Studies on Glycogen Synthesis in Pigeon Liver Homogenates

GLYCOGEN SYNTHESIS FROM GLUCOSE MONOPHOSPHATES AND URIDINE DIPHOSPHATE GLUCOSE

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(Received 1 May 1967)

Comparative time-course studies of glycogen synthesis from glucose 6-phosphate, glucose 1-phosphate and UDP-glucose show that glucose 1-phosphate forms glycogen at an initial rate faster than that obtained with glucose 6-phosphate and UDP-glucose. After 5 min. the rates from glucose monophosphates are considerably slower. 2,4-Dinitrophenol decreases glycogen synthesis from both glucose monophosphates, whereas arsenate and EDTA increase glycogen synthesis from glucose 1-phosphate and inhibit the reaction from glucose 6-phosphate, galactose and galactose 1-phosphate. Mitochondria-free pigeon liver cytoplasmic fraction forms less glycogen from glucose monophosphates than does the whole homogenate. 2-Deoxyglucose 6-phosphate inhibits glycogen synthesis from glucose monophosphates. Glycogen formation from UDP-glucose is relatively unaffected by dinitrophenol, by arsenate, by EDTA, by 2-deoxyglucose 6-phosphate and by the removal of mitochondria from the whole homogenate.

In the preceding paper (Nigam & Fridland, 1967), conditions for the maximum incorporation of glucose into glycogen by the pigeon liver homogenates were established and the effects of a number of factors on glycogen synthesis were described. The present paper deals with the incorporation of [^{14}C]glucose from [$1\text{-}^{14}\text{C}$]glucose 6-phosphate, [$1\text{-}^{14}\text{C}$]glucose 1-phosphate and UDP-[$1\text{-}^{14}\text{C}$]glucose into glycogen by the homogenate system. The study was initiated to shed light on the position of these intermediates in glycogen synthesis in homogenates and the routes they follow for their transformation into glycogen.

MATERIALS AND METHODS

Chemicals. [$1\text{-}^{14}\text{C}$]Glucose 6-phosphate, [$1\text{-}^{14}\text{C}$]glucose 1-phosphate and [$1\text{-}^{14}\text{C}$]galactose were obtained from New England Nuclear Corp., Cambridge, Mass., U.S.A. [$1\text{-}^{14}\text{C}$]Galactose 1-phosphate, UDP-[$1\text{-}^{14}\text{C}$]glucose and UDP-[$1\text{-}^{14}\text{C}$]galactose were products of International Nuclear and Chemical Corp., City of Industry, Calif., U.S.A. Non-radioactive fine chemicals were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. All other chemicals were of reagent grade purity obtained from commercial sources.

Animals. Sprague-Dawley rats (150–200 g.) and pigeons were obtained from Canadian Breeding Laboratories, St Constant, Quebec, Canada. Food and water were supplied to animals *ad libitum*.

Preparation of liver homogenates. Rats or pigeons were

killed by decapitation and were allowed to bleed. Liver homogenates (20%) were prepared in cold 0.25 M-sucrose as described in the preceding paper (Nigam & Fridland, 1967).

Incubation procedure and procedure for the isolation of glycogen. These were the same as described in the preceding paper (Nigam & Fridland, 1967). The amount of each labelled substrate added was 5 $\mu\text{moles/ml.}$ of reaction mixture and contained approx. 45000 counts/min. The substrates were added in 0.05 ml. volume after dissolution in 0.25 M-sucrose.

Determination of glucose 6-phosphate and glucose 1-phosphate. Glucose phosphates were determined in suitable samples of neutral HClO_4 -treated digests. The reaction mixture contained (in 3 ml.): glycylglycine buffer, pH 7.4, 150 μmoles ; MgCl_2 , 15 μmoles ; cysteine hydrochloride, pH 7.0, 30 μmoles ; NADP^+ , 3 μmoles ; glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 1 unit. The increase in E_{340} after the addition of glucose 6-phosphate dehydrogenase gave the amount of glucose 6-phosphate. The further increase in E_{340} after the addition of phosphoglucomutase (EC 2.7.5.1) gave the amount of glucose 1-phosphate in the digest.

Assay of phosphoglucomutase. Phosphoglucomutase activity was determined as described in the preceding paper (Nigam & Fridland, 1967).

Fractionation of liver homogenates. Liver homogenates were prepared in 0.25 M-sucrose containing MgCl_2 (3 mM). The homogenates were fractionated by two procedures. Crude separation into nuclear-mitochondrial and post-mitochondrial fractions was effected by centrifuging the homogenate at 5000g for 10 min. at 0° in a Spinco model L ultracentrifuge. Fractionation of the liver homogenate

into nuclear, mitochondrial, microsomal and soluble fractions was carried out by the method described by Umbreit, Burris & Stauffer (1964).

RESULTS

Glycogen synthesis from glucose, glucose 6-phosphate, glucose 1-phosphate and UDP-glucose. Fig. 1 shows the rates of glycogen synthesis from glucose, glucose 6-phosphate, glucose 1-phosphate and UDP-glucose. Both glucose monophosphates were converted into glycogen at an initial fast rate. During this period, the rate of glycogen synthesis from glucose 1-phosphate was higher than that from UDP-glucose. After 5 min. the rates of glycogen synthesis from glucose monophosphates diminished significantly. It was also observed that, after 10 min., only a trace of glucose 1-phosphate could be detected in the reaction digests and the concentration of glucose 6-phosphate had decreased to one-tenth the initial concentration of the glucose monophosphate added. Glycogen synthesis from glucose and UDP-glucose increased with increase in the time of incubation such that for later periods (after 15 min.) the rate was higher from glucose and UDP-glucose than that obtained from glucose monophosphates.

Rat liver homogenates do not give sizable incorporation of glucose into glycogen (Nigam & Fridland, 1964). When glucose monophosphates and UDP-glucose were used in place of glucose, it was observed that rat liver homogenates formed only 8–10% of glycogen from glucose monophosphates and up to 40% from UDP-glucose compared with that formed by the pigeon liver homogenates with the respective substrates (Table 1). Prior addition of rat microsomal fraction decreased the

synthesis of glycogen from glucose monophosphates in the pigeon liver homogenates. Pigeon liver microsomal fraction did not exhibit this property. The addition of rat microsomal fraction to pigeon liver homogenate had no effect on glycogen synthesis from UDP-glucose (not shown).

Effect of 2,4-dinitrophenol on glycogen synthesis from glucose monophosphates and UDP-glucose. Dinitrophenol is known to abolish glycogen synthesis from glucose in the pigeon liver homogenates (Nigam & Fridland, 1964). Formation of glycogen from glucose 6-phosphate was also greatly decreased by dinitrophenol (Table 2). On the other hand, with glucose 1-phosphate and UDP-glucose as substrates, the decreases due to dinitrophenol were 60–70% and 10% respectively (Table 2). Addition of UTP (5 μ moles) along with phosphoenolpyruvate (5 μ moles) did not increase glycogen synthesis from the glucose monophosphates either in the presence or absence of dinitrophenol.

Effects of sodium arsenate and EDTA on glycogen synthesis from glucose 6-phosphate and glucose 1-phosphate. Although sodium arsenate (5 mM) greatly decreased the incorporation of glucose into glycogen (Nigam & Fridland, 1967), it had a slight stimulatory effect on glucose incorporation from glucose 1-phosphate into glycogen (Table 3). On the other hand, glycogen synthesis from glucose 6-phosphate was diminished on the addition of arsenate to the homogenate. There was no effect of arsenate on glycogen synthesis from UDP-glucose. When galactose or galactose 1-phosphate was used as glycogen precursor, their incorporation into glycogen was also decreased by arsenate. Addition of EDTA (5 mM) in place of arsenate gave exactly the same result.

In subsequent experiments, assay of phosphoglucomutase activity was carried out in the absence

Fig. 1. Time-course of glycogen synthesis from glucose (○), glucose 6-phosphate (▲), glucose 1-phosphate (■) and UDP-glucose (●) by pigeon liver homogenate. Experimental conditions and composition of the digests are given in the Materials and Methods section. The concentration of each substrate was 5 mM. The values are averages of those obtained in two experiments.

Table 1. Glycogen synthesis from glucose monophosphates and UDP-glucose by rat and pigeon liver homogenates

Experimental conditions are given in Fig. 1 and in the Materials and Methods section. The concentration of each substrate was 5 mM. Results are expressed as means $\pm$ S.D. The numbers in parentheses represent numbers of animals as well as experiments.

	Glycogen formed (μ moles of glucose equiv./30 min./80 mg. wet wt. of liver)	
	Rat (4)	Pigeon (6)
Glucose 6-phosphate	0.21 $\pm$ 0.05	2.0 $\pm$ 0.4
Glucose 1-phosphate	0.35 $\pm$ 0.05	3.1 $\pm$ 0.5
UDP-glucose	1.50 $\pm$ 0.20	3.9 $\pm$ 0.3

Table 2. *Effect of dinitrophenol on glycogen synthesis from glucose monophosphates and UDP-glucose by pigeon liver homogenate*

Experimental conditions are given in the Materials and Methods section. The concentration of each substrate was 5 mM. Additions of substances were made after dissolution in 0.25 M-sucrose. The volume of the digest was 1.0 ml. in each case. Results are expressed as means $\pm$ s.d. of those obtained in three experiments.

Substrate	Addition	Glycogen formed (μ moles of glucose equiv./30 min./ 80 mg. wet wt. of liver)
Glucose 6-phosphate	None	2.1 $\pm$ 0.30
Glucose 6-phosphate	UTP (5 mM) + phosphoenolpyruvate (5 mM)	2.2 $\pm$ 0.30
Glucose 6-phosphate	Dinitrophenol (0.25 mM)	0.3 $\pm$ 0.05
Glucose 6-phosphate	Dinitrophenol (0.25 mM) + UTP (5 mM) + phosphoenolpyruvate (5 mM)	0.3 $\pm$ 0.05
Glucose 1-phosphate	None	2.9 $\pm$ 0.30
Glucose 1-phosphate	Dinitrophenol (0.25 mM)	0.8 $\pm$ 0.10
Glucose 1-phosphate	UTP (5 mM) + phosphoenolpyruvate (5 mM)	3.0 $\pm$ 0.30
Glucose 1-phosphate	Dinitrophenol (0.25 mM) + UTP (5 mM) + phosphoenolpyruvate (5 mM)	0.7 $\pm$ 0.10
UDP-glucose	None	4.0 $\pm$ 0.40
UDP-glucose	Dinitrophenol (0.25 mM)	3.8 $\pm$ 0.40

Table 3. *Effects of arsenate and EDTA on glycogen synthesis from glucose monophosphates, UDP-glucose, galactose and galactose 1-phosphate by pigeon liver homogenate*

Experimental conditions are described in the Materials and Methods section. Substances were added after dissolution in 0.25 M-sucrose. EDTA was neutralized to pH 7.0. The volume of the digest was 1 ml. The concentration of glucose 6-phosphate, glucose 1-phosphate and UDP-glucose was 5 mM and that of galactose and galactose 1-phosphate was 2 mM. The first three substrates contained approx. 45 000 counts/min. and the last two approx. 100 000 counts/min. The results are means $\pm$ s.d. of those obtained in three experiments.

Substrate	Addition	Glycogen formed (μ moles of glucose equiv./30 min./ 80 mg. wet wt. of liver)
Glucose 1-phosphate	None	3.1 $\pm$ 0.40
Glucose 1-phosphate	Arsenate (5 mM)	3.7 $\pm$ 0.20
Glucose 1-phosphate	EDTA (5 mM)	4.0 $\pm$ 0.25
Glucose 6-phosphate	None	2.0 $\pm$ 0.40
Glucose 6-phosphate	Arsenate (5 mM)	0.25 $\pm$ 0.05
Glucose 6-phosphate	EDTA (5 mM)	0.15 $\pm$ 0.04
UDP-glucose	None	3.6 $\pm$ 0.20
UDP-glucose	Arsenate (5 mM)	3.7 $\pm$ 0.20
UDP-glucose	EDTA (5 mM)	3.6 $\pm$ 0.25
Galactose	None	0.005 $\pm$ 0.003
Galactose	Arsenate (5 mM)	0.0
Galactose	EDTA	0.0
Galactose 1-phosphate	None	0.15 $\pm$ 0.002
Galactose 1-phosphate	Arsenate (5 mM)	0.005 $\pm$ 0.001
Galactose 1-phosphate	EDTA (5 mM)	0.004 $\pm$ 0.001

and presence of arsenate and EDTA. It was found that arsenate (5 mM) inhibited phosphoglucomutase activity by 50% and that EDTA (5 mM) inhibited it by 60–75%.

Glycogen synthesis from glucose monophosphates and UDP-glucose in post-mitochondrial fraction of pigeon liver. Table 4 shows the amount of glycogen synthesized from glucose 6-phosphate, glucose 1-phosphate and UDP-glucose by post-mitochondrial fraction of pigeon liver. The effects of arsenate, EDTA, dinitrophenol and AMP on glycogen synthesis by this fraction are also presented. It was observed that glycogen formation from the glucose monophosphates was lower in this fraction as compared with the whole homogenate. Addition of either arsenate or EDTA increased the incorporation of glucose from glucose 1-phosphate into glycogen twofold, whereas it decreased glycogen formation from glucose 6-phosphate. EDTA had no effect, whereas arsenate slightly stimulated glycogen synthesis from UDP-glucose. On the other hand, AMP stimulated glycogen formation from the glucose monophosphates and inhibited slightly the reaction with UDP-glucose as the substrate.

Effect of 2-deoxyglucose 6-phosphate on glycogen synthesis from glucose monophosphates and UDP-glucose. 2-Deoxyglucose 6-phosphate (5 and 10 mM) was inhibitory to glycogen synthesis from glucose monophosphates in the whole homogenate (Table 5) as well as in the post-mitochondrial liver fraction (not shown), whereas it had a slight stimulatory effect on glycogen synthesis from UDP-glucose.

Table 4. *Glycogen synthesis from glucose monophosphates and UDP-glucose by post-mitochondrial fraction of pigeon liver*

Procedure for the isolation of post-mitochondrial fraction of pigeon liver and experimental conditions are described in the Materials and Methods section. The concentration of each substrate was 5mM. Results are means $\pm$ s.d. of those obtained in three experiments.

Substrate	Cell fraction	Addition	Glycogen formed (μ moles of glucose equiv./30 min./ 80 mg. equiv. wet wt. of liver)
Glucose 6-phosphate	Homogenate	None	1.8 $\pm$ 0.20
Glucose 6-phosphate	Post-mitochondrial	None	0.5 $\pm$ 0.05
Glucose 6-phosphate	Post-mitochondrial	Arsenate (5 mM)	0.05 $\pm$ 0.0
Glucose 6-phosphate	Post-mitochondrial	EDTA (5 mM)	0.05 $\pm$ 0.0
Glucose 6-phosphate	Post-mitochondrial	AMP (5 mM)	0.7 $\pm$ 0.03
Glucose 1-phosphate	Homogenate	None	2.9 $\pm$ 0.30
Glucose 1-phosphate	Post-mitochondrial	None	1.0 $\pm$ 0.50
Glucose 1-phosphate	Post-mitochondrial	Arsenate (5 mM)	3.9 $\pm$ 0.20
Glucose 1-phosphate	Post-mitochondrial	EDTA (5 mM)	4.1 $\pm$ 0.25
Glucose 1-phosphate	Post-mitochondrial	AMP (5 mM)	3.1 $\pm$ 0.32
UDP-glucose	Homogenate	None	3.4 $\pm$ 0.30
UDP-glucose	Post-mitochondrial	None	3.1 $\pm$ 0.30
UDP-glucose	Post-mitochondrial	Arsenate (5 mM)	3.5 $\pm$ 0.35
UDP-glucose	Post-mitochondrial	EDTA (5 mM)	3.0 $\pm$ 0.40
UDP-glucose	Post-mitochondrial	AMP (5 mM)	2.5 $\pm$ 0.25

Table 5. *Effect of 2-deoxyglucose 6-phosphate on glycogen synthesis from glucose monophosphates and UDP-glucose by pigeon liver homogenates*

Experimental conditions are given in the Materials and Methods section. The concentration of each substrate was 5mM. Results obtained in two experiments are presented.

Substrate	Addition	Glycogen formed (μ moles of glucose equiv./30 min./ 80 mg. wet wt. of liver)	
		Expt. 1	Expt. 2
Glucose 6-phosphate	None	1.90	2.20
Glucose 6-phosphate	2-Deoxyglucose 6-phosphate (5 mM)	0.60	0.70
Glucose 6-phosphate	2-Deoxyglucose 6-phosphate (10 mM)	0.35	0.40
Glucose 1-phosphate	None	2.80	3.30
Glucose 1-phosphate	2-Deoxyglucose 6-phosphate (5 mM)	0.90	1.05
Glucose 1-phosphate	2-Deoxyglucose 6-phosphate (10 mM)	0.54	0.60
UDP-glucose	None	3.00	3.40
UDP-glucose	2-Deoxyglucose 6-phosphate (5 mM)	3.20	3.50
UDP-glucose	2-Deoxyglucose 6-phosphate (10 mM)	3.20	3.60

DISCUSSION

The present study shows that glucose monophosphates and UDP-glucose can be transformed into glycogen in pigeon liver homogenate by independent routes. Since the initial rate of glycogen formation from glucose phosphates is higher than is obtained with UDP-glucose, conversion of glucose phosphates into UDP-glucose is not a prerequisite for glycogen synthesis from glucose phosphates. However, since glucose 1-phosphate is rapidly converted into UDP-glucose (Zancan & Hers, 1965) it is likely that a part of glucose 1-phosphate forms

glycogen after conversion into UDP-glucose. The sum of the two conversions (glucose 1-phosphate $\rightarrow$ glycogen; glucose 1-phosphate $\rightarrow$ UDP-glucose $\rightarrow$ glycogen) would therefore give the total amount of glycogen formed from glucose 1-phosphate in the pigeon liver homogenate.

The inhibition of glycogen synthesis from glucose 1-phosphate by dinitrophenol suggests that aerobic energy is required for the synthesis of glycogen by one or both routes. If it is presumed that aerobic energy is required mainly for UTP generation and subsequent formation of UDP-glucose, the UDP-glucose pathway would account for 60–70% of the

synthesized glycogen from glucose 1-phosphate in pigeon liver homogenate. Since dinitrophenol is more inhibitory to glycogen formation from glucose 6-phosphate, it is possible that transformation of glucose 6-phosphate into glucose 1-phosphate is an energy-dependent reaction. Energy may be required for pushing the unfavourable equilibrium (95:5 in favour of glucose 6-phosphate formation) towards glucose 1-phosphate formation.

The controlling effect of phosphoglucosyltransferase is further exemplified by the action of arsenate and EDTA on glycogen synthesis from glucose 6-phosphate and glucose 1-phosphate. Inhibition of phosphoglucosyltransferase by the two compounds leads to loss in glycogen synthesis from glucose 6-phosphate and stimulation from glucose 1-phosphate. It is therefore apparent that, at least in the absence of aerobic energy, glucose 6-phosphate cannot form glycogen without its prior conversion into glucose 1-phosphate. Further, a twofold increase in glycogen synthesis in post-mitochondrial fraction after phosphoglucosyltransferase inhibition indicates that phosphorylase can accomplish significant glycogen synthesis, and only the rapid transformation of glucose 1-phosphate into glucose 6-phosphate limits glycogen synthesis by this enzyme. Similarly, during glycogen degradation by phosphorylase, removal of glucose 1-phosphate would be essential for the continued breakdown of glycogen. Since glucose 6-phosphate is also an inhibitor of AMP-dependent phosphorylase *b* (Fridland & Nigam, 1965), its concentration is likely to play a role in both synthesis and breakdown of glycogen by phosphorylase.

Arsenate and EDTA both inhibit phosphoglucosyltransferase, and arsenate is also known to uncouple oxidative phosphorylation. Since incorporation of galactose and galactose 1-phosphate into glycogen by pigeon liver homogenate is inhibited by both compounds, it is reasonable to conclude that EDTA in some manner affects the availability of oxidative energy for nucleoside diphosphate sugar function. This conclusion is supported by the fact that phosphoglucosyltransferase and phosphorylase are not involved in glycogen synthesis from galactose and galactose 1-phosphate.

The actions of rat liver microsomal fraction and 2-deoxyglucose 6-phosphate are similar. Both inhibit glycogen synthesis in pigeon liver homogenate from glucose monophosphates without inhibiting glycogen formation from UDP-glucose. It is therefore indicated that both not only inactivate phosphorylase but also inhibit UDP-glucose formation.

The doubtful position of glucose 6-phosphate as revealed in investigations with intact rat diaphragm

(Beloff-Chain *et al.* 1964), rat liver slices (Figuerola & Pfeifer, 1964) and intact Novikoff ascites-hepatoma cells (Nigam, 1967) is not resolved by investigations with pigeon liver homogenate. Although the rates of glycogen formation from glucose and glucose 6-phosphate are different at different periods of incubation, evidence that glucose 6-phosphate is a much inferior precursor of glycogen than glucose has not been obtained. It is possible that in investigations with intact tissues incubation with glucose 6-phosphate results in its breakdown at the cell surface and not enough substrate is available for UDP-glucose- and glycogen-forming enzymes. On the other hand, glucose 6-phosphate, formed from glucose by aerobic ATP, may be compartmentalized and readily available for glycogen synthesis. Theoretical studies by London (1966) have indeed suggested 'compartmentation' of glucose 6-phosphate and glucose 1-phosphate as an essential requirement for glycogen synthesis in model systems.

Contrary to the above interpretation it is also possible that yet another pathway exists in the cell to transform glucose into UDP-glucose without glucose 6-phosphate formation as an intermediate step. Such a view deserves serious consideration because of the low specific activity of glucose 6-phosphate (compared with that of UDP-glucose) obtained in liver after injection of [^{14}C]glucose into rats (Threlfall, 1966). Smith, Taylor & Whelan (1967) have recently proposed a set of reactions for glucose 1-phosphate synthesis involving glucose 1-phosphate kinase and dismutase that by-pass glucose 6-phosphate formation.

This work was supported by grants to Dr A. Cantero, Director of the Research Laboratories, from the National Cancer Institute of Canada. I am indebted to Miss Huguette Basilières for technical assistance.

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