Studies on Glycogen Synthesis in Pigeon Liver Homogenates

INCORPORATION OF HEXOSE INTO GLYCOGEN

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Liver homogenates of avian species, but not of mammals, form glycogen from glucose, mannose, fructose and galactose. Incorporation of labelled glucose, fructose and mannose, but not of labelled galactose, into glycogen is diluted isotopically by unlabelled glucose. Except for fructose, glycogen formation from other substrates by pigeon liver homogenates compares favourably with that from the same substrates in pigeon liver slices. Optimum conditions for glycogen synthesis from glucose by pigeon liver homogenate are: medium of incubation, 0.175 M-sucrose–45 mM-potassium chloride–15 mM-glycylglycine buffer, pH 7.5; concentration of substrate, 15 mM; concentration of tissue, less than 120 mg./ml.; temperature of incubation, 37–43°; atmosphere, oxygen. Uncouplers of oxidative phosphorylation, Ca^{2+} , EDTA, PP_i, 2-deoxyglucose 6-phosphate and microsomal fraction of rat liver are inhibitory to glycogen synthesis from glucose. Starvation of pigeons for 24 and 48 hr. leads to a slight stimulation of glycogen synthesis in their liver homogenates as compared with fed controls. Pigeon liver homogenates can be separated into subcellular fractions that on reconstitution can synthesize glycogen. All the enzymes of the glycogen pathway except soluble high- K_m glucokinase are present in pigeon liver.

Previous work in this Laboratory has shown that liver homogenates of pigeon can synthesize glycogen from glucose at rates similar to those observed for pigeon liver slices (Nigam & Fridland, 1964). The discovery of such a system has offered the possibility of a detailed investigation on the process of glycogen formation and its regulation. In the present work, an attempt has been made to establish the conditions for maximal formation of glycogen from glucose in the pigeon liver homogenate and to study factors that influence glycogen synthesis.

MATERIALS AND METHODS

Chemicals. [$1\text{-}^{14}\text{C}$]Glucose was purchased from Merck and Co., Montreal, Quebec, Canada. [$1\text{-}^{14}\text{C}$]Fructose, [$1\text{-}^{14}\text{C}$]galactose, [$1\text{-}^{14}\text{C}$]mannose and [$1\text{-}^{14}\text{C}$]glucosamine hydrochloride were products of New England Nuclear Corp., Cambridge, Mass., U.S.A. Fine chemicals and enzymes 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.), young

rabbits (2–3 lb.), pigeons, chickens, ducks and frogs were obtained from Canadian Breeding Laboratories, St Constant, Quebec, Canada. Rats were kept on a diet of Purina Labena Chow and pigeons on Pigeon Chow; both diets were obtained from Ralston Purina Co., St Louis, Mo., U.S.A. Unless otherwise stated food and water were provided to the animals *ad libitum*. Chickens and ducks (approx. 3 months old) and adult frogs were used the same day they were obtained from the commercial source. Most of the work with the animals was done without ascertaining their sex, but some studies with pigeons and rats of different sexes have failed to reveal any sex-related difference in glycogen synthesis from glucose in liver homogenate.

Preparation of liver homogenates for incorporation studies. Animals were killed by decapitation and were allowed to bleed. Livers were removed and kept in a beaker over ice. Portions (2 g.) of the liver were chopped with scissors and transferred to a Potter–Elvehjem-type homogenizer with a thick glass tube and a Teflon pestle. The livers were homogenized for 3 min. at 0–5° in either cold 0.15 M-KCl in 50 mM-glycylglycine buffer, pH 7.4, or cold 0.25 M-sucrose. The homogenate was diluted to 10 ml. with the homogenizing medium. The liver homogenates were used 3–5 min. after they were prepared.

Incubation procedure for glucose incorporation into glycogen. Unless otherwise stated, liver homogenate (0.4 ml.) was transferred to 20 ml. beakers containing 0.3 ml. of 0.15 M-KCl in 50 mM-glycylglycine buffer, pH 7.5.

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Activator or inhibitor was added after dissolution in 0.25M-sucrose or buffered KCl solution (0.25ml.). After equilibration for 2–3 min. at room temperature, a known amount (usually 10 μ moles) of labelled sugar (0.05ml., containing either 50 000 or 100 000 counts/min.) was added and the beakers were transferred to a Dubnoff metabolic shaker maintained at 37°. The apparatus was flushed with 100% O₂ and the beakers were shaken at 10–20 strokes/min.

Isolation of glycogen. To the reaction digests an equal volume of 60% (w/v) KOH was added to stop the reaction. The contents were transferred to tubes, and the beakers were rinsed with 0.5 ml. of 30% (w/v) KOH and the rinsing solutions transferred to the appropriate tubes. Glycogen was isolated and its ¹⁴C radioactivity measured as described by Nigam (1967a).

Hydrolysis of isolated glycogen. The identity of isolated glycogen as glucose polymer was established by dialysis of the glycogen solution for 36 hr. at 5°, hydrolysis to glucose by N-H₂SO₄ at 100° for 1 hr., neutralization of the solution with N-NaOH and preparation of the glucose phenyllosazone (Vogel, 1956). A 90% recovery of the radioactivity observed initially in the glycogen was usually obtained in the osazone.

Content of glucose and glycogen in pigeon liver. Determination of glucose in pigeon liver revealed that it contained 5.0–7.0 μ moles/g. wet wt. Because the liver contains glucosyl-oligosaccharides, glucose was determined by its transformation into glucose 6-phosphate with ATP and hexokinase (EC 2.7.1.1) and estimation of glucose 6-phosphate with glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and NADP⁺ (Nigam, 1966). In calculating the amount of glycogen synthesized by liver homogenates, the amount of endogenous glucose was taken into consideration. It was also observed that, during glycogen synthesis in homogenates, the concentration of endogenous glucose decreased by 10–20%.

The concentration of glycogen in the liver of fed pigeons varied in the range 70–200 μ moles of glucose equiv./g. wet wt. This concentration did not change greatly during incubation of the liver homogenates.

Estimation of synthesized glycogen. The number of μ moles of glucose equiv. of glycogen formed in experiments with labelled precursors is given by multiplying the counts/min. in isolated glycogen (after correction for Na₂SO₄ effect and the background) by the number of μ moles of sugar present and dividing this value by the counts/min. in the labelled sugar added, the radioactivity of which was measured under the same conditions but in the absence of Na₂SO₄.

Measurement of oxygen uptake. The rate of respiration in liver homogenate was measured by the conventional Warburg technique (Umbreit, Burris & Stauffer, 1964) after a preincubation period of 5 min. Sugar solution was tipped from the side arm into the main chamber after preincubation. The fluid volume was 2.5 ml. in every case. The CO₂ was absorbed by 0.2 ml. of 20% (w/v) KOH in the centre well with a Whatman no. 1 filter-paper strip inserted in it. Glycogen synthesis was measured concurrently in the homogenate from the same liver, incubated with labelled glucose as described above in a 1 ml. digest in a 20 ml. beaker on a Dubnoff shaker at 37° in air. Thus a rough comparison between the rate of respiration and the rate of glycogen synthesis was obtained.

Preparation and incubation of liver slices. Liver slices were prepared as described by Elliott (1955). They were

suspended in the same medium that was used for homogenization of the liver and incubated under the same conditions.

Assay of enzymes. Hexokinase (EC 2.7.1.1) and glucokinase (EC 2.7.1.2) activities were measured at 0.5 mM- and 100 mM-glucose concentration respectively by the procedure of Viñuela, Salas & Sols (1963).

Phosphoglucosmutase (EC 2.7.5.1) activity was determined by spectrophotometric determination of glucose 6-phosphate formed from glucose 1-phosphate. The digests contained: glucose 1-phosphate, pH 7.4 (20 mM), glycylglycine buffer, pH 7.4 (50 mM), MgCl₂ (5 mM), cysteine, pH 7.5 (10 mM), NADP⁺ (1 mM), glucose 6-phosphate dehydrogenase (2 units) and tissue supernatant in a total of 3 ml. Increase in extinction was measured every 30 sec. for 3 min. Correction was made for the contribution of 6-phosphogluconate dehydrogenase by deducting the rate of NADP⁺ reduction with 6-phosphogluconic acid as substrate.

UDP-glucose pyrophosphorylase activity was measured by the method of Villar-Palasi & Lerner (1960).

UDP-glucose- α -glucan glucosyltransferase (EC 2.4.1.11) activity was determined by the method of Leloir & Goldemberg (1960). The enzyme was measured both in the absence and presence of glucose 6-phosphate.

Phosphorylase (EC 2.4.1.1) activity was measured by the method of Sutherland (1955) in liver homogenates prepared in 0.25 M-sucrose containing KF (0.1 M). The phosphorylase activity was estimated in the presence and absence of AMP as described by Nigam (1967a).

Glucose 6-phosphatase (EC 3.1.3.9) activity was measured by the method of Swanson (1955).

Fractionation of liver homogenates. Liver homogenates prepared in iso-osmotic buffered KCl were fractionated into nuclear-mitochondrial and cytoplasmic fractions by centrifugation of the homogenate at 700g for 10 min. at 0° in the International centrifuge. The nuclear-mitochondrial fraction obtained was washed twice with buffered KCl solution (half the volume of the original homogenate) and the washings were combined with the cytoplasmic fraction. Mixing of the two fractions gave reconstituted homogenates that effectively formed glycogen from glucose. Rat liver homogenates prepared in 0.25 M-sucrose were fractionated by the method described by Umbreit *et al.* (1964).

When pigeon liver homogenates were prepared in iso-osmotic sucrose and were fractionated by the conventional technique (Umbreit *et al.* 1964), recombination of various fractions gave preparations that were inactive in forming glycogen. The procedure was therefore slightly modified. Addition of MgCl₂ (3 mM) to the sucrose homogenate followed by its fractionation gave reconstituted homogenates that had glycogen-synthesizing capacity. However, in the presence of salt (MgCl₂) heavy mitochondria sedimented with the nuclear fraction. In all reconstitution studies, therefore, addition of nuclear fraction was necessary for glycogen synthesis to be obtained.

RESULTS

Table 1 shows the extent of incorporation of [¹⁴C]glucose into glycogen when pigeon liver was homogenized in various media. Iso-osmotic sodium chloride buffered with glycylglycine was only slightly

Table 1. *Glycogen synthesis from glucose by pigeon liver homogenates in various media*

For Expts. 1 and 2, pigeon liver homogenates (20%) were prepared in cold iso-osmotic KCl or NaCl in 50 mm-glycylglycine buffer, pH 7.4, and for Expts. 3, 4 and 5 in cold iso-osmotic sucrose. A 0.4 ml. sample of homogenate was transferred to 20 ml. beakers and diluted to 0.95 ml. with KCl-glycylglycine (Expt. 1), NaCl-glycylglycine (Expt. 2), sucrose (Expt. 3), sucrose and KCl-glycylglycine (Expt. 4) and sucrose and NaCl-glycylglycine (Expt. 5) to give the concentrations indicated. After equilibration at room temperature for 3 min., 10 μ moles of [1- 14 C]glucose (0.05 ml.) dissolved in the medium of the homogenate were added. The reaction digests were incubated at 37° in 100% O₂ for 30 min. The results are given as means $\pm$ s.d. obtained for glycogen synthesis in four experiments of each kind.

Expt. no.	Composition of incubation mixture	Glycogen formed (μ moles of glucose equiv./30 min./80 mg. wet wt. of liver)
1	0.15 M-KCl-50 mm-glycylglycine, pH 7.4	1.05 $\pm$ 0.03
2	0.15 M-NaCl-50 mm-glycylglycine, pH 7.4	0.90 $\pm$ 0.02
3	0.25 M-Sucrose	0.26 $\pm$ 0.01
4	0.175 M-Sucrose-50 mm-KCl-15 mm-glycylglycine, pH 7.4	1.50 $\pm$ 0.05
5	0.175 M-Sucrose-50 mm-NaCl-15 mm-glycylglycine, pH 7.4	1.28 $\pm$ 0.04

inferior to similarly buffered iso-osmotic potassium chloride. Sucrose (0.25 M) in the absence of ionizable salt also permitted incorporation of glucose into glycogen to a small extent. However, incorporation was increased about fivefold when either buffered iso-osmotic potassium chloride or sodium chloride was also added. The final concentration of the salt that gave maximum incorporation was 50 mm.

Subsequently, when liver homogenates of various species were tested for their capacity to incorporate [14 C]glucose into glycogen, sizable incorporation was obtained only with the livers of the avian species. Although the data in Table 2 are given for approx. 15 mm-glucose (endogenous and added), incorporation of glucose into glycogen by homogenates of mammalian species was not obtained even at higher (40 mm) glucose concentration.

As shown in Table 3, 14 C from 14 C-labelled glucose, galactose, mannose and arabinose was incorporated to the same extent in the homogenate as in the slice. Only the incorporation of 14 C from [14 C]fructose into glycogen fell off by about one-half on homogenization of the liver. Measurable incorporation of 14 C from labelled galactose and

Table 2. *Glycogen synthesis from glucose by liver homogenates of different species of animals*

The digests contained: 0.4 ml. of liver homogenate in iso-osmotic sucrose, 0.3 ml. of 0.15 M-KCl in 50 mm-glycylglycine buffer, pH 7.4, and 0.25 ml. of iso-osmotic sucrose. After equilibration for 3 min. at room temperature, labelled glucose (10 μ moles) dissolved in 0.05 ml. of iso-osmotic sucrose was added and the beakers containing the digest were transferred to a Dubnoff metabolic shaker maintained at 37° and flushed with O₂. The incubation time was 30 min. The values are means $\pm$ s.d. Where only two analyses were carried out both values are given.

Species	No. of animals	Glycogen formed (μ moles of glucose equiv./30 min./80 mg. wet wt. of liver)
Mouse	3	0.006 $\pm$ 0.003
Rat	12	0.007 $\pm$ 0.004
Rabbit	2	0.01, 0.015
Chicken	3	0.60 $\pm$ 0.2
Duck	2	1.1, 1.2
Pigeon	12	1.3 $\pm$ 0.42
Frog	3	0.003 $\pm$ 0.0

Table 3. *Glycogen synthesis from different sugars in pigeon liver slices and homogenates*

Pigeon liver homogenate was prepared in iso-osmotic KCl in glycylglycine buffer, pH 7.4. Slices from the same liver were prepared as described in the Materials and Methods section. Slices and homogenate were incubated with labelled hexose under the same conditions. Glycogen was isolated and its 14 C radioactivity measured after 45 min. of incubation. The amount of radioactivity added was approx. 100 000 counts/min./ml. of reaction mixture for each substrate.

Labelled substrate	Concn. of substrate (mm)	Glycogen formed (μ moles of glucose equiv./45 min./200 mg. wet wt. of liver)	
		Slices	Homogenate
[1- 14 C]Arabinose	0.2	0.005	0.003
[U- 14 C]Fructose	10	1.2	0.70
[1- 14 C]Galactose	2	0.05	0.075
[1- 14 C]Glucosamine	5	—	0.002
[1- 14 C]Glucose	40	2.8	3.3
[1- 14 C]Mannose	10	0.90	1.08

arabinose was observed only when substrates with high specific activity were used, showing that they were poor substrates for glycogen synthesis. [1- 14 C]Glucosamine was not incorporated into glycogen by the pigeon liver homogenate.

Kinetic studies

Effect of substrate concentration. The amount of glycogen synthesized from glucose increased on

increasing the glucose concentration. Optimum glycogen synthesis was observed at 15mM-glucose (the range used was 5–45mM). With higher concentration of glucose there was slight inhibition of glycogen synthesis. With 10 μ moles of hexose added, the rates of incorporation from glucose, fructose, mannose and galactose were 0.6, 0.05, 0.2 and 0.05 μ mole of glucose equiv. of glycogen/min./g. wet wt. of liver respectively. These values include the effect of isotopic dilution of fructose and mannose by glucose. No correction factor was necessary for galactose, since incorporation of [14 C]galactose into glycogen was not diluted isotopically by glucose, as described in the following paragraph.

Although galactose served as a precursor of glycogen, it was probably incorporated into glycogen without its conversion into a glucose metabolite. This was borne out when addition of unlabelled glucose failed to decrease the incorporation of 0.1 μ mole of [14 C]galactose into glycogen. On the other hand, incorporation of 14 C from [14 C]-glucose, [14 C]fructose or [14 C]mannose decreased on the addition of unlabelled glucose owing to isotopic dilution (Fig. 1).

Effect of concentration of homogenate, pH and temperature. Incorporation of glucose into glycogen increased linearly from 0.3 to 2.5 μ moles of glucose as the tissue concentration was raised from 20 to 120mg. wet wt. of liver/ml. Beyond 120mg. wet wt. of tissue/ml. there was no increase in the amount of glycogen formed. Optimum glycogen synthesis was obtained at pH 7.5. The amounts of glycogen formed at pH 5.6, 6.5, 8.0 and 8.4 were 10, 27, 10 and 0% respectively of that formed at pH 7.5

Fig. 1. Incorporation of 14 C from 0.1 μ mole of each of 14 C-labelled glucose (●), galactose (■), mannose (▲) and fructose (□) in the presence of increasing amounts of unlabelled glucose. About 50000–55000 counts/min. of each labelled sugar were added. Experimental conditions are described in Table 2. The values are averages of those obtained in two experiments.

(2.2 μ moles of glucose equiv.). The ability of pigeon liver homogenates to synthesize glycogen increased considerably as the temperature was raised from 10° to 20°. Inhibition was not observed at temperatures as high as 43°.

Energy requirement for glycogen synthesis. Two types of experiments were performed to establish the requirement of oxidative energy for the polymerization of glucose. First, oxygen uptake and the incorporation of glucose into glycogen were measured as a function of time (Fig. 2). Oxygen uptake was linear up to 30 min. and then slowly fell off. During the same period, incorporation of glucose into glycogen was linear for 45 min. and no further increase occurred thereafter. The second approach consisted in blocking oxidative phosphorylation by uncouplers and measuring glucose incorporation into glycogen. 2,4-Dinitrophenol (0.25mM), antimycin A (0.1mM) and sodium azide (0.5mM), as well as sodium arsenate (5mM), effectively blocked synthesis of glycogen. Addition of ATP, UTP and an ATP-generating system to dinitrophenol-treated homogenate failed to give glycogen synthesis from glucose, nor did activation of glycogen synthesis occur when ATP, UTP and ATP-generating system were added to liver homogenates.

Effects of various activators and inhibitors

Effects of glucose 6-phosphate and 2-deoxyglucose 6-phosphate. Increasing concentrations of the

Fig. 2. Oxygen uptake (○) and glycogen synthesis (●) by pigeon liver homogenates. Details of the measurement of oxygen uptake are given in the Materials and Methods section and those of the measurement of glycogen synthesis in Table 2. The values are averages of those obtained in three experiments of each kind.

Fig. 3. Inhibition of glycogen synthesis from glucose by 2-deoxyglucose 6-phosphate in the presence (■) and absence (●) of 3mm-glucose 6-phosphate. The values are not corrected for glucose liberated on dephosphorylation of glucose 6-phosphate by glucose 6-phosphatase of pigeon liver homogenate. Experimental conditions are described in Table 2. The values are averages of those obtained in two experiments.

glucose 6-phosphate were added to the pigeon liver homogenate system synthesizing glycogen from [^{14}C]glucose. Incorporation was slightly stimulated by low concentrations of glucose 6-phosphate, but concentrations higher than 5mm decreased the radioactivity in the isolated glycogen.

This effect was related to the liberation of glucose from glucose 6-phosphate by glucose 6-phosphatase. When similar concentrations of unlabelled glucose were added, the radioactivity in the isolated glycogen was also decreased to about the same extent. The situation was different when 2-deoxyglucose 6-phosphate was employed. Fig. 3 shows the loss in the ability of the homogenate to synthesize glycogen from glucose in the presence of increasing concentrations of 2-deoxyglucose 6-phosphate. Further, glucose 6-phosphate was not able to overcome the inhibition due to 2-deoxyglucose 6-phosphate. Since 2-deoxyglucose 6-phosphate is not a precursor of glycogen, its inhibition of glycogen synthesis could be due to the inhibition of an enzyme or the energy-generating system. 2-Deoxyglucose was not an inhibitor of the glycogen-synthesizing system of pigeon liver.

Effects of P_i and PP_i . P_i inhibited only slightly the incorporation of [^{14}C]glucose into glycogen (Fig. 4). Concentrations as high as 10mm gave only a 20% inhibition. PP_i inhibited strongly (80%) at 10mm concentration.

Effects of Mg^{2+} , Ca^{2+} and EDTA. Fig. 5 shows the

Fig. 4. Effect of P_i (●) and PP_i (■) on glycogen synthesis from glucose by pigeon liver homogenates. The pH of P_i and PP_i solutions was adjusted to 7.4. To keep the volume of the digest to 1 ml., the amount of 0.25M-sucrose was decreased. Experimental conditions were the same as described in Table 2. The values are averages of those obtained in two separate experiments.

Fig. 5. Inhibition of glycogen synthesis from glucose by pigeon liver homogenate by MgCl_2 (●), CaCl_2 (■) and EDTA (▲). The substances were dissolved in iso-osmotic sucrose and adjusted to pH 7.4 if necessary. Experimental details are described in Table 2. The values are averages of those obtained in three experiments.

inhibitory effect of Mg^{2+} and Ca^{2+} and the chelating agent EDTA when increasing concentrations were added during the synthesis of glycogen from [^{14}C]glucose. Magnesium chloride at 5mm gave 80% inhibition, and at 10mm gave 95% inhibition (not shown). Calcium chloride (2.0mm) and 1.0mm-EDTA gave nearly complete inhibition of glycogen synthesis.

Effect of starvation

Although it has been shown that glucokinase activity in rat liver supernatant is decreased on starvation of the animals (Viñuela *et al.* 1963) and that the concentration of glucose 6-phosphate, an activator of UDP-glucose- α -glucan glucosyltransferase, is also decreased, starvation of pigeons for 24 and 48 hr. failed to decrease glycogen synthesis in their liver homogenates (Fig. 6). Further, addition of glucose 6-phosphate caused no greater increase in glycogen synthetase in starved animals as compared with fed controls (Fig. 6). The concentration of glucose 6-phosphate in the liver of fed pigeon was $1.9 \mu\text{moles/g. wet wt. of tissue}$, which was decreased to 0.99 and $0.9 \mu\text{mole of glucose 6-phosphate/g. wet wt. of tissue}$ after 24 and 48 hr. of starvation respectively. The concentration of glycogen was also decreased to one-thirtieth of that in the liver of fed pigeon after 48 hr. of starvation.

Measurement of glucokinase and hexokinase activities in rat and pigeon liver supernatant fractions

These activities were assayed by the same procedure in both rat and pigeon liver supernatants as described by Viñuela *et al.* (1963). Pigeon liver contained hexokinase but not glucokinase activity (Table 4). Rat liver contained both enzymes. To ascertain if an inhibitor of glucokinase was maintained in the pigeon liver, assays of glucokinase and hexokinase were performed on a mixture of rat and pigeon liver supernatants. The mixture gave the same activity for glucokinase as that obtained with the same amount of rat liver supernatant.

Fig. 6. Glycogen synthesis from glucose in liver homogenates of fed (●), 24 hr.-starved (■) and 48 hr.-starved (▲) pigeons. The incubations were carried out in the presence of increasing concentrations of unlabelled glucose 6-phosphate. The values are corrected for glucose 6-phosphate hydrolysed to glucose during the 30 min. incubation. Experimental details are given in Table 2. The values are averages of those obtained in three experiments.

Assays for the activities of phosphoglucumutase, UDP-glucose pyrophosphorylase, UDP-glucose- α -glucan glucosyltransferase and glucose 6-phosphatase revealed that these enzymes were present in the pigeon liver (Table 4). The rate-limiting enzyme in pigeon liver is the hexokinase. Whether hexokinase is present only in the supernatant fraction or whether mitochondria also contain the enzyme has not been ascertained.

Reconstitution of the homogenate after fractional centrifugation

It has been repeatedly observed during this investigation that the time required for fractionation and reconstitution of the homogenate to its original concentration invariably leads to serious diminution or complete loss of the activity for glycogen synthesis from glucose. A simple fractionation into nuclear fraction (containing nuclei and heavy mitochondria) and post-nuclear fraction generally gives a satisfactory homogenate on reconstitution. Table 5 shows the results obtained when such fractionation was carried out with the potassium chloride-glycylglycine homogenates of both rat and pigeon liver and the fractions were intercombined. It was observed that crude nuclear and post-nuclear fractions were unable alone to form glycogen from glucose. When the two fractions from pigeon liver were combined, most of the activity was restored. When pigeon liver nuclear fraction was replaced by rat liver nuclear fraction, the activity was decreased slightly. However, addition of rat post-nuclear fraction to the pigeon nuclear fraction gave an inactive system. It thus became apparent that one reason for the inability of rat liver homogenate to synthesize

Table 4. Activity profile of enzymes related to glycogen metabolism in pigeon liver

Enzymes were assayed as described in the Materials and Methods section. The results are given as means $\pm$ s.e.m. of three to five determinations.

Enzyme	Product formed ($\mu\text{moles/min./g. wet wt. of liver}$)
Hexokinase	0.52 ± 0.04
Glucokinase	< 0.05
Phosphoglucumutase	44.0 ± 10
UDP-glucose pyrophosphorylase	25.0 ± 3.2
Glucose 6-phosphatase	10.2 ± 2.8
UDP-glucose- α -glucan glucosyltransferase (no glucose 6-phosphate)	5.8 ± 2.2
UDP-glucose- α -glucan glucosyltransferase (+ glucose 6-phosphate)	10.0 ± 1.5
Phosphorylase (no AMP)	8.3 ± 1.5
Phosphorylase (+ AMP)	12.2 ± 1.0

Table 5. *Glycogen synthesis by reconstituted rat and pigeon liver homogenates*

Rat or pigeon liver homogenates (20%) were prepared in iso-osmotic KCl in 50mM-glycylglycine buffer, pH 7.5. Homogenates were centrifuged at 700g for 10min. in the International centrifuge at 0°. Supernatant was removed with a syringe and the precipitate was washed twice with buffered KCl solution. The washings were mixed with the supernatant. The precipitate was suspended in half the original volume of the homogenate. In reconstitution appropriate volumes of the two separate fractions were mixed to give the same composition as the original homogenate. Incubation was carried out with 10 μ moles of [14 C]glucose (approx. 50000 counts/min.) for 30min. and glycogen was isolated and its 14 C radioactivity measured as described in the Materials and Methods section. The means $\pm$ s.e.m. obtained in four or five experiments are given.

Cell fraction, homogenate or reconstituted homogenate	[14 C]Glucose incorporated into glycogen (counts/min./ml. of digest)
Rat liver homogenate	240 $\pm$ 14
Pigeon liver homogenate	6920 $\pm$ 440
Pigeon liver cytoplasmic fraction (PC)	580 $\pm$ 12
Rat liver cytoplasmic fraction (RC)	174 $\pm$ 10
Pigeon liver nuclear fraction (PN)	409 $\pm$ 97
Rat liver nuclear fraction (RN)	56 $\pm$ 5
PC+PN	5725 $\pm$ 175
PC+RN	3100 $\pm$ 110
RC+PN	386 $\pm$ 14

glycogen could be the presence of an inhibitor in the post-mitochondrial fraction of rat liver.

The rat liver homogenate was fractionated by the conventional technique (Umbreit *et al.* 1964). Glucose incorporation into glycogen by pigeon liver homogenate was examined in the presence of nuclear, mitochondrial, microsomal and soluble fractions of rat liver. The glycogen-synthesizing system of pigeon liver was inactivated by 80% by the rat microsomal fraction. Addition of pigeon microsomal fraction (prepared exactly in the same fashion as the rat microsomal fraction) did not affect glycogen synthesis by the pigeon liver homogenate.

Attempts were directed towards separating the pigeon liver homogenate prepared in iso-osmotic sucrose containing magnesium chloride (3mM) into various cellular fractions and studying the requirement of each fraction in glycogen synthesis from glucose. It appears likely that, when Mg^{2+} was present, the heavy mitochondria also sedimented along with the nuclear fraction. The results obtained are shown in Table 6. All fractions appear to be required for glycogen synthesis. Omission of

Table 6. *Glycogen synthesis in pigeon liver cell fractions and reconstituted homogenate*

A 10% pigeon liver homogenate was prepared in iso-osmotic sucrose containing $MgCl_2$ (3mM). It was separated into cellular fractions as described by Umbreit *et al.* (1964). Sedimented fractions were suspended in appropriate volumes of 0.25M-sucrose-3mM- $MgCl_2$ solution and reconstituted to give the same composition of each fraction as is contained in 80mg. wet wt. of liver. The time of incubation was 30min. at 37° in O_2 with 10 μ moles of [14 C]glucose (approx. 50000 counts/min.). The values are means $\pm$ s.e.m. of those obtained in three experiments.

Cell fraction	Radioactivity in isolated glycogen (counts/min.)
Nuclear (N)	< 20
Mitochondrial (M)	< 20
Microsomal (Ms)	< 20
Supernatant (S)	< 35
N + S	1785 $\pm$ 175
N + M + S	2130 $\pm$ 200
N + M + Ms + S	3080 $\pm$ 132
N + M + Ms (rat) + S	340 $\pm$ 34
M + Ms + S	750 $\pm$ 36
Ms + S	< 60
Pigeon liver homogenate	5760 $\pm$ 240
Pigeon liver homogenate (aged for 3 hr. at 0°)	433 $\pm$ 14
Pigeon liver homogenate + 3mM- $MgCl_2$ (aged for 3 hr.)	3210 $\pm$ 102

any one fraction brought about a decrease in the incorporation of glucose into glycogen. Omission of the supernatant fraction led to a complete loss of glycogen synthesis.

DISCUSSION

The demonstration of glycogen synthesis in pigeon liver homogenate provides a rare instance where a biologically active polymer has been synthesized from its building unit in a cell-free system. Since the reaction in the homogenate occurs with as much efficiency as is observed for the liver slice, the system offers the opportunity of determining the course of the reaction taken by the starting precursor before its polymerization. It can also be helpful in ascertaining the locus of action of substances that regulate the concentration of glycogen in the liver. It is noteworthy that homogenates of pigeon liver, but not of rat liver, can also synthesize glucose from lactate (Krebs, Dierks & Gascoyne, 1964).

Glycogen in pigeon liver homogenates can be synthesized from a number of metabolizable sugars. Glucose is the most suitable precursor, followed by mannose, fructose and galactose in decreasing order

of priority. Arabinose, the only pentose tried, and glucosamine, the only amino sugar tried, form little glycogen. There were some noteworthy differences among various hexoses. First, fructose was a poorer substrate than either glucose or mannose in the homogenate, although with liver slices it formed significant amounts of glycogen; secondly, incorporation of ^{14}C from ^{14}C -labelled galactose into glycogen was not diluted isotopically in the presence of unlabelled glucose. The reason for the difference in glycogen synthesis from fructose in liver slice and liver homogenate is not clear. It may be either that the presence of fructokinase in the liver channels a major portion of available ATP to fructose 1-phosphate formation or that fructose 6-phosphate formed does not equilibrate readily with glucose 6-phosphate. So far as glycogen formation from galactose is concerned, either galactose is incorporated as the galactose molecule into the glucose chains of glycogen or the polysaccharide formed from galactose is not glycogen although it sediments with the glycogen fraction. The presence of galactose in hydrolysates of purified preparations of glycogen from the livers of chicks given galactose (Nordin & Hanson, 1963) suggests that the former is true and that the enzymes participating in the transformation of galactose into glycogen are different from those for glucose.

The effect of substrate concentration on glycogen synthesis, as well as the absence of high- K_m glucokinase from the soluble fraction, excludes participation of glucokinase in glycogen synthesis by pigeon liver homogenates. The maximum rates of glycogen synthesis in pigeon liver homogenate (0.6–0.9 μmole of glucose transformed into glycogen/min./g. of liver) are slightly higher than the activity of hexokinase in the soluble part of pigeon liver (0.52 μmole of glucose phosphorylated/min./g. of liver), suggesting either that additional hexokinase activity is associated with a particulate fraction or that a pathway not involving hexokinase is operative for glycogen synthesis in the pigeon liver homogenates.

In confirmation of the results of Robbins, Traut & Lipmann (1959), glycogen synthesis in liver homogenates is supported only by aerobically produced ATP. Exogenously added ATP and UTP neither activate glycogen synthesis nor do they replace aerobically produced ATP. It is not clear why Mg^{2+} and Ca^{2+} inhibit glycogen synthesis. The inhibitory role of EDTA, however, seems to be related to its inhibition of phosphoglucomutase as well as of UDP-glucose formation. The mode of action of EDTA is discussed in the following paper (Nigam, 1967b).

Synthesis of glycogen in the pigeon liver homogenate occurs in the absence of added P_i . It is apparent that high turnover rates of P_i must be

maintained to supply the demands for aerobic ATP. Addition of P_i results in only a slight lowering of glycogen synthesis, showing that adverse P_i /glucose 1-phosphate ratios for glycogen synthesis (Leloir & Cardini, 1962) do not necessarily result in significant glycogen breakdown by phosphorylase. On the other hand, incomplete inhibition of glycogen synthesis by PP_i suggests that, when UDP-glucose is removed in the UDP-pyrophosphorylase reaction, the resulting glucose 1-phosphate is rerouted to form glycogen through the phosphorylase.

The stimulatory effect of glucose 6-phosphate on glycogen synthesis from glucose is consistent with an activation of UDP-glucose- α -glucan glucosyltransferase (Leloir & Goldemberg, 1960). Higher concentrations of glucose 6-phosphate decrease glycogen formation owing to the isotopic dilution of labelled glucose with unlabelled glucose formed by hydrolysis of glucose 6-phosphate. Although 2-deoxyglucose 6-phosphate is also an activator of glycogen synthesis (Fridland & Nigam, 1965), its inhibition of glycogen synthesis shows that it acts on a locus other than the UDP-glucose- α -glucan glucosyltransferase reaction. Since 2-deoxyglucose 6-phosphate inhibits glycogen synthesis not only from glucose but also from glucose 6-phosphate and glucose 1-phosphate (see Nigam, 1967b), its action is probably mediated by inhibition of UDP-glucose formation. Conversion of 2-deoxyglucose 6-phosphate into 2-deoxyglucose 1-phosphate and later into UDP-2-deoxyglucose (Heredia, De La Fuente & Sols, 1964) is suggested as a possible reaction that may interfere with the utilization of UTP for glycogen synthesis.

Increase in glycogen synthesis in livers of starved pigeons shows the existence of yet another controlling factor in glycogen synthesis. Since starvation lowers the liver glycogen concentration and the glycogen-synthesizing enzymes (UDP-glucose- α -glucan glucosyltransferase and phosphorylase) are solubilized (Luck, 1961), it is possible that these enzymes become available in a more active form. The loss of glycogen perhaps overcomes the decreased activation of UDP-glucose- α -glucan glucosyltransferase due to the low content of glucose 6-phosphate in the livers of starved pigeons. Danworth (1965) in independent studies on glycogen synthesis in muscle has also suggested a regulatory role of glycogen concentration on glycogen synthesis.

The homogenate system of pigeon liver is sensitive, in some instances unpredictably, to the effects of aging and reconstitution. Addition of Mg^{2+} combats partially the loss of activity due to aging of the homogenate. This happens despite the fact that addition of Mg^{2+} to fresh homogenates decreases glycogen synthesis. Fractionation studies have revealed that all cellular components are

required for glycogen synthesis. There also appears to be a close balance between various components such that the addition of one does not greatly enhance glycogen formation. Studies on reconstitution have been helpful in revealing the existence of an inhibitor of glycogen synthesis in the microsomal fraction of rat liver. Since rat liver slices form glycogen, it is obvious that homogenization of the rat liver follows the appearance of the inhibitor. In this context, phosphatases of microsomal fraction that inactivate phosphorylase and UDP-glucose- α -glucan glucosyltransferase become the prime suspects. It is to be recognized that such enzymes, in spite of their unknown nature at present, may play a prominent role in the regulation of glycogen metabolism in the liver.

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