Effects of D-Glucosamine and Glucose Analogues on Glycogen Biosynthesis *in vitro*

By M. KONO AND J. H. QUASTEL

McGill-Montreal General Hospital Research Institute, 3619 University Street, Montreal, Canada

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The observation that glucose phosphorylation in brain is inhibited competitively by D-glucosamine and N-acetyl-D-glucosamine (Harpur & Quastel, 1949) was followed by extensive work on the effects of these amines and their analogues, e.g. 2-deoxy-D-glucose, on glucose transport across cell membranes (Nakada, Morita & Wick, 1955; Woodward & Hudson, 1955), glycolysis (Harpur & Quastel, 1949; Woodward, 1952; Woodward, Cramer & Hudson, 1953; Woodward & Hudson, 1953, 1954; Tower, 1958), respiration (Woodward & Hudson, 1954; Tower, 1958) and tissue growth (Ely, Tull & Hard, 1952; Woodward, 1952; Ely, Tull & Schanen, 1953; Quastel & Cantero, 1953; Woodward & Hudson, 1953; Ball, Wick & Sanders, 1957; Bernstein & Black, 1959). Srinivasan & Quastel (1958), however, observed that D-glucosamine or N-acetyl-D-glucosamine stimulates the formation of oligosaccharides containing the respective amino sugar by yeast invertase, yeast lactase and extracts of *Aerobacter aerogenes*. These two different modes of action of glucose analogues have led us to an investigation of effects of glucose analogues on the metabolism of glycogen. Spiro (1958) has reported on the strong inhibitory effects of D-glucosamine and N-acetyl-D-glucosamine on glycogen synthesis in slices of rat liver, an inhibition of non-specific hexokinase by these glucose analogues being presented as the mechanism of inhibition of glycogen synthesis.

In the present paper, the effects of D-glucosamine, N-acetyl-D-glucosamine and 2-deoxy-D-glucose on glycogen synthesis from glucose and pyruvate, on the influx of glucose into the liver cell, on enzymes concerned with glycogen metabolism and on phosphorylase activity in liver slices are described, with special reference to the mode of action of these glucose analogues on glycogen biosynthesis.

MATERIALS AND METHODS

Animals. Male rats of a local breed weighing 150–250 g. were used. They were normally fed with Purina Chow *ad lib.* but were starved for 13–14 hr. before use.

Glycogen synthesis in liver slices. When glycogen synthesis from glucose was studied, liver slices, weighing approximately 200 mg./flask, were first preincubated without glucose for 20 min. and then incubated for a further

60 min. in an atmosphere of O₂ + CO₂ (95:5) with glucose at 37° in small Erlenmeyer flasks containing 2 ml. of the potassium-rich medium used by Hastings, Teng, Nesbitt & Sinex (1952). Glucose was added, to give a final concentration of 30 mM, at the beginning of incubation. When glycogen synthesis from pyruvate was studied, the incubation medium described by Buchanan, Hastings & Nesbitt (1949) was used except that the concentration of pyruvate was increased to 60 mM. Conditions for incubation were the same as those used when glycogen synthesis from glucose was investigated. Effects of D-glucosamine hydrochloride, N-acetyl-D-glucosamine and 2-deoxy-D-glucose, purchased from the Nutritional Biochemical Corp., were studied by adding solutions of these substances to the medium at the beginning of the incubation at the concentrations indicated in each Table.

Estimation of glycogen. Glycogen was isolated and purified by the combined methods of Good, Kramer & Somogyi (1933) and van der Kleijk (1951). The reaction was stopped by adding potassium hydroxide to give a final concentration of 25% (w/v). The contents of flasks were transferred to glass centrifuge tubes and digested for 30 min. in a boiling-water bath. The solution was cooled, 1.5 vol. of 95% (v/v) ethanol was added, the mixture was boiled briefly and cooled in an ice bath. Glycogen was sedimented by centrifuging. The sediments were dissolved in 1.0 ml. of water and once more 1.5 vol. of ethanol was added, the mixtures were boiled and the glycogen was sedimented by centrifuging. The glycogen was then dissolved in 1.0 ml. of cold 5% (w/v) trichloroacetic acid, after which 1.5 ml. of cold ethanol was immediately added and the purified glycogen was centrifuged down. Glycogen was estimated after hydrolysis in N-hydrochloric acid as glucose by the method of Somogyi (1952) by using Nelson's (1944) chromogenic solution. Results are expressed as mg. of glucose/hr./g. of wet tissue.

D-Glucosamine 6-phosphate. D-Glucosamine 6-phosphate was synthesized according to the method of Brown (1955). D-Glucose 1,6-diphosphate was synthesized from sucrose and inorganic phosphate, by using yeast according to the method of Leloir & Paladini (1957).

Measurement of influx of glucose into liver slices in the presence of glucose analogues. Liver slices weighing about 200 mg. were incubated at 37° in 2.0 ml. of the potassium-rich medium of Hastings *et al.* (1952) in the presence of 30 mM-[¹⁴C₆]glucose (about 1 µC/flask) with or without added glucose analogues. After certain periods of incubation in an atmosphere of O₂ + CO₂ (95:5), the slices were removed from the medium, quickly washed with ice-cold water and immersed in a solution containing 0.3 ml. of 5% (w/v) zinc sulphate and 0.7 ml. of water. They were then homogenized with a Potter-Elvehjem homogenizer and 0.3 ml. of 0.3N-barium hydroxide was added to the homo-

genate. The volume was made up to 5.0 ml. with water. Suitable samples of the protein-free supernatant fluids obtained after centrifuging were plated on aluminum planchets and their radioactivities were counted. The readings were corrected for self-absorption and converted into mg. of glucose. The results were expressed as mg. of glucose/g. of tissue.

Preparation of crude liver enzymes. Crude liver extracts, as a source of phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase and phosphorylase, were prepared as described by Villar-Palasi & Lerner (1960). The extract used for uridine diphosphate glucose pyrophosphorylase was further centrifuged at 18 000g for 40 min. to obtain a clear supernatant fluid. A particle-free extract of liver was prepared as a source of liver hexokinase according to the method of Crane & Sols (1955). Uridine diphosphate glucose-glycogen glucosyltransferase was prepared according to the procedure of Leloir & Goldemberg (1960).

Assay of enzyme activities. Hexokinase was assayed by the method described by Crane & Sols (1955). D-Glucose remaining in the reaction medium was estimated with D-glucose oxidase (McComb & Yushok, 1958). When 2-deoxy-D-glucose was present in the medium, the amount of D-glucose was obtained by calculation after subtraction of the amount of 2-deoxy-D-glucose which was estimated as described by Stoll & Kreis (1933) and Kiesow (1959). Phosphoglucomutase was assayed by the method described by Najjar (1955) except that the volume of each component was halved. Activities of uridine diphosphate glucose pyrophosphorylase and uridine diphosphate glucose-glycogen glucosyltransferase were determined as described by Munch-Petersen (1955) and Leloir & Goldemberg (1960) respectively. All experiments were carried out in duplicate, usually several times, and the mean results $\pm$ S.E.M. are given. The experimental deviation from the mean rarely exceeded 10%.

Measurement of phosphorylase activity in liver slices. Incubation of liver slices was carried out as described for glycogen synthesis in liver slices, except that the period of incubation was 30 min. instead of 60 min. The reaction was stopped by immersing the flasks in crushed ice. In accordance with the method of Rall, Sutherland & Wosilait (1956), slices were removed from the medium, washed quickly in cold 0.9% sodium chloride and homogenized in 3 ml. of ice-cold 1 mM-EDTA in 0.1M-sodium fluoride, pH 6.1. The resulting homogenate was centrifuged at 8000g for 10 min. and 0.1 ml. samples of the supernatant fluid were assayed for phosphorylase activity as described by Sutherland (1955). The phosphorylase activity of the slices was expressed as μ moles of inorganic phosphate released/10 min./g. of tissue.

RESULTS

Diminution of glycogen synthesis from glucose by D-glucosamine, N-acetyl-D-glucosamine, 2-deoxy-D-glucose and D-glucosamine 6-phosphate

The effects of D-glucosamine, N-acetyl-D-glucosamine, 2-deoxy-D-glucose and D-glucosamine 6-phosphate on glycogen synthesis in the presence of rat-liver slices from glucose were studied at various concentrations (that of glucose being 30 mM) (Table 1). All the compounds tested strongly diminish glycogen synthesis, D-glucosamine 6-phosphate being the most effective. This gives a diminution of about 90% at a concentration of 1.1 mM, the ratio of glucose to glucose 6-phosphate being 27:1. Both D-glucosamine and N-acetyl-D-glucosamine diminish glycogen synthesis to about the same extent, producing at a concentration of

Table 1. *Effects of glucose analogues on glycogen synthesis from glucose by rat-liver slices*

Glucose and analogues were added 20 min. after preincubation at 37° in potassium-rich medium containing about 200 mg. of slices. The final concentration of glucose was 30 mM. Incubation was done at 37° for a further 60 min. after the additions. The gas phase was O₂ + CO₂ (95:5). The values for the increase in glycogen are means $\pm$ S.E.M. from six experiments. The values for initial glycogen refer to the amount of glycogen found in the slices at the end of preincubation period without added glucose (six experiments).

Analogue	Concn. of analogue (mM)	Increase in glycogen (mg./g. of tissue/hr.)	Inhibition (%)	Initial glycogen (mg./g. of tissue)
D-Glucosamine	0	0.72 $\pm$ 0.07	0	0.02-0.11
	0.13	0.57 $\pm$ 0.03	21	
	0.5	0.46 $\pm$ 0.08	36	
	1	0.26 $\pm$ 0.06	64	
	5	0.24 $\pm$ 0.04	67	
	10	0.10 $\pm$ 0.08	85	
N-Acetyl-D-glucosamine	0	1.71 $\pm$ 0.36	0	0.13-0.69
	1	0.74 $\pm$ 0.07	57	
	10	0.19 $\pm$ 0.07	89	
2-Deoxy-D-glucose	0	0.74 $\pm$ 0.21	0	0.25-0.61
	2	0.74 $\pm$ 0.23	0	
	5	0.48 $\pm$ 0.10	35	
	15	0.22 $\pm$ 0.06	70	
	30	0.02 $\pm$ 0.01	98	
D-Glucosamine 6-phosphate	0	0.74 $\pm$ 0.09	0	0.02-0.11
	0.54	0.38 $\pm$ 0.02	49	
	1.08	0.08 $\pm$ 0.03	89	

1 mM a diminution of glycogen synthesis of approximately 60 %, and at one of 10 mM a diminution of 90 %. 2-Deoxy-D-glucose has apparently the least inhibitory effect.

Diminution of glycogen synthesis from pyruvate by D-glucosamine and 2-deoxy-D-glucose

Glycogen synthesis from pyruvate in liver slices is also diminished by glucose analogues (Table 2). When D-glucosamine, at concentrations varying from 0.5 mM to 20 mM, is added to the incubation medium containing 60 mM-pyruvate, no net in-

crease in glycogen is observed. However, the presence of the amino sugar increases the rate of breakdown of glycogen owing to its effect on phosphorylase in liver slices (see below). Thus the observed decrease in glycogen may be due to the fact that the breakdown of glycogen is too fast to be counteracted by the synthesis of glycogen. 2-Deoxy-D-glucose also diminishes glycogen synthesis from pyruvate but to a lesser extent. Diminutions of 64 and 76 % were observed at concentrations of 1 and 10 mM respectively (Table 2).

Diminution of glycogen synthesis from glucose by L-glutamine, L-glutamic acid, α -oxoglutaric acid and ammonium chloride

Pogell & Gryder (1957) and Gryder & Pogell (1960) showed that glucosamine 6-phosphate is formed from hexose 6-phosphate and glutamine by an enzyme obtained from rat liver. In view of their findings, the effects of L-glutamine, and metabolically related compounds, were studied with special reference to glycogen synthesis from glucose in liver slices (Table 3). All of the compounds tested diminish glycogen synthesis from glucose. L-Glutamine, at a concentration of 50 mM, diminishes glycogen synthesis by only 22 %, but 10 mM-L-glutamic acid, 20 mM- α -oxoglutaric acid and 10 mM-ammonium chloride diminish it by 26–28, 72 and 96 % respectively. A mixture of equimolar concentrations of ammonium chloride and L-glutamic acid gives a diminution whose value is intermediate between the values obtained in the presence of each of these compounds.

Effects of glucose analogues on glucose influx. Table 4 shows that the influx of glucose into rat-

Table 2. *Effects of D-glucosamine and 2-deoxy-D-glucose on glycogen synthesis from potassium pyruvate by rat-liver slices*

Pyruvate and analogues were added 20 min. after preincubation at 37° in potassium-rich medium containing about 200 mg. of slices. The final concentration of pyruvate was 60 mM. Incubation was carried out at 37° for a further 60 min. after the additions. The gas phase was O₂ + CO₂ (95:5). The values for the increase in glycogen and the initial glycogen are the means of duplicate results. The values for initial glycogen refer to the amount of glycogen found in the slices at the end of preincubation period without added glucose.

Analogue	Concn. of analogue (mM)	Increase in glycogen (mg./g./hr.)	Initial glycogen (mg./g.)
D-Glucosamine	0	1.14	2.74
	0.5	-0.03	
	2	-0.64	
	5	-0.90	
	20	-0.65	
2-Deoxy-D-glucose	0	0.53	1.29
	1	0.19	
	10	0.13	

Table 3. *Inhibition of glycogen synthesis from glucose in rat-liver slices by L-glutamine, L-glutamic acid, α -oxoglutaric acid and ammonium chloride*

Glucose (30 mM) and the test compounds in the concentrations indicated were added 20 min. after preincubation at 37° in potassium-rich medium containing about 200 mg. of slices. Incubation was carried out for a further 60 min. after the additions. The gas phase was O₂ + CO₂ (95:5). The values for the increase in glycogen are the means of duplicate results. The values for the initial glycogen refer to the amount of glycogen found in the slices at the end of the preincubation period without added glucose.

Expt. no.	Compound	Concn. (mM)	Increase in glycogen (mg./g./hr.)	Inhibition (%)	Initial glycogen (mg./g. of tissue)
1	L-Glutamine	0	1.22	0	0.99
		50	0.95	22	
		100	0.29	76	
2	L-Glutamic acid	0	1.52	0	1.11
		10	1.12	26	
		25	0.31	80	
		50	-0.11	(107)	
3	α -Oxoglutaric acid	0	2.30	0	0.08
		20	0.64	72	
	NH ₄ Cl	10	0.33	96	
	L-Glutamic acid	10	1.65	28	
	NH ₄ Cl and L-glutamic acid	10	1.19	48	

Table 4. *Effects of glucose analogues on entry of glucose from the medium into rat-liver slices*

The incubation mixture contained [^{14}C]glucose (approx. $1\text{ }\mu\text{C}/\text{flask}$) and about 200 mg. of slices in 2 ml. of potassium-rich medium. Incubation was carried out at 37° in an atmosphere of $\text{O}_2 + \text{CO}_2$ (95:5). Glucose was estimated from the radioactivity values. Values for the free glucose in slices are mean values from duplicate experiments.

Expt. no.	Component	Free glucose in slices (mg./g.)			
		0 min.	15 min.	30 min.	60 min.
1	90 mM-Glucose	0.66	10.5	11.2	12.4
	90 mM-Glucose + 90 mM-D-glucosamine	—	10.9	12.7	13.0
2	30 mM-Glucose	0.53	—	4.22	—
	30 mM-Glucose + 30 mM-D-glucosamine	—	—	4.39	—
3	30 mM-Glucose	0.45	2.89	3.46	3.14
	30 mM-Glucose + 30 mM-N-acetyl-D-glucosamine	—	2.83	3.33	3.09
4	30 mM-Glucose	0.41	—	3.08	—
	30 mM-Glucose + 90 mM-N-acetyl-D-glucosamine	—	—	2.57	—
5	30 mM-Glucose	0.33	2.46	2.56	2.95
	30 mM-Glucose + 30 mM-2-deoxy-D-glucose	—	2.30	2.68	2.98
6	30 mM-Glucose	0.41	—	3.08	—
	30 mM-Glucose + 30 mM-2-deoxy-D-glucose	—	—	2.90	—
	30 mM-Glucose + 90 mM-2-deoxy-D-glucose	—	—	2.95	—

Table 5. *Effects of glucose analogues on glucose phosphorylation by liver hexokinase*

The reaction mixture contained: 0.1 ml. of tris-EDTA-histidine buffer, pH 7.4; 0.1 ml. of 0.75 M-ATP and 0.04 M- MgCl_2 ; 0.05 ml. of 0.05 M-glucose; 0.2 ml. of extract; 0.05 ml. of glucose analogue solution; the final volume was 0.5 ml. (i.e. the final concn. of glucose was 5 mM). Incubation was carried out at 30° for 60 min. The values for the glucose phosphorylated are the means $\pm$ S.E.M. from six experiments.

Analogue	Concn. of analogue (mM)	Glucose phosphorylated ($\mu\text{moles/hr.}$)
D-Glucosamine	0	0.96 ± 0.08
	5	0.94 ± 0.06
	10	0.98 ± 0.08
	20	0.86 ± 0.06
	40	0.64 ± 0.07
N-Acetyl-D-glucosamine	0	0.91 ± 0.03
	5	0.92 ± 0.05
	20	0.86 ± 0.03
	50	0.67 ± 0.08
2-Deoxy-D-glucose	0	1.01 ± 0.02
	5	1.03 ± 0.02
	20	1.11 ± 0.07
	40	1.09 ± 0.08

liver slices almost reaches its maximum in the first 15 min. of incubation and only a slight change in the amount of glucose in the slices is observed in the next 45 min. of incubation. Little effect on the amount of glucose found in the slices is observed when the three glucose analogues are added to the incubation medium at various concentrations (Table 4). The influx of glucose into the slices is not affected by the presence of these glucose analogues. When the ratio of glucose to the analogue is increased, no effect on the amount of free glucose

found in the slice is observed. However, a slight decrease in glucose is found when 90 mM-N-acetyl-D-glucosamine is added to the medium containing 30 mM-glucose, the degree of apparent inhibition observed being about 16 %.

Effects of glucose analogues on enzymes concerned in glycogen metabolism: (a) Hexokinase. The liver extract used as hexokinase preparation had no glucose-6-phosphatase activity and its adenosine-triphosphatase activity was such that it decomposed approximately only about one-tenth of the ATP added to the medium during the period of incubation. Table 5 shows that the three glucose analogues have no inhibitory effects at relatively low concentrations on glucose phosphorylation by liver. At higher concentrations D-glucosamine and N-acetyl-D-glucosamine inhibit hexokinase to a small extent. 2-Deoxy-D-glucose is not an inhibitor of this enzyme even at relatively high concentrations.

(b) *Phosphoglucomutase.* Table 6 summarizes the effects of glucose analogues on phosphoglucomutase. Substantial inhibition of the reaction is observed only when D-glucosamine or N-acetyl-D-glucosamine is added to the medium at relatively high concentrations. Neither of these analogues, at lower concentrations, nor 2-deoxy-D-glucose, has any inhibitory effect on phosphoglucomutase.

(c) *Uridine diphosphate glucose pyrophosphorylase.* 1.7 mM-N-Acetyl-D-glucosamine (at a concentration representing a ratio of substrate to inhibitor of 1:4) has no effect on this enzyme, whereas 0.3 mM-D-glucosamine slightly stimulates the reaction (Fig. 1). 2-Deoxy-D-glucose (at a concentration representing a ratio of substrate to inhibitor of 1:3) is not inhibitory and glucosamine 6-phosphate, like D-glucosamine, stimulates the reaction slightly (Fig. 2).

(d) *Uridine diphosphate glucose-glycogen glucosyl-transferase*. Table 7 shows that D-glucosamine is very inhibitory even at low concentrations. At a concentration of 2.5 mM, which represents a substrate to inhibitor ratio of 1:0.5, the inhibition of this enzyme is 26%; with 20 mM-D-glucosamine,

Table 6. *Effects of glucose analogues on liver phosphoglucomutase*

The reaction mixture contained: 0.05 ml. of 0.02M-glucose 1-phosphate; 0.05 ml. of 7.5 mM-MgSO₄; 0.05 ml. of 0.1M-cysteine, pH 7.5; 0.05 ml. of diluted extract; 0.05 ml. of glucose analogue solution; the final volume was 0.25 ml. (i.e. the final concn. of glucose 1-phosphate was 4 mM). Incubation was carried out at 30° for 5 min. The values for the glucose 6-phosphate formed are the means $\pm$ S.E.M. from four experiments.

Analyse	Concn. of analogue (mM)	Glucose 6-phosphate formed (μ mole/5 min.)
D-Glucosamine	0	0.241 $\pm$ 0.006
	2	0.270 $\pm$ 0.008
	4	0.258 $\pm$ 0.002
	8	0.259 $\pm$ 0.008
	40	0.134 $\pm$ 0.017
N-Acetyl-D-glucosamine	0	0.326 $\pm$ 0.020
	2	0.309 $\pm$ 0.007
	4	0.299 $\pm$ 0.001
	8	0.306 $\pm$ 0.004
	40	0.246 $\pm$ 0.003
2-Deoxy-D-glucose	0	0.226 $\pm$ 0.005
	1	0.238 $\pm$ 0.020
	4	0.211 $\pm$ 0.010
	8	0.224 $\pm$ 0.004
	20	0.207 $\pm$ 0.010

Fig. 1. Effects of D-glucosamine and N-acetylglucosamine on liver uridine diphosphate glucose pyrophosphorylase. The reaction mixture contained: 0.2 ml. of diluted liver extract; 0.05 ml. of muscle phosphoglucomutase; 0.1 ml. of 0.06 mM-glucose 1,6-diphosphate; 0.5 ml. of 50 mM-tris-maleate buffer, pH 7.8; 0.05 ml. of 15 mM-NADP; 0.1 ml. of 12 mM-UDP-glucose; 0.01 ml. of glucose 6-phosphate dehydrogenase (2 mg./0.5 ml.); 0.1 ml. of 10 mM-D-glucosamine or 50 mM-N-acetyl-D-glucosamine; 0.1 ml. of 0.6M-MgCl₂; the final volume was 30 ml. The reaction was started by adding 0.1 ml. of 30 mM-sodium pyrophosphate at zero time at room temperature. A, Control; B, N-acetyl-D-glucosamine; C, D-glucosamine.

the inhibition is 77%. Neither N-acetyl-D-glucosamine nor 2-deoxy-D-glucose is an inhibitor of this enzyme.

(e) *Phosphorylase*. Phosphorylase is inhibited by D-glucosamine and 2-deoxy-D-glucose at relatively high concentrations (Table 8), but N-acetyl-D-glucosamine has no effect on the enzyme at the concentrations tested.

Variation of effects of glucose on the phosphorylase activity with time of incubation

Fig. 3 (which gives results found in a typical experiment) shows that a rapid decrease in the phosphorylase activity is observed, during the first 20 min. of incubation, when liver slices are incubated in the absence of glucose. However, little further change in the activity occurs during the next 30 min. of incubation. When glucose is added to the medium after the first 20 min. of incubation, a further decrease in phosphorylase activity takes place (Fig. 3). Phosphorylase activity, after 50 min. of incubation, is 31 and 13% of that at the beginning of incubation in the absence and in the presence of glucose respectively.

Effects of glucose analogues and certain other compounds on the phosphorylase activity of liver slices

When either D-glucosamine or N-acetyl-D-glucosamine together with glucose are present in the

Fig. 2. Effects of 2-deoxyglucose and glucosamine 6-phosphate on liver uridine diphosphate glucose pyrophosphorylase. The reaction mixture contained: 0.2 ml. of diluted liver extract; 0.05 ml. of muscle phosphoglucomutase; 0.1 ml. of 0.06 mM-glucose 1,6-diphosphate; 0.5 ml. of 50 mM-tris-maleate buffer, pH 7.8; 0.05 ml. of 15 mM-NADP; 0.1 ml. of 12 mM-UDP-glucose; 0.01 ml. of glucose 6-phosphate dehydrogenase (2 mg./0.5 ml.); 0.1 ml. of 40 mM-2-deoxyglucose or 5 mM-glucosamine 6-phosphate; 0.1 ml. of 0.6M-MgCl₂; the final volume was 30 ml. The reaction was started by adding 0.1 ml. of 30 mM-sodium pyrophosphate at zero time at room temperature. A, Control for glucosamine 6-phosphate; B, glucosamine 6-phosphate; C, control for 2-deoxyglucose; D, 2-deoxyglucose.

Table 7. *Effects of glucose analogues on liver uridine diphosphate glucose-glycogen glucosyltransferase*

The reaction mixture contained: 0.01 ml. of 25 mM-UDP-glucose; 0.01 ml. of 0.05M-glucose 6-phosphate; 0.01 ml. of particulate glycogen fraction in 0.75M-glycine buffer, pH 8.5, containing EDTA (25 mM) and glycogen (40 mg./ml.); 0.01 ml. of glucose analogue solution; the final volume was 0.05 ml. (i.e. the final concn. of UDP-glucose was 5 mM). Incubation was done at 37° for 5 min. The values for the UDP formed are the means $\pm$ s.e.m. from six experiments (with D-glucosamine) or four experiments (with N-acetyl-D-glucosamine or 2-deoxy-D-glucose).

Analogue	Concn. of analogue (mM)	UDP formed (μ mole/5 min.)	Inhibition (%)
D-Glucosamine	0	0.066 $\pm$ 0.001	0
	2.5	0.049 $\pm$ 0.001	26
	5	0.033 $\pm$ 0.002	50
	10	0.024 $\pm$ 0.004	64
	20	0.015 $\pm$ 0.001	77
N-Acetyl-D-glucosamine	0	0.065 $\pm$ 0.001	0
	2.5	0.063 $\pm$ 0.004	3
	10	0.065 $\pm$ 0.002	0
	20	0.062 $\pm$ 0.002	4
2-Deoxy-D-glucose	0	0.052 $\pm$ 0.004	0
	2.5	0.053 $\pm$ 0.001	0
	5	0.056 $\pm$ 0.005	0
	10	0.060 $\pm$ 0.001	0
	20	0.048 $\pm$ 0.004	8

Table 8. *Effects of glucose analogues on liver phosphorylase*

The reaction mixture contained: 0.05 ml. of a mixture of 0.128M-glucose 1-phosphate, 1.6% glycogen, 0.13M-NaF and 4 mM-AMP, pH 6.1; 0.05 ml. of diluted extract in 0.1M-NaF; 0.1 ml. of glucose analogue solution; the final volume was 0.2 ml. (i.e. the final concn. of glucose 1-phosphate was 32 mM). Incubation was done at 30° for 5 min. The values for the inorganic phosphate are the means $\pm$ s.e.m. from two experiments.

Analogue	Concn. of analogue (mM)	Inorganic phosphate formed (μ mole/10 min.)
D-Glucosamine	0	0.467 $\pm$ 0.010
	25	0.451 $\pm$ 0.005
	50	0.401 $\pm$ 0.002
	100	0.309 $\pm$ 0.001
N-Acetyl-D-glucosamine	0	0.418 $\pm$ 0.003
	12.5	0.420 $\pm$ 0.006
	25	0.429 $\pm$ 0.003
2-Deoxy-D-glucose	0	0.453 $\pm$ 0.004
	25	0.367 $\pm$ 0.004
	50	0.341 $\pm$ 0.010
	100	0.295 $\pm$ 0.005
		0.276 $\pm$ 0.001

Fig. 3. Variation of liver phosphorylase activity with incubation time and effect of glucose. The reaction mixture contained 200 mg. of liver slices in the high-potassium medium. The temperature of incubation was 37°. The gas phase was O₂ + CO₂ (95:5). Phosphorylase activity was assayed at appropriate intervals as described in the text. A, No glucose added; B, glucose (final concn. 30 mM) added to the medium at the time indicated by the arrow.

medium, the phosphorylase activity of the slices is maintained at a higher level than in the absence of these analogues. Table 9 (column A) shows that the phosphorylase activity is 39 and 71 % higher in the presence of 40 mM-D-glucosamine and 40 mM-N-acetyl-D-glucosamine respectively than in their absence. N-Acetyl-D-glucosamine is more effective in maintaining the higher phosphorylase activity than is D-glucosamine. On the other hand, 2-deoxy-D-glucose decreases the phosphorylase

activity to a slight extent below that of the control, in contrast with the change occurring in the presence of glucose. The effects of D-glucosamine and N-acetyl-D-glucosamine are more marked in the absence of glucose (Table 9, columns B, C and D). L-Glutamine, L-glutamic acid, α -oxoglutaric acid and ammonium chloride are also effective in maintaining the higher level of the activity (which is higher in the absence than in the presence of glucose).

Table 9. *Effects of glucose analogues and other compounds on the phosphorylase activity of liver slices*

Slices (approx. 200 mg./flask) were preincubated in the potassium-rich medium for 20 min. at 37° without addition. At the end of this period, glucose (30 mM) and test compounds (40 mM), either alone or in combination, were added and the incubation was further continued for 30 min. The phosphorylase content was determined as described in the text. The gas phase was O₂ + CO₂ (95:5).

Addition	Phosphorylase activity (μmoles of inorganic phosphate liberated/10 min./g. of tissue)			
	In the presence of glucose (A)	In the absence of glucose		
		Expt. 1 (B)	Expt. 2 (C)	Expt. 3 (D)
None (initial)*	51.6 ± 6.6	75.9 ± 1.8	38.5 ± 0.9	53.5 ± 3.3
None (control)	28.7 ± 3.5	59.4 ± 2.5	48.5 ± 1.5	51.4 ± 0.8
D-Glucosamine	40.9 ± 2.5	93.0 ± 2.5	—	—
N-Acetyl-D-glucosamine	49.0 ± 6.6	—	64.0 ± 0.3	—
2-Deoxy-D-glucose	27.1 ± 2.5	—	—	37.4 ± 0.4
L-Glutamic acid	43.6 ± 2.9	87.5 ± 1.4	—	—
L-Glutamine	39.7 ± 1.5	87.1 ± 1.6	—	—
NH ₄ Cl	38.7 ± 1.2	83.0 ± 1.2	—	—
α-Oxoglutaric acid	34.7 ± 4.1	—	—	—

* The initial phosphorylase content of the slices was determined at the end of the 20 min. preincubation.

It has been mentioned that glucose causes a decrease in the phosphorylase activity (Fig. 3). The effects of glucose analogues and of L-glutamic acid, L-glutamine and ammonium chloride on the phosphorylase activity of liver slices in the absence of glucose were studied (Table 9). The activities of phosphorylase observed in the presence of the test compounds are much higher than the corresponding values obtained in the presence of glucose. However, the percentage increases in the activities are not very different from each other.

DISCUSSION

Spiro (1958) has proposed a mechanism to explain the inhibition of glycogen synthesis in rat-liver slices that involves hexokinase as the site of inhibition by glucose analogues. However, the experimental results obtained in the present study indicate that hexokinase cannot play a major role in the suppression of glycogen synthesis by glucose analogues. First, when pyruvate is the source of glycogen, the production of the latter is inhibited by D-glucosamine and 2-deoxy-D-glucose. As pyruvate is converted into hexose phosphate by the reverse reactions of the glycolytic cycle before it is transformed into glycogen, hexokinase is apparently not involved. Secondly, L-glutamine, L-glutamic acid, α-oxoglutaric acid and ammonium chloride also inhibit glycogen synthesis from glucose. These compounds, at the concentrations used, do not seem to affect hexokinase activity. Moreover, hexokinase activity is not inhibited significantly by D-glucosamine, N-acetyl-D-glucosamine and 2-deoxy-D-glucose at ratios of glucose to analogue concentrations which inhibit strongly glycogen synthesis from glucose.

Influx of glucose into the liver cell is not affected by the three glucose analogues tested, except for N-acetyl-D-glucosamine which slightly diminishes glucose transport at a concentration of 90 mM. The amount of inhibition of glucose transport by N-acetyl-D-glucosamine does not explain the marked inhibition of glycogen synthesis by this compound in liver slices. The conclusion that glucose analogues do not affect the entry of glucose into liver slices is expected from the results obtained by Cahill, Ashmore, Earle & Zottu (1958) which show that liver cells are freely permeable to glucose and also to other sugars of similar molecular weight.

Hexokinase, phosphoglucosmutase, uridine diphosphate glucose pyrophosphorylase and phosphorylase are not inhibited by glucose analogues sufficiently to explain the diminution of glycogen synthesis by these analogues. Most of these enzymes are affected slightly by the analogues only at relatively high concentrations. Uridine diphosphate glucose-glycogen glucosyltransferase is the only enzyme which is inhibited significantly by D-glucosamine at relevant concentrations. However, the other two glucose analogues have no effect on this enzyme. Inhibitions of 26 and 50 % are observed in the presence of 2.5 mM- and 5 mM-D-glucosamine respectively in a medium containing 5 mM-uridine diphosphate glucose. Even these extents of inhibition are insufficient to account for the marked inhibition of glycogen synthesis in liver slices by this amino sugar as, at a ratio of substrate to analogue of 3:1, D-glucosamine inhibits glycogen synthesis from glucose by 85 %.

The diminution of glycogen synthesis, however, may be accounted for by the fact that a higher phosphorylase activity than the normal is maintained when liver slices are incubated in the presence

of D-glucosamine and N-acetyl-D-glucosamine, the latter being about twice as effective as the former. L-Glutamine, L-glutamic acid, α -oxoglutaric acid and ammonium chloride, which are inhibitors of glycogen synthesis, have a similar effect also on the phosphorylase activity. The difference between the phosphorylase activities is sufficient to account for the decrease in the amount of glycogen. It may be calculated from the experimental results that liver contains phosphorylase and uridine diphosphate glucose-glycogen glucosyltransferase with activities corresponding to 200 μ moles of substrate affected/hr./g. of tissue and 50 μ moles of substrate affected/hr./g. of tissue respectively. [Villar-Palasi & Lerner (1960) obtained a value of 300 μ moles/hr./g. of tissue for phosphorylase, and Leloir, Olavarria, Goldemberg & Carminatti (1959) 187 μ moles/hr./g. of tissue for uridine diphosphate glucose-glycogen glucosyltransferase.] As phosphorylase activity drops to about 20% of the initial activity after 50 min. of incubation, this enzyme activity then corresponds to 40 μ moles of substrate affected/hr./g. of tissue. If the activity of uridine diphosphate glucose-glycogen glucosyltransferase is not altered during this period, the difference between the activities of these two enzymes, 10 μ moles of substrate affected/hr./g. of tissue, will account for the net increase in glycogen in 1 hr. The value is in good agreement with the rate of glycogen synthesis, 1.0–2.0 mg. of glucose affected/hr./g. of tissue, as 10 μ moles of glucose is equivalent to 1.8 mg. of glucose. The presence of 40 mM-N-acetyl-D-glucosamine increases the phosphorylase activity by about 70% (Table 9, column A). Consequently the phosphorylase activity corresponds approximately to 68 μ moles of substrate affected/hr./g. of tissue. Because this activity is slightly higher than that of uridine diphosphate glucose-glycogen glucosyltransferase (50 μ moles of substrate affected/hr./g. of tissue), no glycogen synthesis is observed. A similar explanation can be applied to D-glucosamine when the inhibition of uridine diphosphate glucose-glycogen glucosyltransferase by this amino sugar is considered.

Rall, Sutherland & Berthet (1957) found that the formation of active phosphorylase from dephosphophosphorylase is stimulated by adenosine 3',5'-phosphate. Rall & Sutherland (1958) also observed that L-glutamic acid stimulates the formation of this cyclic nucleotide. This finding implies that L-glutamic acid will increase the phosphorylase activity through the formation of the cyclic nucleotide. These authors also found that pyruvate and fumarate are about as effective as L-glutamate in this connexion and that anaerobiosis reduces the formation of the cyclic nucleotide. The stimulatory effects are explained by the maintenance, in the liver preparation, of ATP, the precursor of the cyclic

nucleotide. Kleinzeller & Rybova (1957), in their studies of glycogen synthesis in brain-cortex slices, have confirmed that the presence of L-glutamate diminishes glycogen synthesis (McIlwain & Tresize, 1956) and have shown also that high concentrations of K⁺ ion in the medium bathing the brain slices diminish glycogen synthesis. The latter effect is correlated with the well-known accelerating action of high concentrations of K⁺ ion on brain aerobic glycolysis. The glutamate effect is attributed to utilization of ATP (Acs, Balazs & Straub, 1953) in the synthesis of L-glutamine. In the present study, L-glutamine, L-glutamic acid, α -oxoglutaric acid and ammonium chloride maintained higher phosphorylase activities than in their absence in slices of liver. These compounds may stimulate the production of adenosine 3',5'-phosphate by a mechanism similar to that postulated for L-glutamic acid, i.e. the maintenance, in liver, of ATP. Conceivably D-glucosamine and N-acetyl-D-glucosamine may act by stimulation of the formation of adenosine 3',5'-phosphate from ATP. The behaviour of 2-deoxy-D-glucose towards phosphorylase activity differs from that of D-glucosamine or of N-acetyl-D-glucosamine although it inhibits glycogen synthesis in liver slices like the latter glucose analogues. Possibly 2-deoxy-D-glucose 6-phosphate (Sols & Crane, 1954) is implicated in its activity.

The term 'inhibition', when referred to glycogen biosynthesis, does not seem appropriate, at any rate in liver, where the effects of D-glucosamine, N-acetyl-D-glucosamine, L-glutamine, L-glutamic acid, α -oxoglutaric acid and ammonium chloride are concerned. The major effect of these compounds is not so much an inhibition of enzymic reactions involved in glycogen synthesis as an elevation of phosphorylase activity leading to increased breakdown of glycogen. Apparently large inhibitions of glycogen synthesis from pyruvate are brought about by D-glucosamine (Table 1). This phenomenon may be interpreted as being due to the sum of two separate events: synthesis and degradation of glycogen. The activity of phosphorylase is so high compared with that of uridine diphosphate glucose-glycogen glucosyltransferase that a net decrease in glycogen content is observed.

SUMMARY

1. The synthesis of glycogen from glucose in liver slices is strongly diminished by D-glucosamine, N-acetyl-D-glucosamine, 2-deoxy-D-glucose and D-glucosamine 6-phosphate at low concentrations. L-Glutamine, L-glutamic acid, α -oxoglutaric acid and ammonium chloride also diminish glycogen synthesis from glucose in the same tissue.

2. Glycogen synthesis from pyruvate is diminished by D-glucosamine and 2-deoxy-D-glucose.

3. The glucose analogues tested affect neither glucose influx across the liver-cell membrane nor, at low concentrations, the activities of four enzymes concerned in glycogen metabolism (hexokinase, phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase and phosphorylase). A fifth enzyme, uridine diphosphate glucose-glycogen glucosyltransferase, is considerably affected by D-glucosamine.

4. The phosphorylase activity of liver slices incubated in the presence of the compounds mentioned above, except for 2-deoxy-D-glucose, is higher than that found in the control slices. As phosphorylase catalyses the breakdown of glycogen, this effect on phosphorylase activity is sufficient to account for the apparent inhibition of glycogen synthesis.

5. The possible mechanisms of action of the amino sugars and other substances on glycogen biosynthesis are discussed.

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