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Synthesis of Glycogen from Uridine Diphosphate Glucose in Liver*

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The synthesis of glycogen from uridine diphosphate glucose was first obtained with a liver enzyme (1). Subsequently special attention was directed to the muscle enzyme (2) which has also been studied by several workers (3–6).

The main purpose of this investigation was to extend the original studies with the liver enzyme, and in particular to determine whether it is activated by glucose 6-phosphate and to study its intracellular localization. Further studies on assay, purification and optimal conditions are also reported in this paper.

EXPERIMENTAL

Methods

Reagents—Most of the methods were as described previously (2). UDP-glucose was prepared as described by Pontis *et al.* (7). Pyruvate kinase was obtained as described previously (8) and dialyzed at 4° against distilled water. The water was renewed every 3 hours until a precipitate appeared. This precipitate was removed by centrifugation. The enzyme was stored at −15° in 0.1 M $MgSO_4$. The suitable dilution was ascertained by testing known amounts of UDP.

Glycogen was prepared by the usual KOH method and is referred to as KOH-glycogen. Soluble starch was obtained from Mallinckrodt Chemical Works, St. Louis, Missouri. Galactose-6-P was freed from glucose-6-P by treating it with a glucose 6-phosphatase preparation from liver. After incubation, proteins were coagulated by heating; inorganic phosphate was removed as the insoluble Ba salt, and Ba galactose-6-P was precipitated with 3 volumes of ethanol and was washed and dried. After the treatment the amount of glucose-6-P in the sample was 0.26% as measured with glucose-6-P dehydrogenase and TPN. Glucosamine-6-P was a gift from Dr. S. Roseman.

Analytical Methods—Nucleic acids were estimated as described by Littlefield *et al.* (9). Glycogen was measured after digestion with KOH and ethanol precipitation, by a modification of the iodine method (10). Phosphorylase was determined by measuring the amount of inorganic phosphate liberated from 0.016 M glucose-1-P, in the presence of 0.001 M 5′-AMP, 0.04 M cysteine (11), citrate buffer of pH 5.9 and 0.03 M potassium fluoride (12). The results are expressed as μmoles of inorganic phosphate.

Protein was estimated according to Lowry *et al.* (13), usually after trichloroacetic acid precipitation because large amounts of glycogen produced turbidity which interfered in direct determinations. Plasma albumin, precipitated in the same fashion, was used as a standard. Amylase activity was measured as previously reported (2), in the presence of 0.01 M chloride and at pH 7.2; the reducing power was expressed as μmoles of glucose. Glucose 6-phosphatase was estimated according to Swanson (14), and results indicated as μmoles of inorganic phosphate.

Estimation of UDP—The assay method previously reported (8) has been improved in collaboration with I. Algranati and E. Cabib. The procedure is as follows: To the unknown solution, standards and blanks are added (*a*) 0.025 ml of 0.01 M phosphopyruvate (usually as the cyclohexyl ammonium salt) in 0.4 M KCl, and (*b*) 0.025 ml of pyruvate kinase diluted in 0.1 M $MgSO_4$. After incubating 15 minutes at 37°, 0.15 ml of 0.1% dinitrophenylhydrazine in 2 N HCl is added. After 5 minutes 0.2 ml of 10 N NaOH and 1.1 ml of 95% ethanol are added, and the samples are mixed and centrifuged. The optical density of the supernatant fluid at 520 mμ is then measured.

The improvements consist (*a*) in carrying out the pyruvate kinase reaction in the presence of K^+ (15). In this manner a more dilute enzyme can be used so that substances such as AMP no longer interfere, (*b*) in adding ethanol so that glycogen is precipitated and turbidity avoided.

Test for Glycogen Synthetase—The composition of the standard reaction mixture was as follows: (in μmoles) 0.25 of UDP-glucose, 0.5 of glucose-6-P, 7.5 of glycine buffer of pH 8.5, 0.25 of EDTA,[1] 0.4 mg of glycogen, and enzyme; the total volume was 0.05 ml. The incubation time was 5 to 10 minutes and the temperature 37°. The reaction was stopped by heating and UDP was estimated as described above. Cysteine (0.003 M) was added when samples of purified enzyme were tested since with these preparations it produced about 50% activation.

Preparation of Liver Extracts—The rats were given 30% sucrose solution orally *ad libitum* during 5 to 6 hours and killed by a blow in the neck. The liver was homogenized in 3 volumes of 0.25 M sucrose containing 0.001 M EDTA. The homogenate was then centrifuged. The supernatant fluid obtained after 10 minutes at 2000 × *g* is referred to as "crude extract." The crude extracts did not lose activity when stored a month or more at −15°.

RESULTS

Intracellular Localization of Enzyme—When liver homogenates were fractionally centrifuged in order to separate the different

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[1] The abbreviation used is: EDTA, ethylenediaminetetraacetate.

cell constituents most of the activity was recovered in the small particle fraction. This fraction could be subdivided in two parts: The microsomes which formed a brownish loosely packed layer, and the "particulate glycogen" which appeared as a transparent pellet firmly packed at the bottom of the tube. These two fractions could be easily separated by inverting the tube thus allowing the microsomes to drain down. An experiment in which the different unwashed fractions were analyzed is shown in Table I. It may be observed that the activity of glycogen synthetase is parallel to the glycogen content. Thus the ratio of these two quantities was approximately constant for the different fractions whereas the specific activity was highest in the particulate glycogen. In relation to the original supernatant a 25-fold increase in specific activity was achieved. The distribution of phosphorylase was somewhat different from that of glycogen synthetase, in that more activity was found in the final supernatant. The adsorption of phosphorylase to glycogen has been observed by Sutherland and Wosilait (16).

Glucose 6-phosphatase was concentrated mainly in the microsome fraction together with the nucleic acid. In fact glucose 6-phosphatase is considered to be a microsome localized enzyme (17).

Purification of Enzyme—As mentioned above, by separation of the "'particulate glycogen" a 20- to 30-fold increase in specific activity is obtained. Hardly any activity could be extracted by washing the glycogen particles with saline buffer but as shown in Table II considerable activity was recovered in the supernatant fluid when the washing solutions contained KOH-glycogen. Soluble starch did not help in the extraction of the activity but did increase the amount of inactive protein extracted. A simple procedure for the purification of the enzyme was developed with the use of these facts. A typical preparation was as follows: Crude extract (3 ml) was centrifuged for 10 minutes at 25,000 $\times$ *g* in head No. 295 of an International centrifuge PR-1, and the supernatant fluid and microsome fraction were sucked out. The remaining glycogen pellet ("unwashed glycogen") was then suspended in 0.3 ml of saline buffer (0.15 M KCl, 0.001 M EDTA, 0.01 M Tris of pH 7.1, 0.001 M glucose-6-P) containing 50 mg per ml of soluble starch, and centrifuged for 10 minutes. The supernatant fluid was discarded, the precipitate was washed again with 0.3 ml of the same solution and centrifuged. The remaining pellet was resuspended in 0.3 ml of the saline buffer ("washed glycogen"). As shown in Table III, a 300-fold increase in specific activity was obtained. The yield was about 12%. Evidently such an increase in specific activity is obtained when the latter is calculated on the basis of protein concentration but this would not be true if dry weight were used in the calculation since most of the glycogen remains with the enzyme. Table III also shows that phosphorylase is considerably decreased and amylase increased.

The purified preparations lost activity rather rapidly, even when kept at −15° in the presence of glucose-6-P (about 70% in 24 hours). Some protection was obtained by the addition of cysteine, albumin or heated liver extracts. Furthermore in the purified preparations cysteine produced a 48% increase in activity at 0.003 M concentration.

TABLE I

Fractional centrifugation of liver extract

The liver homogenate was centrifuged 10 minutes at 700 $\times$ *g*. Precipitate discarded. The supernatant fluid was centrifuged 10 minutes at 5,000 $\times$ *g*. The precipitate is referred to as mitochondria. The supernatant fluid was centrifuged 10 minutes at 25,000 $\times$ *g*. The upper part of the supernatant fluid was sucked off (final supernatant), the middle part was discarded. The tube was then inverted thus allowing the small particle fraction to pour out (microsomes). A transparent pellet remained at the bottom of the tube (particulate glycogen). The different precipitates were suspended in sucrose-EDTA solution (volume about 0.1 of original supernatant) and tested without washing.

	UDP formation	Glycogen content	Protein content	Ratio		Phosphorylase	Ratio: Phosphorylase/glycogen	Amylase	Glucose-6-phosphatase	Nucleic acid
				UDP/glycogen	UDP/protein					
	μmoles/min/ml	*mg/ml*	*mg/ml*			*μmoles/min/ml*		*μmoles/min/ml*	*μmoles/min/ml*	*mg/ml*
Supernatant fluid 700 $\times$ *g*	0.79	18.7	28	0.042	0.028	2.55	0.14	0.25	1.47	0.92
Mitochondrial fraction	0.48	12.9	30	0.037	0.016	0.42	0.033	0.22	1.50	1.19
Microsomal fraction	0.66	15.5	28	0.042	0.024	1.05	0.068	0.21	2.7	1.50
Particulate glycogen	1.74	53.0	2.5	0.033	0.7	4.15	0.079	0.21	0.5	0.10
Supernatant fluid 25,000 $\times$ *g*	0.21	3.7	18	0.057	0.012	1.05	0.28	0.042	0.35	0.36

TABLE II

Effect of washing particulate glycogen with different solutions

The glycogen pellets from 2 ml of crude extract in each case were washed twice with 0.2 ml of 0.15 M KCl containing 0.001 M EDTA, 0.01 M Tris buffer of pH 7.1 and 0.006 M glucose-6-P. The pellets were then suspended in 0.2 ml of the same solution with the additions indicated and centrifuged after 10 minutes at 0°.

Washing solution	Glycogen synthetase activity	
	Supernate	Precipitate
	μmoles UDP/min/ml	
KCl, EDTA, Tris, glucose-6-P.....	~0.06	1.35 (2.2)*
Same + KOH-glycogen (50 mg/ml)..	0.84 (0.90)	0.84 (3.3)
Same + soluble starch (50 mg/ml)...	~0.09	1.32 (7.0)
None.....	—	1.81 (2.2)

* Numbers in parenthesis represent specific activities.

TABLE III

Purification of enzyme

Preparation as described in text. The sample of unwashed glycogen was obtained in a parallel run. Tests for glycogen synthetase were carried out in the presence of 0.003 M cysteine.

		Protein content	UDP formation	Specific Activity	Phosphorylase	Amylase
	ml	*mg/ml*	*μmoles/ml/min*		*μmoles/ml/min*	*μmoles/ml/min*
Crude extract.....	3	32	0.95	0.03	2.08	0.35
Unwashed glycogen....	0.3	2.4	1.5	0.63	1.8	0.55
Washed glycogen.....	0.3	0.12	1.2	10.0	0.15	0.52

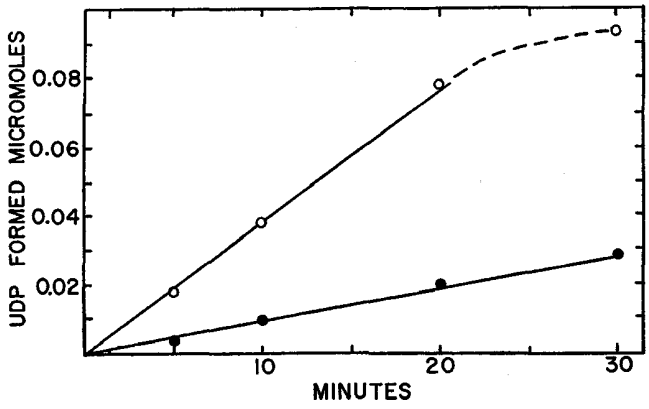

FIG. 1. Time course of the reaction: UDP formation was measured after incubation under standard conditions. Lower and upper curves with 0.8 μg and 3.2 μg of protein respectively. The washed particulate glycogen was used as the enzyme preparation.

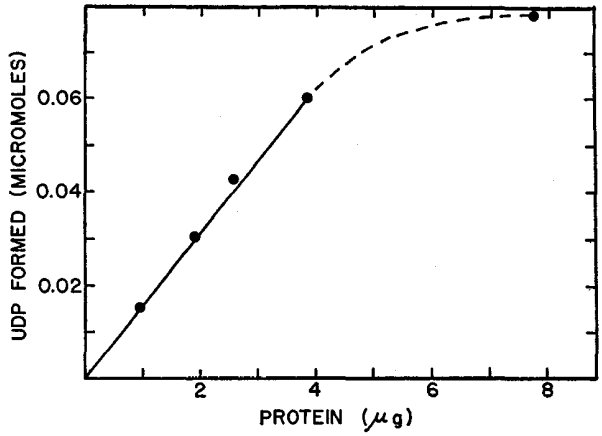

FIG. 2. Relation between UDP formation and enzyme concentration. Conditions as in Fig. 1.

Kinetics—Within certain limits the relationship between UDP formation and time or enzyme concentration is linear. Thus as shown in Figs. 1 and 2, a straight line was obtained when the UDP formed did not exceed 0.06 μmoles. The tests for enzyme activity were always carried out within these limits, the incubation time being 5 or 10 minutes.

The effect of changing the concentration of UDP-glucose is shown in Fig. 3. The value obtained for K_m was 4.8×10^{-4} M and does not differ significantly from the value of 5×10^{-4} M reported (2) for the rat muscle enzyme.

pH Optimum—As shown in Fig. 4 the pH optimum was found to be about 8.4, that is, the same as for the muscle enzyme (2). It may be observed that more activity was obtained with 0.15 M glycine buffer than with 0.075 M Tris-maleate.

Action of Glucose-6-P—As in the case of the muscle enzyme, addition of glucose-6-P led to an increase in UDP formation. A 4- to 15-fold increase in activity was obtained in different preparations. A curve showing the relationship between glucose-6-P concentration and activity is shown in Fig. 5. The K_m calculated from such data is 6×10^{-4} M. Fig. 5 also shows the action of the other substances which gave an activation. The results indicated that galactose-6-P has an activating action although smaller than that of glucose-6-P.

It may be observed in Fig. 5 that the addition of fructose-6-P

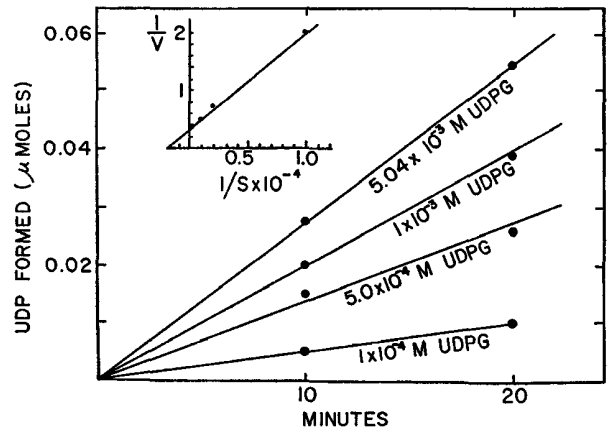

FIG. 3. The effect of changing UDP-glucose concentration.

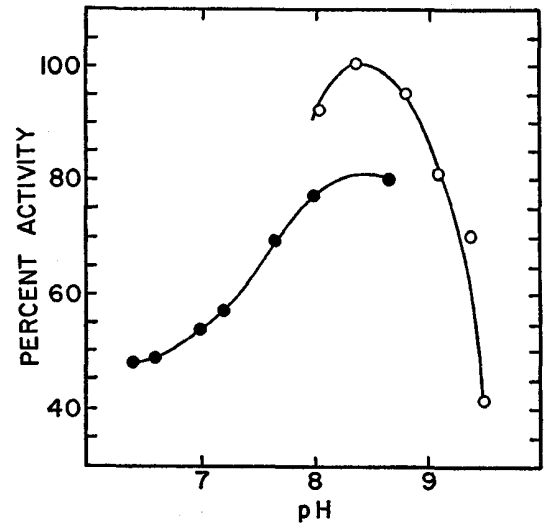

FIG. 4. pH optimum. O——O, 0.075 M Tris maleate buffer. ●——●, 0.15 M glycine buffer. Conditions as in Fig. 1. The results are expressed as percentage of maximal activity.

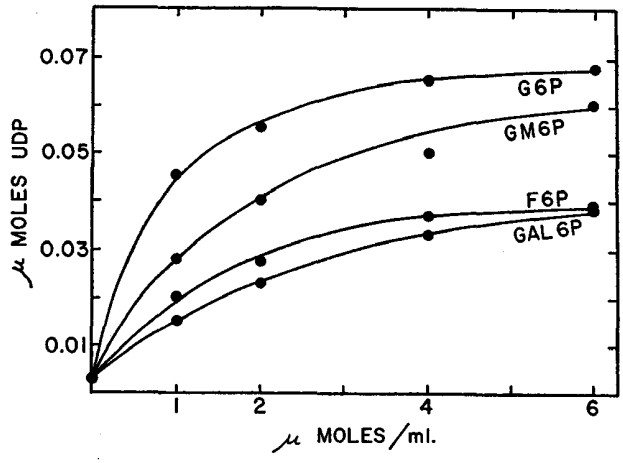

FIG. 5. The action of glucose-6-phosphate and other substances on UDP formation. GM-6-P, glucosamine-6-P, F-6-P, fructose-6-P, Gal-6-P, galactose-6-P.

TABLE IV

Action of glucose-6-P on glucose-C^{14} incorporation

Complete system as described in text (with cysteine) except that the samples corresponding to the left column contained 0.19 μmole of C^{14}-labeled UDP-glucose with 7500 counts per minute. The glycogen was treated with KOH, precipitated and washed with ethanol, and counted. The values for samples at t = 0 were subtracted.

	Glycogen	UDP formed
	c.p.m.	*μmoles*
Complete system	1470	~0.09
No glucose-6-phosphate	230	0.019

also produced some activation. Although the glycogen synthetase used was contaminated with small amounts of phosphohexose isomerase, it can be concluded that fructose-6-P is not an intermediate in glucose-6-P activation but it remains unsettled whether it has some action *per se*. As to the glucosamine-6-P sample, it had been obtained by chemical phosphorylation of glucosamine so that it is very unlikely that it could be contaminated with glucose-6-P.

In order to ascertain whether the increased formation of UDP in the presence of glucose-6-P corresponded in fact to an increased glycogen formation, an experiment was carried out measuring the transfer of glucose-C^{14} from UDP-glucose to glycogen. The results are shown in Table IV, where it may be observed that glucose-6-P increases the incorporation of radioactivity into the glycogen.

Many other substances were tested as activators with negative results. These were: glucose, galactose, maltose, sucrose phosphate, acetylglucosamine-6-P, glucose-1-P, glucose-1,6-diphosphate, α-methyl-glucoside, AMP, trehalose phosphate, trehalose, α- and β-glycerophosphates, D-xylose, D-ribose, D-arabinose, lactose, cellobiose, gentiobiose and salicin.

Glucose-6-P was found to protect the enzyme from inactivation. Thus as shown in Table V samples preincubated at 37° in the presence of glucose-6-P were considerably more active than those preincubated with water or buffers.

Miscellaneous Experiments—Some experiments were carried out in order to detect the reversibility of glycogen synthetase. The tests consisted in incubating the enzyme with glycogen and UDP and measuring UDP-glucose with UDP-glucose dehydrogenase. All the results were negative.

The action of some inhibitors was investigated. Glucose at 0.1 M concentration produced 45% inhibition. Trehalose phosphate at 0.01 M concentration inhibited 59% and UMP at 0.005 M concentration, 50%. α-Methyl-glucoside produced no effect. Magnesium ions were not found to activate the enzyme and it seems likely that the increases in activity which have been reported may be due to an activation of phosphoglucomutase which in impure preparations would lead to the formation of glucose-6-P.

TABLE V

Action of different substances on stability of enzyme

Samples of enzyme (unwashed glycogen fraction) were preincubated 10 minutes at 37° in 4 volumes of the solutions indicated and immediately tested.

Solution added for preincubation	UDP formed in 10 min
	μmoles
Water	0.005
Tris buffer pH 7.1, 0.02 M	0.003
Glucose-6-phosphate, 0.01 M	0.065
Plasma albumin	0.013
Heated liver extracts	0.063
Control (not preincubated)	0.073

DISCUSSION

The experiments reported show that on centrifugation of liver extracts, glycogen synthetase sediments together with the glycogen. Thus it can be separated together with the "particulate glycogen" first described by Lazarow (18). This type of glycogen has been studied further (19, 20) and preparations have been obtained which contain less than 0.005% protein (20). The molecular weight has been estimated to be 2×10^7 to 2×10^8 while that of KOH-glycogen is about 3×10^6 (21).

Glycogen synthetase appears to be bound to the "particulate glycogen" as an enzyme-substrate complex since on addition of KOH-glycogen followed by centrifugation considerable amounts of enzyme remain in the supernatant fluid. Robbins *et al.* (4) had already suggested that binding to the glycogen might explain the ready sedimentability of the enzyme in muscle. The firm binding to the substrate may explain the apparent particulate character of other polysaccharide synthesizing enzymes.

No new information has been obtained on the mechanism of the activation by glucose-6-P. The latter substance was found to have a protective action on the enzyme and it cannot be decided with certainty if the apparent activation is due to protection or to real activation.

The concentration of glucose-6-P necessary for half maximal activation is 6×10^{-4} M and the concentration in liver ranges from 0.5×10^{-4} to 6×10^{-4} moles per kg of wet liver (22) so that it seems likely that glucose-6-P concentration *in vivo* should be important in the regulation of glycogen synthetase activity.

SUMMARY

The formation of glycogen from uridine diphosphate glucose has been studied in liver extracts. On fractional centrifugation of liver homogenates the enzyme was found to sediment together with the glycogen. By washing the glycogen precipitate the specific activity could be increased up to 300-fold.

The liver enzyme, like that of muscle, was found to be activated by glucose-6-phosphate at physiological concentration, and also by glucosamine-6-phosphate and galactose-6-phosphate.

Studies on the method of assay and pH optimum are reported.

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