Glycogen Metabolism in Novikoff Ascites-Hepatoma Cells

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A study of the enzymes of the glycogen pathway in Novikoff ascites hepatoma shows that glycogen synthetase has the lowest activity and that the tumour contains no high- K_m soluble glucokinase. However, incubation of tumour cells with metabolizable sugars *in vitro*, or intraperitoneal administration of glucose into the tumour-bearing rat, results in glycogen accumulation by the tumour cells. Glycogen synthesis in the tumour is supported by aerobically produced ATP but is decreased anaerobically and by uncouplers of oxidative phosphorylation. Absence of P_i from the incubation medium increases glycogen synthesis and decreases glycolysis. The optimum temperature for glycogen synthesis is 37°. The capacity of the intact tumour cell to degrade deposited glycogen is low, but is accelerated by 2,4-dinitrophenol. Tumour homogenates prepared after osmotic shock do not incorporate [^{14}C]glucose into glycogen. The glucose moiety of glucose 1-phosphate and of UDP-glucose is incorporated into glycogen by the homogenates and the incorporation of glucose 1-phosphate is greatly enhanced by AMP. Glucose 6-phosphate is a poor precursor of glycogen in the homogenate system, probably because it inhibits activation of phosphorylase *b* by AMP.

Variations in the amounts of enzymes and other constituents in tumours have been directly or indirectly related to tumour formation. One of the most important findings was that tumours have a relatively high glycolytic activity. This led to Warburg's (1956) proposition that damage to tissue respiration is a cause of cancer.

In recent years, however, tumours (Morris hepatomas) have been described that respire normally and have low glycolytic activity (Aisenberg & Morris, 1961). In spite of the relatively normal behaviour of the 'minimal-deviating' hepatomas, they have, like other tumours, a low content of glycogen. The study of formation and storage of glycogen therefore offers a promising area of investigation.

In this Laboratory, work since 1962 has been directed towards the study of the regulation of glycogen metabolism in both normal and tumour tissues. Previous studies on the enzyme activities in a variety of tumours (Nigam, MacDonald & Cantero, 1962), and on the intracellular distribution of glycogen synthetase and phosphorylase in Novikoff ascites hepatoma (Nigam, 1962*a*), have suggested a low capacity to form glycogen and to degrade it. Recent work on glycogen synthesis by Novikoff ascites-hepatoma cells (Nigam, 1965), however, tends to modify the previous conclusions. The present paper describes the requirements for

glycogen synthesis by Novikoff ascites-tumour cells and the effect of regulatory factors on glycogen deposition.

MATERIALS AND METHODS

Chemicals. Most of the chemicals used in the present investigation were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., and from Calbiochem Inc., Los Angeles, Calif., U.S.A. All other chemicals were of reagent-grade purity available from commercial sources. [^{14}C]Glucose was purchased from Merck and Co., Montreal, Canada. [^{14}C]Fructose, [^{14}C]galactose, [^{14}C]glucosamine hydrochloride, [^{14}C]glucose 6-phosphate and [^{14}C]glucose 1-phosphate were obtained from New England Nuclear Corp., Cambridge, Mass., U.S.A. [^{14}C]Glycogen and UDP-[^{14}C]glucose were products of International Chemical and Nuclear Corp., City of Industry, Calif., U.S.A.

Animals. Sprague-Dawley rats weighing 150–200g. and pigeons of various ages and weights were obtained locally from Robidoux Farm. The rats were kept on a diet of Labena Chow and the pigeons on Purina Pigeon Chow; both diets were obtained from Ralston Purina Co., St Louis, Mo., U.S.A. Food and water were available to animals *ad libitum*.

Isolation of glycogen. Glycogen was isolated from tissues and reaction mixtures by digestion with KOH and precipitation with ethanol as described by Robbins, Traut & Lipmann (1959). The precipitation of glycogen was carried out in the presence of 0.1 ml. of saturated Na_2SO_4 instead of 0.2 ml. used by Robbins *et al.* (1959).

Estimation of ^{14}C in glycogen. Where incorporation of

¹⁴C into glycogen was to be ascertained 5mg. of unlabelled rabbit-liver glycogen was added after KOH digestion. The isolated glycogen was dissolved in 2ml. of warm distilled water and 1ml. of the solution was plated on aluminium planchets (5cm.² in area) and dried. The ¹⁴C was measured by a Picker Nuclear Counter with an ultra-thin window. The samples had negligible self-absorption in the range of glycogen usually present in the samples (2.5–3mg./ml.). It was essential, however, to correct for absorption due to the presence of 0.05ml. of saturated Na₂SO₄ solution/ml. of the sample. This reduced the counts by 25%. Since all samples contained the same amount of Na₂SO₄, the same correction factor was applied throughout.

Identification and chemical estimation of glycogen. The identity of isolated glycogen as a glucose polymer was established by dialysis of the glycogen solution against distilled water at 5° for 36hr., hydrolysis to glucose by 1N-H₂SO₄ at 100° for 1hr., neutralization by 1N-NaOH and preparation of glucose phenylosazone (Vogel, 1956). About 80–90% of the ¹⁴C initially in the glycogen was recovered in the osazone. The glycogen was hydrolysed in acid and estimated as glucose (Nelson, 1944).

Determination of glucose. Solutions containing glucose were treated with Ba(OH)₂-ZnSO₄ (Ashwell, 1957) before chemical determination by Nelson's (1944) method or before the preparation of the osazone and measurement of its radioactivity.

Isolation of lactic acid and measurement of its radioactivity. Lactic acid was isolated from digests from which proteins and sugar phosphates had been removed by treatment with Ba(OH)₂-ZnSO₄. To a known volume of the supernatant carrier lactic acid was added and the solution was acidified and extracted with 2vol. of ether over a 72hr. period. The ether extract was evaporated *in vacuo* and the residue was dissolved in a known volume of water. The lactic acid was further purified by chromatography on a Dowex 1 (Cl⁻ form) column as described by Kinochita, Masurat & Helfant (1955). The ¹⁴C in the lactic acid was determined after its oxidation to acetaldehyde and preparation of the dimedone derivative as described by Rahman & Kerly (1961). Chemical estimation of lactic acid was carried out by the method of Barker (1957).

Determination of ATP. ATP was measured in the incubation mixture, which contained (μmoles/3ml.): glycylglycine buffer, pH7.4, 150; MgCl₂, 15; EDTA, 10; glucose, 30; NADP⁺, 3; glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 1 unit. The increase in extinction after the addition of glucose 6-phosphate dehydrogenase gave a measure of the amount of glucose 6-phosphate that was present. The additional increase in extinction at 340mμ after the addition of hexokinase (EC 2.7.1.1) reflected the amount of ATP in the digest. An increase of 0.207 represented 0.1 μmole of ATP/3ml. of digest.

Determination of ADP. ADP was estimated with crystalline pyruvate kinase (EC 2.7.1.40) and the trisodium salt of phosphoenolpyruvate with lactate dehydrogenase (EC 1.1.1.27) and NADH as the indicator system. The digest was the same as described for the estimation of C₃ intermediates but contained no ADP (Nigam, 1962b). Prior addition of NADH and lactate dehydrogenase removed any trace of pyruvate present. The decrease in extinction at 340mμ obtained after the addition of pyruvate kinase gave the amount of ADP. A decrease of 0.207 represented 0.1 μmole of ADP/3ml. of digest.

Determination of AMP. This was done by the method of Munche-Petersen & Kalckar (1957) by following the decrease in extinction resulting from the deamination of AMP by adenylate deaminase. The assay mixture contained (μmoles/3ml.): succinate buffer, pH6.1, 750; MgCl₂, 20; adenylate deaminase, 50 μg. A decrease of 0.02 in extinction was given by deamination of 1 μg. of AMP/ml.

Assay of hexokinase and glucokinase activities. These measurements were made at 5mm- and 100mm-glucose concentration respectively by employing the procedure described by Viñuela, Salas & Sols (1963).

Assay of phosphoglucomutase activity. This was carried out by the spectrophotometric estimation of glucose 6-phosphate (Crane & Sols, 1955) formed from glucose 1-phosphate. The digest contained: glucose 1-phosphate pH7.4, 0.02M; glycylglycine buffer, pH7.4, 0.05M; MgCl₂, 0.005M; cysteine, pH7.5, 0.01M; NADP⁺, 0.001M; glucose 6-phosphate dehydrogenase, 2 units; tissue supernatant; the total volume was 3ml. Increase in extinction was measured every 30sec. for 3min. Correction was made for the contribution of 6-phosphogluconate dehydrogenase by deducting from the rate of NADP⁺ reduction the rate when 6-phosphogluconic acid was used as the substrate.

Assay of UDP-glucose-pyrophosphorylase activity. This was carried out by the method of Villar-Palasi & Lerner (1960).

Assay of UDP-glucose-α-glucan-glucosyltransferase (EC 2.4.1.11) activity. This was carried out in digests similar to that described by Leloir & Goldemberg (1960). The amount of ¹⁴C incorporated into glycogen from UDP-[¹⁴C]glucose, both in the presence and absence of glucose 6-phosphate, was estimated.

Assay of phosphorylase activity. This was carried out by measuring the ¹⁴C incorporated into glycogen after incubation of [U-¹⁴C]glucose 1-phosphate in a digest similar to that described by Sutherland (1955) for the assay of phosphorylase activity. It contained: [U-¹⁴C]glucose 1-phosphate, pH6.1, 0.03M (100000 counts/min.); glycogen, 5mg.; KF, 0.1M; AMP, pH6.7, 0.005M; tissue homogenate, 0.2ml.; the total volume was 1ml. The incorporation into glycogen was measured both in the presence and absence of AMP. Homogenates for the assay of phosphorylase activity were made in 0.25M-sucrose containing 0.1M-KF and 0.005M-MgCl₂ to avoid loss of activity due to the action of phosphorylase phosphatase.

Preparation of tumour cells. Sprague-Dawley rats received transplants of Novikoff ascites hepatoma cells (50 × 10⁶–70 × 10⁶). After 4–7 days the animals were killed and the ascitic fluid was collected in 4vol. of ice-cold tris wash-medium as described by Wu & Racker (1959) and the livers were excised. The tumour-cell suspension was centrifuged at 80g for 4–6min. at 0° in the International refrigerated centrifuge. The sedimented cells were washed with the ice-cold medium four or five times to free them of erythrocytes and polymorphonuclear leucocytes. The coagulated blood, which formed a streak on the side of the centrifuge tube, was removed with a syringe. During the washing the cells were mixed with the fresh medium by gentle shaking. Since the ascitic-cell suspension, obtained after the transplantation of Novikoff ascites-hepatoma cells to rats, was invariably haemorrhagic, it was essential to wash the tumour cells. The washed tumour cells were suspended in tris incubation medium as described by Wu & Racker (1959) and centrifuged at 12000g for 8min. in

a graduated centrifuge tube to ascertain their volume: 1 ml. of packed cells (uncorrected for raffinose space) usually contained 300×10^6 – 400×10^6 cells.

Preparation of liver homogenates. The livers were homogenized in 0.25 M-sucrose. A Potter–Elvehjem homogenizer with a Teflon pestle and a thick-walled glass tube was used. After the tissue had been minced with a pair of scissors, a known amount (2g.) was transferred to a pre-cooled homogenizing tube and 4–6 ml. of cold iso-osmotic sucrose was added. The liver was homogenized for 3 min. and the homogenate was made up to 10 ml. with iso-osmotic sucrose. During homogenization the tube was immersed in ice-water mixture. Sucrose homogenates were used to study the incorporation of [^{14}C]glucose into glycogen. For enzymic studies liver homogenates were prepared in 0.25 M-sucrose containing 0.001 M-EDTA. Hexokinase, glucokinase, phosphoglucomutase and UDP-glucose-pyrophosphorylase activities were measured in the supernatant solution obtained after centrifugation of the homogenate in a Spinco model L ultracentrifuge for 1 hr. at 100000g at 0°. UDP-glucose- α -glucan-glucosyltransferase activity was measured in the whole homogenate. For the assay of phosphorylase activity homogenates were prepared in 0.25 M-sucrose containing 0.1 M-KF and 0.005 M-MgCl₂.

Preparation of tumour-cell homogenates. The homogenizing procedure described for liver usually gave no more than 10–20% breakage of the tumour cells. The procedure used for homogenization of tumour cells was the same as used by Hawtree & Silk (1960) for the homogenization of Ehrlich ascites-tumour cells. Packed tumour cells (1 vol.) were suspended in 4 vol. of quarter-strength tris wash medium. The suspension was left in the cold for 10 min. and was shaken intermittently. The cells were later sedimented by centrifugation at 0°. Treatment with hypo-osmotic medium doubled the original volume of the tumour cells. To the swollen cells, an equal volume of cold water was added and the suspension was transferred to a tight-fitting Potter–Elvehjem homogenizer. The cells were homogenized in the cold for 2 min. Immediately after homogenization an equal volume of 0.5 M-sucrose alone or 0.5 M-sucrose containing 0.002 M-EDTA or 0.5 M-sucrose containing 0.2 M-KF and 0.01 M-MgCl₂ was added. By counting the number of nuclei and the number of intact cells it appeared that 85–95% breakage of the tumour cells occurred. The free cells and a large number of nuclei were then removed by centrifugation of the homogenate at 1200g for 8 min. at 0°. Sucrose homogenates were used for incorporation studies, sucrose–EDTA homogenate for UDP-glucose- α -glucan-glucosyltransferase assay, 100000g sucrose–EDTA supernatants for the assay of hexokinase, phosphoglucomutase and UDP-glucose pyrophosphorylase, and sucrose–KF–MgCl₂ homogenates for the assay of phosphorylase.

Incubation procedure and procedures for the estimation of glycogen synthesized during the incorporation of [^{14}C]hexose into glycogen by the tumour cells. These were similar to those described previously (Nigam, 1965).

Tumour-cell suspensions (0.9 ml. containing 0.2–0.25 ml. of packed cells) were incubated, with 5–10 μ moles of labelled sugar dissolved in 0.05–0.10 ml. of incubation medium, in 20 ml. beakers placed in a Dubnoff shaker at 36–38° and flushed with oxygen. At intervals the reaction was stopped by the addition of an equal volume of 60% KOH. Glycogen was isolated and its radioactivity

measured. The glucose equivalent of glycogen formed was obtained by multiplying the counts/min. in the isolated glycogen by the μ moles of sugar added and dividing this value by the counts/min. in the labelled sugar added.

Incorporation of labelled glucose or glucose phosphates into glycogen by the homogenates was measured as follows. To 0.4–0.5 ml. of tissue homogenate was added 0.3 ml. of 0.15 M-KCl buffered with 0.05 M-glycylglycine, pH 7.4. Substrate and cofactors were dissolved in 0.25 M-sucrose. Rat-liver or pigeon-liver fractions were added in 0.3 ml. of 25 M-sucrose containing 0.005 M-MgCl₂. The volume in each case was made up to 1.2 ml. with 0.25 M-sucrose. The digests were incubated in 20 ml. beakers for 20 min. and later glycogen was isolated and counted for its ^{14}C content.

Rates of glycogen synthesis, lactate production and glucose utilization by the tumour cells during sequential addition of [^{14}C]glucose. These rates (Fig. 3; see the Results section) were measured as follows.

Tumour-cell suspensions (0.9 ml. containing approx. 0.17 ml. of packed cells) were incubated in 15–20 ml. beakers. At zero time 0.5 μ mole of [^{14}C]glucose was added to each tube. After 1 min. the digests in three of the beakers were inactivated by heat and a further 0.5 μ mole of [^{14}C]glucose was added to each of the remaining tubes. The inactivated digests were used for the estimation of ^{14}C in the isolated glycogen and the chemical determination of lactate and glucose. After another 1 min. of incubation, three more digests were inactivated and glucose was added to each of the remaining nine tubes. This procedure was continued for 5 min. Glucose solutions of increasing strength but containing the same amount of ^{14}C were used to minimize volume changes.

Fractionation of pigeon-liver homogenate. Nuclear-mitochondrial and soluble fractions were obtained as follows.

Pigeon-liver homogenates (20%) were prepared in 0.25 M-sucrose containing 0.005 M-MgCl₂. The homogenate was centrifuged for 10 min. at 6000g at 0–5° to obtain the nuclear-mitochondrial sediment. The supernatant was centrifuged at 105000g for 30 min. The soluble fraction was removed with a syringe, leaving a microsomal pellet. The nuclear-mitochondrial fraction was suspended in a total of 7.0 ml. of 0.25 M-sucrose containing 0.005 M-MgCl₂, just before the reaction was started. The activity of the reconstituted homogenate is maintained better by the addition of MgCl₂ to iso-osmotic sucrose. In the presence of salt a heavy mitochondrial sediment with the nuclear fraction was obtained on centrifugation at 1200g in the International centrifuge. In all the experiments therefore both nuclear and mitochondrial fractions were sedimented together and added as such to the reaction digests.

RESULTS

Activities of enzymes of glycogen metabolism in Novikoff ascites hepatoma and in liver

To evaluate the capacity of Novikoff ascites-tumour cells to synthesize glycogen, individual enzymes related to glycogen metabolism were assayed in the tumour extract. Table 1 gives the results and comparative values for normal rat liver and the liver of the tumour-bearing animal (the

Table 1. *Activities of glycogen-forming enzymes*

Experimental details are given in the text. Mean values $\pm$ S.D. of three to five analyses are presented.

Enzyme	Conversion of substrate (μ moles/hr. per g. wet wt. of liver or per ml. of packed tumour cells)		
	Normal rat liver	Liver of tumour-bearing rat	Tumour
Hexokinase	24 $\pm$ 8	25 $\pm$ 3	130 $\pm$ 15
Glucokinase	65 $\pm$ 12	50 $\pm$ 15	< 0.5
Phosphoglucumutase	2250 $\pm$ 280	2130 $\pm$ 230	85 $\pm$ 35
UDP-glucose pyrophosphorylase	3450 $\pm$ 250	3080 $\pm$ 400	1528 $\pm$ 120
UDP-glucose- α -glucan glucosyltransferase (without glucose 6-phosphate)	55 $\pm$ 25	45 $\pm$ 10	29 $\pm$ 9
UDP-glucose- α -glucan glucosyltransferase (with glucose 6-phosphate)	237 $\pm$ 24	212 $\pm$ 18	70 $\pm$ 12
Phosphorylase (without AMP)	345 $\pm$ 38	295 $\pm$ 20	8 $\pm$ 5
Phosphorylase (with AMP)	381 $\pm$ 63	312 $\pm$ 37	35 $\pm$ 10

Table 2. *Concentration of glucose in the ascitic fluid and the glycogen content of tumour cells before and after intraperitoneal injection of glucose*

Three intraperitoneal injections of 100mg. of glucose in 1ml. of iso-osmotic saline were given to tumour-bearing rats at intervals of 1 hr. At 30min. after the third injection the animals were killed, and the tumour cells were prepared as described in the text. Glycogen was isolated and determined chemically after dialysis.

Expt. no.	Glucose (μ mole/ml. of ascitic fluid)	Glycogen (μ moles of glucose equivalent/ml. of packed cells)
Before glucose injection		
1	0.2	2.0
2	0.25	1.8
3	0.15	1.2
After glucose injection		
1	—	30.0
2	—	28.0
3	—	32.0

last tissue was included since its glycogen content is lower than that of its normal counterpart). In confirmation with the report of Shotton, Donnelly & Weinhouse (1962), it was observed that the tumour supernatant solution did not contain the high- K_m glucose-phosphorylating enzyme (glucokinase), and the activity of hexokinase (the low- K_m enzyme) was higher than the activity of the glucokinase in the liver of either the normal or the tumour-bearing rat. In the liver of the tumour-bearing animal the activity of glucokinase was decreased without any change in hexokinase activity. Other enzymes whose activities were significantly decreased in the tumour were: phosphoglucumutase (94–96% decrease), UDP-glucose

pyrophosphorylase (55% decrease), UDP-glucose- α -glucan glucosyltransferase (66% decrease) and AMP-activated phosphorylase (90% decrease). Although AMP caused only a slight stimulation of phosphorylase activity in liver, the activation was four- to five-fold for the tumour phosphorylase. The livers of the tumour-bearing animals showed small decreases in the activities of all the enzymes estimated.

Glycogen formation by Novikoff ascites hepatoma in vivo

The lack of agreement between the low glycogen content of freshly prepared tumour cells (Table 2) and the synthesis of glycogen from glucose by the intact tumour cells *in vitro* (Nigam, 1965) suggested that the ascitic fluid bathing the cells *in vivo* might either lack the substrate or contain an inhibitor of glycogen synthesis. Accordingly, glucose in the ascitic fluid was measured and also glycogen was estimated in the tumour cells after the intraperitoneal administration of glucose to the tumour-bearing rat. The ascitic fluid contained only traces of glucose, suggesting that limitation of glucose could be the cause of low glycogen accumulation by the tumour *in vivo* (Table 2). This view was confirmed when it was observed that a significant amount of glycogen accumulated in the tumour cells when glucose had been administered in the ascitic fluid (Table 2).

Studies on glycogen synthesis by Novikoff ascites-hepatoma cells in vitro

Rate of glycogen formation. When intact tumour cells were incubated in the synthetic medium of Wu & Racker (1959), the synthesis of glycogen was

Fig. 1. Time-course of glycogen synthesis from glucose, fructose, mannose and galactose by Novikoff ascites-hepatoma cells. Experimental details are given in the Materials and Methods section. The values are the averages of two to six experiments. ▲, Glucose; ●, fructose; □, mannose; ○, galactose.

Fig. 2. Effect of exclusion of P_i from the medium on glycogen synthesis and lactic acid production from glucose by hepatoma cells. The procedure was as given in the Materials and Methods section except that the cell suspension contained 0.16 ml. of packed cells. The values are the averages of two experiments. Δ and ▲, Glucose equivalent of glycogen formed; ○ and ●, lactate produced. Δ and ○, P_i present; ▲ and ●, P_i absent.

observed from all metabolizable sugars with the exception of glucosamine. The rates for glycogen synthesis from galactose, mannose, glucose and fructose were 0.10, 0.53, 0.93 and 1.00 μ mole of glucose equivalent/min./ml. of packed cells respectively (Fig. 1). The rate was linear despite a lowering in pH that occurred (particularly with glucose and fructose as substrate) because of lactic acid production.

Effect of washing of tumour cells on glycogen synthesis from glucose. Laws & Stickland (1963) have reported that washing of Ehrlich ascites-tumour cells leads to a loss of about 25% in ADP and ATP concentrations in the tumour cells. Investigation was therefore carried out to determine if the washed and unwashed tumour cells differed in their capacity to form glycogen from glucose. Since the ascitic-cell suspensions obtained from the intraperitoneal cavity of rats bearing the Novikoff ascites-tumour cells were grossly haemorrhagic, the amount of tumour cells in the suspension was determined after the cells had been washed. Usually a 75–80% recovery was obtained, the loss being accounted for by the removal of polymorphonuclear leucocytes, erythrocytes and coagulated blood, and incidental losses of tumour cells. Measurement of the amounts of glycogen formed from $[^{14}C]$ glucose by tumour cells in fresh ascitic-cell suspension and by washed tumour cells suspended either in tris incubation medium or in cell-free ascitic fluid gave similar values (1.5–2.1 μ moles of glucose equivalent of glycogen formed/10 min./0.2 ml. of packed cells in 1 ml. volume). The time of incubation was kept at 10 min. because longer incubation in ascitic fluid led to aggregation of the tumour cells.

Influence of P_i . Washing of the tumour cells in

P_i -free tris wash medium and their subsequent incubation with $[^{14}C]$ glucose in P_i -free tris incubation medium did not result in a decrease in the amount of glycogen formed. On the contrary, when P_i was added to 0.004 M concentration to such digests, synthesis of glycogen was diminished and lactate production was increased (Fig. 2). However, the degree of inhibition of glycogen synthesis has been observed to vary from 10 to 50% in different tumour preparations.

Effect of uncouplers of oxidative phosphorylation and anaerobiosis on glycogen synthesis. Recent investigations on glycogen synthesis by pigeon-liver homogenates have suggested that ATP alone in aerobic conditions supports glycogen synthesis (Nigam & Fridland, 1964). This view was also supported by experiments with Novikoff ascites-hepatoma cells. Thus preincubation of tumour-cell suspension with uncouplers of oxidative phosphorylation, namely dinitrophenol, antimycin A and sodium azide, and subsequent addition of $[^{14}C]$ glucose resulted in an inhibition of glycogen labelling. Sodium arsenate was also an effective inhibitor, and anaerobic conditions decreased the incorporation of ^{14}C into glycogen (Table 3). The ATP concentration was 1.2 μ moles/ml. of packed cells. On the addition of glucose the concentration was decreased to 0.8 μ mole/ml. and did not rise on further incubation. That of ADP increased slightly from 0.4 to 0.6 μ mole/ml. after glucose addition. The concentration of AMP both in the presence and absence of glucose was too low to be determined accurately. However, addition of dinitrophenol to tumour-cell suspension diminished ATP to a very low concentration, ADP concentration was 0.7

Table 3. *Effect of anaerobiosis and uncouplers of oxidative phosphorylation on glycogen synthesis by the tumour cells*

Experimental conditions were as described in the Materials and Methods section.

Addition	Gas phase	Glycogen formed (μ moles of glucose equivalent/hr./ml. of packed cells)
None	O ₂	39.4
None	N ₂	4.5
Dinitrophenol (0.2mM)	O ₂	1.0
Antimycin A (0.01mM)	O ₂	15.5
Sodium azide (10mM)	O ₂	13.4
Sodium arsenate (10mM)	O ₂	7.5

μ mole/ml. and AMP concentration rose to 0.5–0.6 μ mole/ml. of packed cells. When glucose was added to dinitrophenol-treated cells, the amount of ATP rose to 0.7–0.8 μ mole/ml. ADP remained at 0.4–0.5 μ mole/ml., and the concentration of AMP fell to 0.1–0.2 μ mole/ml. of packed cells. These values fail to support the idea that ATP limitation was responsible for a lack of glycogen formation in the presence of dinitrophenol.

Effect of variation in substrate concentration. In a short-term experiment (5 min. duration) the capacity of the tumour cell to synthesize glycogen was estimated by using different concentrations of [¹⁴C]glucose. It was observed that glycogen formation increased as the glucose concentration was raised from 0.2mM to 5mM. The amount of glycogen formed was equivalent to 0.04, 0.15 and 0.08 μ mole of glucose/ml. when 0.2mM-, 1.0mM- and 5.0mM-glucose concentrations were employed. Further increase in the concentration of glucose resulted in little increase in glycogen formation. This result could have been due either to the existence of a glucokinase of higher K_m (higher than the K_m of hexokinase) in the tumour or to the exhaustion of the substrate when a low concentration of glucose was used. To resolve this question, tumour cells were incubated with 0.5mM-glucose (0.5 μ mole of glucose in 1 ml. of digest) and further glucose (0.5 μ mole) was added every minute to replenish the amount that had been utilized. Under these conditions the analysis of glucose, lactate and glycogen, carried out for every minute of incubation, revealed that 0.18 μ mole of glucose/min./ml. was utilized for glycogen synthesis and 0.25 μ mole/min./ml. for production of lactate (Fig. 3). Thus a continuous increase in glycogen formation occurred if the supply of substrate was maintained. Although glucose molarity remained between 0.5–1.0mM during 5 min. of incubation, the amount of glycogen synthesized was nearly the same as that obtained with 5mM- or 20mM-glucose.

Fig. 3. Rates of glycogen synthesis, lactate production and glucose utilization. Hepatoma-cell suspension was treated with 0.5 μ mole of [¹⁴C]glucose added at 1 min. intervals to restore the concentration of glucose. Experimental details are given in the Materials and Methods section. ●, Glucose utilized; □, lactate produced; ▲, glucose equivalent of glycogen formed.

Effect of temperature. The effect of temperature (Fig. 4) shows that glycogen synthesis is negligible at 0–10° and rises to its maximum at 37°. At 43° significant agglutination of the cells and complete inhibition of glycogen synthesis was observed. However, the amount of lactate produced at 43° was almost the same as that produced at 37°.

Degradation of glycogen. Two methods were employed for this study. In one, a small amount of glucose (0.2 μ mole/ml. of cell suspension) was used as substrate and the ¹⁴C content of isolated glycogen was measured as a function of time. As shown in Fig. 5, glycogen was labelled to its maximum within 0.5 min. and subsequent incubation

was followed by its degradation. In 60 min., the ^{14}C in glycogen fell to half its value at 0.5 min., showing that degradation was slower than synthesis.

Fig. 4. Effect of variation of temperature on glycogen synthesis. Experimental conditions are given in the Materials and Methods section. The cell suspension contained 0.18 ml. of packed cells/ml. Each result is an average of two experiments.

Fig. 5. Degradation of glycogen. Tumour-cell suspension (0.16 ml. of packed cells/ml.) was incubated with a small amount of labelled glucose ($0.2 \mu\text{mole}$ of ^{14}C glucose; 10^5 counts/min.) and radioactivity of glycogen was estimated at intervals.

In the second approach the tumour cells were preincubated with $10 \mu\text{moles}$ of ^{14}C glucose for 10 min., chilled and washed with the incubation medium to free them of glucose and later reincubated for 30 min. The ^{14}C was measured in glucose, lactate and glycogen initially and after 30 min. of incubation (Table 4). The ^{14}C in glycogen dropped to two-thirds of the initial value and appeared predominantly in the lactic acid. When dinitrophenol was added to such digests, the glycogen concentration decreased at a faster rate, lactic acid formation increased and the AMP concentration increased.

Glycogen synthesis in homogenate systems. The discovery that glycogen could be synthesized from glucose by pigeon-liver homogenate (Nigam & Fridland, 1964) suggested a similar study with tumour homogenate. Incubation of the tumour homogenate was carried out with ^{14}C -labelled glucose and glucose phosphates. Tumour and rat homogenates did not incorporate ^{14}C from ^{14}C -glucose and $[1-^{14}\text{C}]$ glucose 6-phosphate, and ^{14}C from $[\text{U}-^{14}\text{C}]$ glucose 1-phosphate was incorporated into the glycogen to a small extent (Table 5). Pigeon-liver homogenates were more active in forming glycogen from the three substrates. Addition of ATP, and UTP alone and in the presence of phosphoglucomutase, cysteine and magnesium chloride, increased the incorporation of glucose into glycogen by the tumour homogenate. The incorporation was increased significantly when the tumour homogenate was supplemented with the crude rat or pigeon nuclear-mitochondrial fraction. Addition of dinitrophenol, as well as washing of this liver fraction twice with 0.25 M -sucrose, led to a loss of its stimulatory activity. Besides, when the washed fractions were incubated with cytoplasmic fraction of pigeon liver (microsomes + soluble fraction) itself, the synthesizing capacity of the reconstituted homo-

Table 4. *Degradation of the glycogen deposited in the tumour cell into lactic acid*

Experimental conditions for the deposition of glycogen in tumour cells were as described in the Materials and Methods section. The time for glycogen deposition in the tumour cells was 15 min. The tumour cells were freed of glucose by washing and reincubated for 30 min. at 37° in O_2 , in the absence and presence of 0.2 mM -dinitrophenol. ^{14}C in glycogen and lactate was estimated after their isolation from the digests. Lactate was measured as dimedone derivative of acetaldehyde.

Expt. no.	Incubation time (min.)	Addition	^{14}C (counts/min. per 0.16 ml. packed cells in 1 ml.)	
			Glycogen	Lactic acid
1	0	None	14500	2500
2	0	None	12200	3000
1	30	None	10100	5300
2	30	None	9200	4500
3	30	Dinitrophenol	750	9100
4	30	Dinitrophenol	700	8700

Table 5. *Incorporation of [^{14}C]glucose and [^{14}C]glucose monophosphates into glycogen by tumour and liver homogenates*

Experimental details are given in the Materials and Methods section. The ranges shown were obtained in four to six experiments of each kind. *, ** and *** indicate that 0.25 mM-dinitrophenol addition caused 0–20%, 50% and 90–95% decrease in the glycogen counts respectively.

Homogenate (concn., w/v)	Additions	^{14}C (counts/min. in isolated glycogen/ digest)
Substrate: [^{14}C]glucose (5 $\mu\text{moles/digest}$; 5×10^4 counts/min.)		
Tumour (10%)	None	100–300*
Tumour (10%)	ATP, UTP (5 $\mu\text{moles each}$)	400–500*
Tumour (10%)	ATP, UTP, Mg^{2+} (5 $\mu\text{moles each}$), neutral cysteine (10 μmoles), phosphoglucomutase (2 units)	400–600*
Tumour (10%)	Pigeon-liver nuclear-mitochondrial fraction (equivalent to that obtained from 80 mg. of pigeon liver)	2000–5000***
None	Pigeon-liver nuclear-mitochondrial fraction (as above)	200–400***
Rat liver (20%)	None	100–300***
Pigeon liver (20%)	None	7000–10000***
Substrate: [^{14}C]glucose 6-phosphate (5 $\mu\text{moles/digest}$; 5×10^4 counts/min.)		
Tumour (10%)	None	200–400*
Tumour (10%)	Phosphoglucomutase, cysteine, Mg^{2+} (as above)	500–700*
Tumour (10%)	AMP (5 μmoles), phosphoglucomutase, cysteine, Mg^{2+}	900–1000*
Tumour (10%)	Pigeon-liver nuclear-mitochondrial fraction (as above)	900–1200***
None	Pigeon-liver nuclear-mitochondrial fraction	700–1000***
Tumour (10%)	Pigeon-liver soluble fraction (equivalent of that obtained from 80 mg. of pigeon liver)	2000–3000*
Rat liver (20%)	None	600–800***
Pigeon liver (20%)	None	8000–10000***
Substrate: [^{14}C]glucose 1-phosphate (5 $\mu\text{moles/digest}$; 5×10^4 counts/min.)		
Tumour (10%)	None	1000–2000*
Tumour (10%)	Glucose 6-phosphate (5 μmoles)	700–900*
Tumour (10%)	AMP (5 μmoles)	3000–5000*
Tumour (10%)	Phosphoglucomutase, cysteine, Mg^{2+} (as above)	800–1100*
Tumour (10%)	Pigeon-liver soluble fraction (as above)	2500–3200*
Tumour (10%)	Pigeon-liver nuclear-mitochondrial fraction (as above)	2000–3000**
None	Pigeon-liver nuclear-mitochondrial fraction (as above)	1200–1500**
Rat liver (20%)	None	1100–1200**
Pigeon liver (20%)	None	15000–16000

genate was 10–25% of that obtained when the nuclear-mitochondrial fraction was used without washing. There was little stimulation of glycogen synthesis from either glucose 6-phosphate or glucose 1-phosphate when tumour homogenates were supplemented with the washed nuclear-mitochondrial fraction, whereas unwashed fractions were active in forming glycogen from the two sugar phosphates and only slightly stimulated by the addition of tumour homogenate. Addition of phosphoglucomutase, cysteine and magnesium chloride slightly increased the incorporation of glucose from glucose 6-phosphate into glycogen by the tumour homogenate but decreased the incorporation of glucose from glucose 1-phosphate into glycogen. AMP significantly stimulated the incorporation of glucose from the 1-phosphate, but less so when glucose 6-phosphate was the precursor.

Incorporation was not affected by the presence of dinitrophenol. Similarly the soluble fraction of pigeon or rat stimulated the incorporation of glucose from the glucose phosphates into glycogen by the tumour homogenate. The soluble fraction itself gave only slight incorporation of glucose from glucose phosphates into glycogen. In most of these experiments the incorporation from glucose 1-phosphate was better than from glucose 6-phosphate. Incorporation from glucose 1-phosphate was also inhibited by glucose 6-phosphate in the tumour homogenate.

DISCUSSION

The data presented in this paper show that Novikoff ascites-hepatoma cells possess a high capacity to transform glucose into glycogen. The

rate of glycogen synthesis obtained with the tumour cells (0.6–1.00 μ mole of glucose equivalent formed/min./ml. of packed cells) compares favourably with the rates obtained for normal rat-liver slice (0.4–1.00 μ mole of glucose equivalent formed/min./g. wet wt. of liver). The demonstration of glycogenesis by a tumour opposes the idea that an enzymic lesion results in low glycogen storage in tumours.

Among the enzymes of the glycogen pathway, the high- K_m glucokinase is absent from the tumour, and the other three enzymes, phosphoglucomutase, UDP-glucose pyrophosphorylase and UDP-glucose- α -glucan glucosyltransferase, have low activities compared with those of rat liver. The activity of UDP-glucose- α -glucan glucosyltransferase is the lowest and is close to the rate of glycogen synthesis from glucose by intact tumour cells. The activity of phosphorylase *a* (active in the absence of AMP) is also low in the tumour, but in the presence of AMP (phosphorylase *a*+*b*) it increases six- to seven-fold.

Novikoff ascites-hepatoma cells form nearly equal amounts of glycogen from glucose and fructose. Mannose yields only one-third as much, and galactose is a poor precursor of glycogen. In my experiments glucosamine was not incorporated into glycogen by the intact tumour cells.

The capacity of the tumour cell to form glycogen depends on at least two factors, namely the concentration of glucose and, to a small extent, on the presence of P_i . Although glycogen synthesis is achieved in the absence of glucokinase, the amount of glucose is so low in the ascitic fluid that a significant accumulation of glycogen by the tumour cells appears unlikely. Further, the presence of approx. 5mm- P_i in the ascitic fluid probably stimulates the conversion of a part of glucose into lactic acid. Since the administration of glucose to the tumour-bearing rat results in the accumulation of glycogen, the effect of P_i can be considered to be of minor consequence. A lack of requirement of externally added P_i for glycogen synthesis is not unusual since pigeon-liver homogenates prepared in the absence of P_i and containing as little as 40mg. of tissue/ml. (P_i concn. 0.2–0.4mm) give maximal rates of glycogenesis from glucose.

Glycogen formation in the absence of glucokinase and almost equal rates of glycogen synthesis at low and high molar glucose concentration argue against the essential requirement of glucokinase for glycogen synthesis, as has been proposed for rat liver by Viñuela *et al.* (1963). Almost equal rates of glycogen synthesis from both glucose and fructose also offer evidence in favour of hexokinase participation. Since other glycogen-storing tissues, namely heart muscle (Mayer & Mayfield, 1964), sheep liver (Ballard & Oliver, 1964), human liver

(G. E. Boxer, personal communication) and pigeon liver (V. N. Nigam & A. Fridland, unpublished work) are also free of glucokinase, the position of this enzyme in glycogen synthesis remains to be ascertained.

Glycogen synthesis by the tumour cell appears to require the presence of oxygen. The action of uncouplers and anaerobic conditions supports this conclusion. The effect of high temperature (43°), which diminishes respiration because of agglutination of the cells, but does not affect glycolysis, supports the idea that oxidative energy is required for glycogen synthesis. Whether the amount of dissolved oxygen is rate-limiting for glycogen synthesis *in vivo* has not been ascertained. It has not been possible to determine to what extent the concentration of ATP regulates glycogen synthesis, since ATP concentration in aerobic conditions and the presence of glucose is only slightly less than it is in the presence of dinitrophenol and glucose. An absolute requirement for aerobic energy for glycogen formation from glucose and glucose 6-phosphate (Nigam & Fridland, 1964) and from glucose 1-phosphate (Zancan & Hers, 1965) in pigeon-liver homogenates has been shown and it is observed that externally added ATP or UTP cannot meet the energy requirement.

Degradation of deposited glycogen in the tumour cell is slow; less than 0.1 μ mole of glycogen/min./ml. of packed cells is obtained. When dinitrophenol is added, an increase in the concentration of AMP occurs and glycogen degradation is accelerated. Since phosphorylase activity is also stimulated by AMP, activation of phosphorylase may be responsible for the degradation of glycogen. Glycogen breakdown in yeast (Stickland, 1956) and in HeLa cells (Wu, 1959) after dinitrophenol addition has also been observed and AMP activation of phosphorylase activity has been suggested for glycogen degradation in HeLa cells. Since degradation of glycogen is followed by its conversion into lactic acid, the process offers a route for the transformation of aerobic energy (used in glycogen synthesis) into glycolytic energy (obtained on glycogenolysis).

Glucose 6-phosphate as well as glucose 1-phosphate appear to be better precursors of glycogen in the phosphorylase reaction than in homogenates supplemented with the nuclear-mitochondrial fraction. Since glucose can form more glycogen than glucose 6-phosphate when the tumour homogenates are supplemented with the mitochondrial fraction, it is questionable whether glucose 6-phosphate formation is essential for glycogen synthesis.

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