three experiments.

Labelled hexose (5 mm)	Addition	Glycogen formed (μ moles of glucose equiv./15 min./ml. of reaction mixture)
Glucose	None	1.6 $\pm$ 0.4
Glucose	Glucose (10 mm)	1.9 $\pm$ 0.6*
Glucose	Fructose (10 mm)	1.4 $\pm$ 0.6
Glucose	Mannose (10 mm)	0.4 $\pm$ 0.2
Fructose	None	0.9 $\pm$ 0.3
Fructose	Glucose (10 mm)	0.2 $\pm$ 0.1
Fructose	Fructose (10 mm)	2.3 $\pm$ 0.2*
Fructose	Mannose (10 mm)	0.1 $\pm$ 0.1
Mannose	None	0.6 $\pm$ 0.3
Mannose	Glucose (10 mm)	0.5 $\pm$ 0.4
Mannose	Fructose (10 mm)	0.6 $\pm$ 0.4
Mannose	Mannose (10 mm)	0.6 $\pm$ 0.2*

* Calculated on the basis of addition of 15 mm substrate/ml. of reaction mixture.

Table 2. *Effect of increase in fructose and glucose concentration on glycogen synthesis and lactate production in the absence and presence of oligomycin and 2,4-dinitrophenol by Novikoff ascites-hepatoma cells*

The reaction mixture consisted of 0.9 ml. of tumour-cell suspension containing 0.2 ml. of packed cells, 0.03 ml. of distilled water, or of 66% ethanol, or of 5 mm-2,4-dinitrophenol soln. in water, or of 14 mm-oligomycin soln. in 66% ethanol, and 0.07 ml. of labelled glucose or fructose soln. (approx. 50 000 c.p.m.) in tris incubation medium. Experimental conditions for incubation, determination of lactate and isolation and determination of glycogen are described in the Materials and Methods section. Results are expressed as means $\pm$ S.D. of those obtained in two to four experiments.

Labelled hexose and concn. (mm)	Addition	Lactate produced (μ moles/15 min./ml. of reaction mixture)	Glycogen formed (μ moles of glucose equiv./15 min./ml. of reaction mixture)
Fructose, 5	Water	1.6 $\pm$ 0.1	1.08 $\pm$ 0.30
Fructose, 5	Ethanol	1.5 $\pm$ 0.3	1.02 $\pm$ 0.24
Fructose, 5	2,4-Dinitrophenol	2.5 $\pm$ 0.5	0.02 $\pm$ 0.0
Fructose, 5	Oligomycin	1.6 $\pm$ 0.3	0.15 $\pm$ 0.04
Fructose, 15	Water	2.4 $\pm$ 0.2	2.0 $\pm$ 0.20
Fructose, 15	Ethanol	2.4 $\pm$ 0.3	2.05 $\pm$ 0.20
Fructose, 15	2,4-Dinitrophenol	5.0 $\pm$ 0.2	0.30 $\pm$ 0.05
Fructose, 15	Oligomycin	2.3 $\pm$ 0.3	0.8 $\pm$ 0.15
Glucose, 5	Water	2.6 $\pm$ 0.2	1.40 $\pm$ 0.30
Glucose, 5	Ethanol	2.3 $\pm$ 0.4	1.35 $\pm$ 0.30
Glucose, 5	2,4-Dinitrophenol	5.1 $\pm$ 0.1	0.04 $\pm$ 0.0
Glucose, 5	Oligomycin	2.6 $\pm$ 0.1	0.12 $\pm$ 0.0
Glucose, 15	Water	2.7 $\pm$ 0.2	1.65 $\pm$ 0.25
Glucose, 15	Ethanol	2.8 $\pm$ 0.2	1.95 $\pm$ 0.32
Glucose, 15	2,4-Dinitrophenol	5.5 $\pm$ 0.0	0.03 $\pm$ 0.0
Glucose, 15	Oligomycin	2.9 $\pm$ 0.2	0.15 $\pm$ 0.02

predictions are borne out by data given in Table 1. Glycogen synthesis from glucose by intact tumour cells is inhibited by mannose but not by fructose; glycogen synthesis from fructose is inhibited by

both glucose and mannose whereas glycogen synthesis from mannose is not susceptible to the inhibitory effect of either glucose or fructose. However, in these experiments glucose failed

Table 3. *Formation of [^{14}C]glycogen from [^{14}C]glucose monophosphates and UDP-[U- ^{14}C]glucose in the absence and presence of unlabelled hexose by aged Novikoff ascites-hepatoma cells*

The reaction mixture consisted of 0.9 ml. of tumour-cell suspension containing 0.4 ml. of aged packed cells, 0.05 ml. of labelled glucose monophosphate or UDP-glucose soln. and 0.05 ml. of unlabelled hexose soln. The hexoses, glucose monophosphates and UDP-glucose were added after dissolution in tris incubation medium. Experimental conditions are described in the Materials and Methods section. Results are expressed as means $\pm$ s.d. of those obtained in three experiments.

Labelled substrate (5 mM)	Addition	Glycogen formed* ($\mu\text{moles of glucose}$ equiv./15 min./ml.) of reaction mixture)
1 Glucose 6-phosphate	None	0.10 $\pm$ 0.06
2 Glucose 6-phosphate	Glucose (10 mM)	0.05 $\pm$ 0.02
3 Glucose 6-phosphate	Fructose (10 mM)	0.12 $\pm$ 0.05
4 Glucose 6-phosphate	Mannose (10 mM)	0.05 $\pm$ 0.01
5 Glucose 1-phosphate	None	0.26 $\pm$ 0.04
6 Glucose 1-phosphate	Glucose (10 mM)	0.16 $\pm$ 0.04
7 Glucose 1-phosphate	Fructose (10 mM)	0.36 $\pm$ 0.10
8 Glucose 1-phosphate	Mannose (10 mM)	0.17 $\pm$ 0.05
UDP-glucose	None	1.2 $\pm$ 0.10
UDP-glucose	Glucose (10 mM)	2.00 $\pm$ 0.05
UDP-glucose	Fructose (10 mM)	1.95 $\pm$ 0.05
UDP-glucose	Mannose (10 mM)	1.90 $\pm$ 0.06

* *P* values: 1 versus 2, < 0.001; 1 versus 3, not significant; 1 versus 4, < 0.001; 5 versus 6, < 0.001; 5 versus 7, < 0.01; 5 versus 8, < 0.01.

to inhibit glycogen synthesis from mannose although its K_m for hexokinase is only 1.7-fold that of the K_m of mannose.

Variations in glycogen synthesis and glycolysis due to hexose concentration and the effects of 2,4-dinitrophenol and oligomycin. It is reasonable to expect that the other major reaction of hexose utilization, namely glycolysis, should be affected by variation in substrate concentration since it also depends on hexose phosphorylation. Data presented in Table 2 show that this is indeed true. Glycogen synthesis and glycolysis from glucose increase slightly and from fructose much more as their concentrations are increased. However, alterations in this behaviour occur for glycogen synthesis from fructose when the reaction is carried out in the presence of 2,4-dinitrophenol or oligomycin. Oligomycin strongly inhibits glycogen synthesis from both glucose and fructose at lower hexose concentration, but its inhibitory effect is partly offset by a higher concentration of fructose but not by a higher concentration of glucose. Glycolysis from both glucose and fructose remains unaltered in the presence of oligomycin. On the other hand, 2,4-dinitrophenol increases lactic acid production from both glucose and fructose, at low as well as at high concentration of the hexoses, to the same extent. Suppression of glycogen synthesis by 2,4-dinitrophenol is again offset slightly by an increased concentration of fructose only. These results suggest that fructose is able to stimulate an enzyme of the glycogen pathway whereas glucose is unable to exert the same influence.

Glycogen synthesis from glucose 6-phosphate, glucose 1-phosphate and UDP-glucose by aged tumour cells in the absence and presence of glucose, fructose and mannose. To establish that an enzyme of the glycogen pathway was differently affected by glucose and fructose, aged tumour cells, which hexose monophosphates and UDP-glucose can enter (Nigam, 1967a), were used and glycogen synthesis from [^{14}C]glucose 6-phosphate, [U- ^{14}C]glucose 1-phosphate and UDP-[U- ^{14}C]glucose was measured in the absence and presence of glucose, fructose and mannose. It was observed (Table 3) that, although the three hexoses stimulated glycogen synthesis from UDP-glucose, the effects of fructose were different from those of glucose and mannose when hexose monophosphates were glycogen precursors. Fructose had no effect on glycogen synthesis from glucose 6-phosphate, but it stimulated that from glucose 1-phosphate, whereas glucose and mannose inhibited glycogen synthesis from both glucose monophosphates.

Glycogen synthesis from labelled nucleosides and the effects of glucose, fructose, oligomycin and 2,4-dinitrophenol. Another method of providing intracellular hexose monophosphate for intact cells that does not involve hexose phosphorylation or aging of the cells is through the use of nucleosides. Glucose 6-phosphate is produced by the steps: nucleoside $\rightarrow$ ribose 5-phosphate $\rightarrow$ fructose 6-phosphate $\rightarrow$ glucose 6-phosphate. Uniformly labelled adenosine and uridine were incubated with intact tumour cells and they were capable of forming small amounts of [^{14}C]glycogen (Table 4). However, the

Table 4. *Formation of glycogen and lactate from uniformly labelled adenosine and uridine by Novikoff ascites-hepatoma cells and the effect of 2,4-dinitrophenol, oligomycin, glucose and fructose*

The digest consisted of 0.8 ml. of tumour-cell suspension containing 0.16 ml. of packed cells, 0.1 ml. of [^{14}C]-adenosine or [^{14}C]uridine (50 mM each; approx. 100 000 c.p.m.) in tris incubation medium, 0.03 ml. of water, or of 66% ethanol, or of 5 mM-2,4-dinitrophenol in water, or of 14 mM-oligomycin in 66% ethanol, and 0.07 ml. of tris incubation medium or of a 0.14 M solution of glucose or fructose in tris incubation medium. Experimental conditions for incubation, determination of lactate and isolation and determination of glycogen are described in the Materials and Methods section. Calculation of glycogen formed is based on ^{14}C present in the ribose moiety of the nucleosides.

Substrate	Addition	Lactate produced ($\mu\text{mole}/15\text{ min.}/\text{ml. of}$ reaction mixture)		Glycogen formed (μmole of glucose equiv./15 min./ml. of reaction mixture)	
		Expt. 1	Expt. 2	Expt. 1	Expt. 2
Adenosine	Water	0.23	0.35	0.04	0.04
Adenosine	Ethanol	0.26	0.29	0.04	0.05
Adenosine	2,4-Dinitrophenol	0.55	0.80	0.01	0.01
Adenosine	Oligomycin	0.24	0.31	0.02	0.01
Adenosine	Glucose	—	—	0.03	0.03
Adenosine	Fructose	—	—	0.07	0.08
Uridine	Water	0.13	0.18	0.016	0.012
Uridine	Ethanol	—	—	0.015	—
Uridine	2,4-Dinitrophenol	0.40	0.51	0.003	0.002
Uridine	Oligomycin	0.16	0.18	0.006	0.005
Uridine	Glucose	—	—	0.014	0.010
Uridine	Fructose	—	—	0.022	0.020

ratio of glycogen formed to lactate produced by nucleosides was much lower than that obtained for labelled hexose. Oligomycin decreased glycogen synthesis by 50% without affecting glycolysis, whereas 2,4-dinitrophenol decreased glycogen synthesis by 80% with increment in lactate production. Glucose decreased glycogen synthesis from the two nucleosides, whereas fructose had a slight stimulatory effect. These results would appear to be similar in quality to those described in Tables 2 and 3.

DISCUSSION

Observations cited in the introduction to this paper indicate that the scheme formulated to demonstrate steps in glycogen synthesis (Leloir & Cardini, 1962) is inadequate for our understanding of the complex process of glycogen formation in intact tissues. It is apparent that the inconsistencies between experimental observations and the present scheme must be resolved either by the discovery of a new pathway or by modification of the existing one. The results reported in this paper favour explanations for these inconsistencies within the framework of the existing pathway of glycogen synthesis, after making certain assumptions. No new facts have emerged that could point to the existence of a new pathway of glycogen synthesis in intact Novikoff ascites-hepatoma cells. On the other hand, there is sufficient evidence to support hexokinase participation in glycogen synthesis,

thereby making it mandatory to seek methods for glycogen formation in intact tissues in ways other than exclusion of hexokinase.

Variation in glycogen synthesis at increasing concentrations of glucose, fructose and mannose show that maximum rates of glycogen synthesis from these sugars are reached at concentrations that parallel their affinity for hexokinase. Thus mannose and glucose, which have low K_m values for hexokinase, attain maximal rates of glycogen synthesis at a much lower concentration (0.2–0.5 mM) than is required for fructose (5 mM), which has a high K_m for hexokinase. Competition experiments also give inhibition of glycogen synthesis from one sugar by the addition of another, as is predicted by the affinities of these sugars for hexokinase (Table 1). At a high concentration (15 mM) of the sugars, the rates of glycogen synthesis follow the order fructose > glucose > mannose, which is the same order as their rates of phosphorylation by hexokinase at saturation (15 mM) concentration of the substrates. Effect of substrate concentration on glycogen synthesis from glucose and fructose also matches the effect of substrate concentration on glycolysis (Table 2), a process well known to require hexose phosphorylation as an initial step.

However, results presented in Tables 3 and 4 also indicate that glucose 6-phosphate added to aged tumour cells or glucose 6-phosphate formed from nucleoside are not metabolized in the same manner as the glucose 6-phosphate formed from hexose. The ratio lactic acid produced/glycogen formed

is higher in the first case than ratios obtained when hexose is used as the substrate. Such disparity in the behaviour of the same product available to the cell in different ways suggests that different intracellular environments are encountered by the product depending on its site of formation or location after its entry into the cell. It would mean compartmentation of glucose 6-phosphate within the cell. Indeed, such a proposition is a favoured argument of several investigators (Segal & Lopez, 1963; Muntz & Vanko, 1962; Nigam & Fridland, 1964; London, 1966; Nigam, 1967a; Landau & Sims, 1967; Threlfall & Heath, 1968) in situations where experimental observations have come in conflict with known reactions of the scheme.

Some support for a difference in the intracellular environment available to glucose 6-phosphate after its entry into aged tumour cells (or to glucose 6-phosphate formed from nucleoside) is provided by observations presented in Tables 3 and 4, which indicate that α -glucan phosphorylase (and not UDP-glucose- α -glucan glucosyltransferase) mediates glycogen synthesis when glucose 6-phosphate is made available to the cell in the above-mentioned ways. Inhibition of glycogen synthesis from exogenously added glucose 1-phosphate by glucose is an observation consistent with an indirect inhibition of phosphorylase by glucose in rat diaphragm (Holmes & Mansour, 1968). The same conclusion was also arrived at by studies on activation of glycogen synthesis from glucose 1-phosphate by AMP and its inhibition by glucose 6-phosphate in aged tumour cells (Nigam, 1967a). However, no reason can be offered for the difference between our observations, showing activation of glycogen synthesis by fructose and an inhibition by mannose (Table 3), and the lack of effect of either fructose or mannose on phosphorylase activity of rat diaphragm reported by Holmes & Mansour (1968). With Novikoff ascites-hepatoma cells fructose has the same effect as is obtained by fructose diphosphate in rat diaphragm (Holmes & Mansour, 1968).

It is also apparent (Table 3) that fructose does not stimulate glycogen synthesis from glucose 6-phosphate although it does so with glucose 1-phosphate as the substrate. This difference could be due to the inhibitory action of glucose 6-phosphate on phosphorylase activity (Fridland & Nigam, 1965) and may be responsible for its poor performance as a glycogen precursor when compared with glucose 1-phosphate. An alternative explanation can be that fructose addition to tumour cells supplies glycolytic intermediates via the fructokinase pathway (Threlfall & Heath, 1968), thus favouring glucose monophosphates added exogenously or those formed from nucleoside to follow a pathway in the direction of glycogen forma-

tion. However, this suggestion does not appear to be valid because Novikoff ascites-hepatoma cells lack fructokinase (V. N. Nigam, unpublished results).

The decreased amount of glycogen obtained in the presence of oligomycin at a low concentration of fructose and a partial recovery of amount of glycogen with a high concentration of fructose (Table 2) can also be described as evidence indicative of compartmentation of hexose monophosphate. It is possible that loss of mitochondrial ATP production (and of UTP production) by oligomycin leads to a loss of glucose 6-phosphate and UDP-glucose formation in one compartment without having any effect on glycogen synthesis or glycolysis from glucose 6-phosphate (formed from glycolytic ATP) in the other. Increase in fructose concentration may enhance phosphorylase activity so that glucose 6-phosphate in the second compartment is more effectively transformed into glycogen.

Although the above evidence indirectly supports compartmentation of glucose 6-phosphate, the actual location of the two compartments in the tumour cell can only be speculated about. Considering that mitochondria are a source of ATP, and contain hexokinase (Rose & Warms, 1967) and nucleoside diphosphokinase (Gevers, 1967), it would seem reasonable to assume that they may participate in the formation of one compartment whereas the cytoplasmic part rich in enzymes of the Embden-Meyerhof pathway would constitute the second compartment.

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