Biochemistry

Volume 15, Number 6 March 23, 1976

Uridine 5′-(5-Thio-α-D-glucopyranosyl pyrophosphate): Chemical Synthesis and Activation of Rat Liver Glycogen Synthetase†

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ABSTRACT: Uridine 5′-(5-thio-α-D-glucopyranosyl pyrophosphate), UDPTG, appears to be a potent activator of rat liver glycogen synthetase *a* even though it is not a substrate. At 1.0 mM, UDPTG causes over 400% activation of glycogen synthetase *a* activity. Activation by UDPTG is accompanied by normalization of the otherwise sigmoidal kinetics for UDPG with glycogen synthetase *a* and a decrease in the apparent K_m for UDPG from approximately 2.0 to 0.62 mM. UDPTG inhibits catalytic activity at higher concentrations. At the concentrations examined, UDPTG has no effect on glycogen synthetase *b* activity. The use of glycogen synthetase free from glycogen synthetase *b* phosphatase and the selective inhibition of glycogen synthetase *b* phosphatase by 100 mM NaF·indicate that conversion of synthetase *b* to *a* is not responsible for the activation. Moreover, the use of the colorimetric assay for glycogen synthetase activity precludes effects of UDPTG on glycogen phosphorylase activity. UDPTG, chemically synthesized in 60% yield, is characterized by chromatographic and electrophoretic procedures, by its uv spectra, and by analysis of products after chemical and enzymatic hydrolysis.

During the past few years, our laboratory has been concerned with the synthesis and biochemical characterization of sugar analogues with sulfur replacing the ring oxygen. These analogues differ chemically and physically only very subtly from their natural counterparts, but possess interesting biochemical properties. Thus, 5-thio-D-glucose is an inhibitor of D-glucose transport in many tissues (Hoffman and Whistler, 1968; Whistler and Lake, 1972; Critchley et al., 1970; Barnett et al., 1970; Hellman et al., 1973), and its 1- and 6-phosphates act as alternate substrates and potent inhibitors of several glycolytic enzymes (Tsai and Whistler, unpublished results). Nucleosides made from 4-thio-D-ribose have novel biochemical properties, as exemplified by the analogues of 5-fluorouridine (Bobek et al., 1975), NADH (Hoffman and Whistler, 1970), cyclic adenosine 3′,5′-monophosphate (Anisuzzaman et al., 1973), and 5′-AMP[1] (Hoffman and Whistler, 1970). We report here the chemical synthesis of the UDPG analogue, uridine 5′-(5-thio-α-D-glucopyranosyl pyrophosphate), and its unique behavior toward rat liver glycogen synthetase.

Experimental Procedure

General Methods and Materials

Qualitative paper chromatography was performed by the descending method on Whatman No. 1 paper and preparative paper chromatography by the descending method on Whatman No. 3 MM paper, using the following irrigants: (A) ethyl alcohol–0.5 M ammonium acetate buffer, pH 3.8 (5:2, v/v); (B) ethyl alcohol–1 M ammonium acetate buffer, pH 7.5 (5:2, v/v) (Paladini and Leloir, 1952); (C) acetone–ethyl alcohol (2:1, v/v). Ascending cellulose thin-layer chromatography was achieved using Eastman Chromagram cellulose sheets.

Qualitative paper electrophoresis was achieved by using Whatman No. 3 MM paper impregnated with 0.05 M phosphate or 0.05 M triethylammonium bicarbonate buffers at pH 7.5 (Porath, 1955). Paper strips were 15 × 30 cm. Voltage was held constant at 400 V using a Beckman Duostat regulated power supply. Ferroin sulfate and the disodium salt of fluorescein were found to be convenient cationic and anionic markers, respectively.

Sugars were visualized by spraying with anisaldehyde reagent (Stahl and Kaltenbach, 1961). Sugar and nucleoside phosphates were visualized by the method of Bandurski and

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[1] Abbreviations used: UDPTG, uridine 5′-(5-thio-α-D-glucopyranosyl pyrophosphate); UDPG, 5-thio-α-D-glucopyranose 1-phosphate; GDP, guanosine diphosphate; 5′ AMP, adenosine 5′-monophosphate; UPML, 4-morpholine *N,N′*-dicyclohexylcarboxamidinium uridine 5′-phosphoromorpholidate; UMP, uridine monophosphate; UDP, uridine diphosphate.

Axelrod (1951). Inorganic salts were determined by spraying with a saturated solution of alizarin in alcohol, followed by 25% ammonium hydroxide (DeVries et al., 1964). Nucleosides, nucleotides, and nucleoside diphosphate sugars were determined by irradiation with a short wavelength ultraviolet light.

NMR's were performed on a Varian T-60A spectrometer. Ultraviolet spectra were determined on a Beckman DB spectrophotometer.

UDPG, UMP, UDP and phosphoenolpyruvate were obtained from Sigma Chemical Company, St. Louis, Missouri, as the sodium salts. Glycogen (type III, from rabbit liver), pyruvate kinase (type II, from rabbit skeletal muscle), and phosphodiesterase (type II, from *Crotalus adamanteus* venom) were also obtained from Sigma. The phosphodiesterase preparation was used as a source of nucleotide pyrophosphatase. D-Glucose 6-phosphate and D-glucose 1-phosphate, as well as pyruvic acid, were all obtained from Calbiochem, Los Angeles, California. UDP-[U-^{14}C]glucose was obtained from Amersham/Searle Corporation, Arlington Heights, Illinois.

Synthetic Methods

5-Thio-α-D-glucopyranose 1-Phosphate (I). 1,2,3,4,5-Penta-*O*-acetyl-5-thio-D-glucopyranose was prepared by the method of Nayak and Whistler (1969), and 5-thio-α-D-glucopyranose 1-phosphate (I) by the method of Whistler and Stark (1970). The identity of the 1-phosphate as well as its anomeric configuration was verified by NMR.

Uridine 5′-(5-Thio-α-D-glucopyranosyl pyrophosphate) (III). 4-Morpholine *N,N′*-dicyclohexylcarboxamidinium uridine 5′-phosphoromorpholidate (II, UPML) was prepared by the method of Moffatt and Khorana (1961), and uridine 5′-(5-thio-α-D-glucopyranosyl pyrophosphate) (III, UDPTG) by the following modifications of the method of Roseman et al. (1961). The modifications in reaction conditions and purification procedures were necessary due to the high lability of 5-thio-D-glucose 1-phosphate and the UDPTG analogue.

5-Thio-α-D-glucopyranose 1-phosphate (I, 140 mg, 0.28 mmol) was passed slowly through a water-jacketed (15 °C) column of Dowex 50W-X2 (H^+, H_2O), 200–400 mesh. The effluent was collected dropwise into a Erlemeyer containing ice-cold anhydrous pyridine. This mixture was evaporated down to 3–6 ml, and then 0.50 ml (about 3 mol equiv) of tri-*n*-octylamine was added. This mixture was evaporated to dryness and co-evaporated four times with anhydrous pyridine to remove all moisture. Separately, 350 mg (0.42 mmol) of the phosphoromorpholidate derivative (II) was dissolved in anhydrous pyridine and co-evaporated four times with anhydrous pyridine. These solutions, containing the sugar and nucleotide derivatives, were then combined and co-evaporated twice more with anhydrous pyridine. The final syrup was dissolved in 3 ml of pyridine and the reaction mixture tightly stoppered, placed in a desiccator containing silica gel, and reacted at 40 °C for 2 days. Paper chromatography or cellulose thin-layer chromatography using irrigant A was used to follow the reaction. Reaction was completed in 2 days, the symmetrical diester of UMP being the most abundant by-product, in addition to small amounts of UMP itself. The reaction was taken to dryness (temperature not exceeding 30 °C) and the residue shaken in a mixture of ice-cold water containing lithium acetate (20% more than the total amines present) and ether. The ice-cold aqueous layer was then extracted with ether, and the combined ether layers were backwashed with ice-water. The aqueous solution of product was then lyophilized and stored at −20 °C.

Table I: Chromatographic Mobilities.

Compound	R_f, Paper Chromatography (A)	R_f, Paper Chromatography (B)	R_f, Cellulose TLC (A)	R_f, Cellulose TLC (B)
UMP	0.41	0.18	0.51	0.12
UDP	0.24	0.10	0.32	0.07
UPML	0.55	0.57	0.74	0.66
UMP diester	0.18	0.15	0.19	0.09
UDPG	0.25	0.25	0.33	0.24
Glc 1-P	0.30	0.10		
UDPTG	0.23	0.20	0.29	0.22
5-Thio-Glc 1-P		0.21	0.68	
5-Thio-Glc			0.72	0.69
Uridine			0.63	0.63

Several methods were employed for the purification of UDPTG. These included preparative paper chromatography, ion-exchange chromatography on Dowex 1-X8, gel filtration on Sephadex G-10 and G-25 fine, and separation on DEAE-cellulose ion-exchange chromatography. Because of the high lability of UDPTG, many of these conventional methods led to decomposition of the product and decreased yields. Paper chromatography was found to offer the mildest conditions.

Preparative paper chromatography was performed using both solvent systems A and B sequentially. The chromatography and all subsequent operations were conducted at 5 °C to minimize decomposition of the analogue. Bands corresponding to UDPTG were cut from the paper and eluted, with stirring, into ice-cold water containing sufficient ammonium hydroxide to make the pH slightly alkaline. The eluted samples were filtered, lyophilized, and stored as the ammonium salt at −20 °C. Excess ammonium acetate was removed by again applying the analogue to paper and eluting with solvent C at 5 °C. UDPTG was obtained in 60% overall yield as calculated from the starting sugar phosphate. When lyophilized and stored under desiccation at −20 °C, the UDPTG was stable for a period of several months.

Analysis of UDPTG. UDPTG was characterized by the same analytical procedures used to verify the identity of UDPG.

The analogue was homogeneous by paper chromatography and cellulose thin-layer chromatography in two solvents, by paper electrophoresis at two pH's (data for pH 3.8 not shown) and by Dowex 1-X8 ion exchange chromatography. Behavior in these systems (Tables I and II) suggest that UDPTG has a higher negative charge density than normal UDPG. Mobility on Dowex 1 ion exchange (Table II) refers to the concentration of lithium chloride (in 0.005 N hydrochloric acid) required for elution.

The uv spectra of UDPG and UDPTG were found to be nearly identical, both displaying maximal adsorption at 262 nm. The molar extinction coefficient for UDPG at 262 nm, pH 2.0, is given as 10.0×10^6 cm^2/mol (Bergmeyer, 1963). The molar extinction coefficient of UDPTG was determined to be 9.6×10^6 cm^2/mol at 262 nm, pH 2.0, and was used for determination of UDPTG concentrations in the enzyme work.

Chemical hydrolysis of UDPTG was conducted in 1 N

Table II: Electrophoretic and Ion Exchange Mobilities.

Compound	Paper Electrophoresis	Dowex 1 Ion Exchange
Fluorescein, disodium	+1.0	
Ferroin sulfate	−1.4	
UMP	+1.7	0.02 N
UDP		0.10 N
UPML	+0.9	0.015 N
UMP diester	+1.3	(0.40 N)
UDPG	+1.3	0.06 N
UDPTG	+1.4	0.10 N

Table III: Chemical and Enzymatic Hydrolysis of UDPTG.

Product	HCl, pH 2	1 N HCl	Nucleotide Pyrophosphatase
UDP	1.09		
UMP		1.05	0.90
Uridine			0.05
5-Thio-Glc	1.12	0.92	
5-Thio-Glc 1-P			0.90
Acidlabile phosphate	1.00	1.00	1.00

hydrochloric acid solution and in hydrochloric acid solution at pH 2 (Caputto et al., 1950). In each case, 1.0 mg of UDPTG was dissolved in 1 ml of the appropriate acid solution and was incubated at 100 °C for 10 min. Each solution was neutralized with ammonium hydroxide and concentrated to dryness. The resulting syrups were dissolved in water and applied to preparative cellulose thin-layer chromatography plates. After chromatography, the various products were eluted and determined quantitatively. 5-Thio-D-glucose was determined by the phenol–sulfuric method (Dubois et al., 1956), and UMP and UDP were determined by their uv adsorption at pH 2.0. Before chromatography, acid-labile phosphate was determined on an aliquot of the reaction mixture by the method of Fiske and Subbarow (1925). This value was taken as a standard for a relative molar ratio comparison. The hydrolysis products and product ratios are shown in Table III and correlate with those seen for UDPG (Caputto et al., 1950).

UDPTG was also hydrolyzed by snake venom nucleotide pyrophosphatase by incubating 1.0 mg of the analogue in 0.1 ml of 0.01 M Tris buffer, pH 7.4, at 37 °C with a sufficient amount of the enzyme to give total hydrolysis within 20 min. The products were quantitatively determined in the same way as for the chemical hydrolysis, except that 5-thio-D-glucose 1-phosphate was determined by the phenol–sulfuric method after hydrolysis to 5-thio-D-glucose. As shown in Table III, hydrolysis by nucleotide pyrophosphatase gave the expected ratios of UMP and 5-thio-D-glucose 1-phosphate (Munch-Petersen et al., 1953). The small amount of uridine in the enzymatic hydrolysis was presumably the result of the small amount of phosphomonoesterase activity reported as being present in the snake venom enzyme preparation.

Enzymatic Methods

Glycogen Synthetase Preparations. UDPTG appears to activate only the *a* form of rat liver glycogen synthetase at concentrations of UDPTG examined (see Results and Discussion). However, only the *b* form of the rat liver enzyme has been highly purified (Lin and Segal, 1973; McVerry and Kim, 1974). Attempts to obtain highly purified rat liver synthetase *a* have been frustrated by spontaneous change of the *a* form, upon isolation, to a D-glucose 6-phosphate dependent form (Kim, personal communication). Synthetase *a* seems, then, to lose its physiological integrity upon extensive purification, and the glycogen pellet enzyme or liver extracts, enriched in the *a* form by pre-treatment of rats with sucrose or glucose (Leloir and Goldemberg, 1962; DeWulf and Hers, 1967a), insulin (Blatt and Kim, 1971), or glucocorticoids (Hornbook et al., 1966; DeWulf and Hers, 1967b; Kreutner and Goldberg, 1967), seem to be the preparations of choice for regulatory studies on the *a* form. Thus, carboxylic acid activation of liver glycogen synthetase *a* and *b* has been characterized using a pellet enzyme preparation (Magner and Kim, 1973), and crude liver extracts have been commonly used to investigate regulation by ATP, D-glucose 6-phosphate, inorganic phosphate, and magnesium (Gold, 1970; DeWulf et al., 1968; Mersmann and Segal, 1967).

Following the methods of Villar-Palasi et al., (1966), we isolated a preparation of glycogen synthetase free from glycogen synthetase *b* phosphatase. Adult male Wistar rats (250 g) were fed 30% sucrose ad libitum for 6 h before sacrifice in order to raise the percent of synthetase *a* in the preparations (Leloir and Goldemberg, 1962). Livers were extracted between 10:00 a.m. and noon, the diurnal peak for synthetase activity (McVerry and Kim, 1972). The phosphatase-free glycogen pellets were suspended in a solution of 30% glycerol in sucrose–EDTA (0.25 M/0.005 M), pH 7.5, and stored at −20 °C.

Assay of Glycogen Synthetase Activity. To assay the possible activity of UDPTG, a modification of the colorimetric assay of Leloir and Goldemberg (1962) was employed. Since this assay measures the formation of UDP, it has the added advantage of being independent of glycogen phosphorylase activity and was used as a control to monitor possible effects of UDPTG on phosphorylase. Two modifications on the assay of Leloir and Goldemberg (1962) were made: reaction was started upon addition of enzyme, rather than substrate, and glycogen synthetase activity was destroyed by heating at 100 °C for 4 min rather than only 1 min. Activity was linear for up to 30 min, depending on the components and reaction conditions.

For all other experiments, glycogen synthetase activity was assayed by following the more specific incorporation of [^{14}C]-D-glucose into glycogen (Villar-Palasi et al., 1966). The standard assay contained UDP-[U-^{14}C]-D-glucose (6000 to 18 000 cpm/assay, depending on the activity being followed), 8 mg/ml of glycogen, 50 mM Tris-HCl, pH 7.8, 5 mM EDTA, and enzyme (usually 80 units) in a total volume of 50 μl. One unit of enzyme is defined as the amount of enzyme necessary for the transfer of 1 pmol of D-glucose from UDPG to glycogen in 1 min. Reaction was initiated upon addition of enzyme and run at 37 °C for 5–10 min. Reaction was stopped by the addition of 0.5 ml of 10% Cl_3CCOOH containing 2 mg/ml of LiBr. If significant protein was present, it was removed by centrifugation. Then 1.5 ml of 95% ethanol was added and the precipitated glycogen was removed by centrifugation. The resulting glycogen pellet was washed twice with 3 ml of 71% ethanol and finally redissolved in 0.5 ml of water and mixed with 10 ml of dioxane cocktail for counting. The cocktail, containing 5 g of 2,5-diphenyloxazole and 100 g of naphthalene in 1 l. of dioxane, was that recommended for use in the Beckman CPM-100 instrument used. The use of 71% ethanol rather

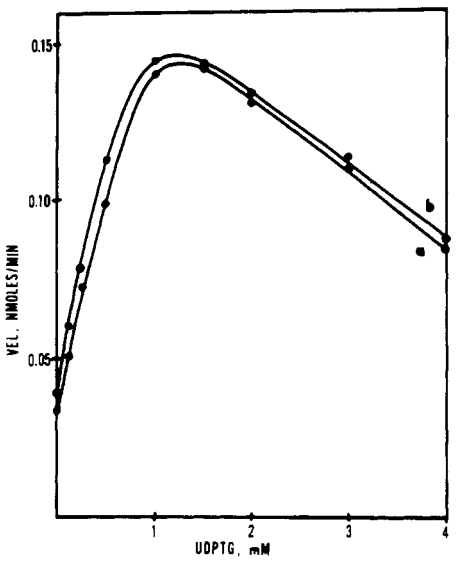

FIGURE 1: Activation of glycogen synthesis by UDPTG. Reaction was followed in the standard assay mixture containing 1.0 mM UDPG in the absence (a) or presence (b) of 100 mM NaF.

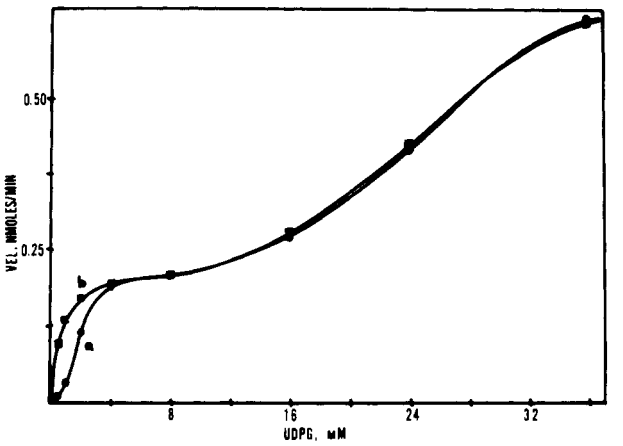

FIGURE 2: Effect of UDPTG on UDPG binding kinetics. Reaction was followed in the standard assay mixture in the absence (a) or presence (b) of 1.0 mM UDPTG.

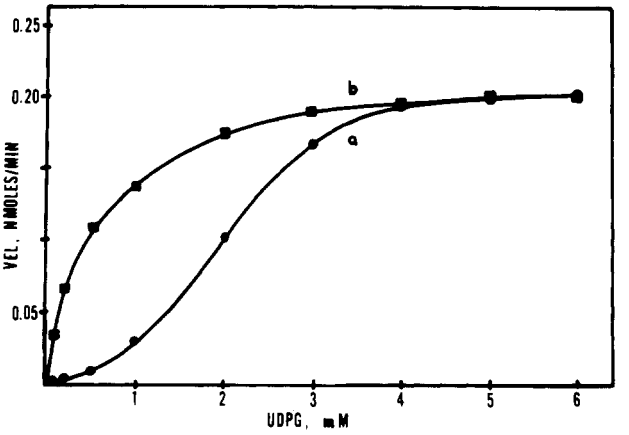

FIGURE 3: Effect of UDPTG on UDPG binding kinetics at low UDPG concentrations. Reaction was followed in the standard assay mixture in the absence (a) or presence (b) of 1.0 mM UDPTG.

than 66%, as reported by Villar-Palasi et al. (1966), led to higher reproducibility and did not lead to precipitation of [^{14}C]UDPG. Protein was determined by the method of Lowry et al. (1951).

Results and Discussion

Interaction of UDPTG with Glycogen Synthetase. UDPTG does not serve as a substrate for rat liver glycogen synthetase, in the presence or absence of D-glucose 6-phosphate, even upon prolonged incubation at concentrations of UDPTG as high as 10 mM. However, screening of UDPTG for inhibitory activity revealed a surprising activation of activity by UDPTG at low concentrations, followed by an inhibition of activity at higher concentrations (Figure 1, curve a). Since the enzyme preparation is free of glycogen synthetase *b* phosphatase, conversion of synthetase *b* to the more active synthetase *a* is not responsible for the activation by UDPTG. To further confirm that no *b* to *a* conversion is occurring, the effect of 100 mM fluoride on the activation was also examined. At pH 7.5, 100 mM fluoride nearly completely inhibits glycogen synthetase *b* phosphatase activity (DeWulf and Hers, 1968). As shown (Figure 1, curve b) fluoride seems to have a slight stimulatory effect on the glycogen synthetase preparation, but had no effect on activation by UDPTG. The slight, independent stimulation of activity by fluoride is probably the result of increased ionic strength, a condition known to stimulate synthetase *a* under certain conditions (DeWulf et al., 1970). An identical activation curve with those in Figure 1 is also observed if glycogen synthetase activity is followed using the colorimetric UDP assay, suggesting that activation is not due to an effect of UDPTG on glycogen phosphorylase activity.

Effect of UDPTG on UDPG Kinetics. One possible explanation of activation by UDPTG would be that UDPTG can replace UDPG in its positive cooperative interactions with the enzyme. Thus, at low concentrations, UDPTG may bind to a few UDPG sites causing stimulation of binding to unoccupied sites. At higher concentrations, however, UDPTG may begin to saturate the UDPG sites, leading to inhibition of catalysis. This type of activation/inhibition action for nonreactive substrate analogues has precedence in the action of aspartate analogues on aspartate transcarbamylase (Gerhart and Pardee, 1963; Gregory and Wilson, 1971) and phosphate analogues on alkaline phosphatase (Kelly et al., 1974). If activation by UDPTG were occurring by binding of UDPTG to the cooperative UDPG sites of glycogen synthetase, one would except UDPTG to normalize the sigmoidal kinetics of the enzyme for UDPG. A decrease in the apparent K_m for UDPG should occur, but no activation by UDPTG should be observed at saturating UDPG concentrations.

The activities of glycogen synthetase *a* and *b* can be distinguished, even in crude liver extracts, by the large difference in their K_m's for UDPG (Mersmann and Segal, 1967). The activity of the pellet glycogen synthetase preparation used in these experiments was examined over a wide range of UDPG concentrations in the presence and absence of 1.0 mM UDPTG (Figure 2).

In the absence of UDPTG, UDPG reaction kinetics (curve a) are biphasic, and sigmoidal saturation curves are observed both at low and high UDPG concentrations. Mersmann and Segal (1967) also report sigmoidal reaction kinetics for UDPG with both synthetase *a* and *b*, with approximate K_m's of 1.5–2.2 and 16–32 mM, respectively. Double reciprocal plots of the data of Figure 2 yield approximate K_m's of 2.0 and 21 mM for synthetase *a* and *b*, in close agreement with the values of Mersmann and Segal (1967). In the presence of 1.0 mM UDPTG (Figure 2,

curve b), UDPG reaction kinetics with glycogen synthetase *a* seem to be normalized, whereas no effect is seen on UDPG reaction kinetics with glycogen synthetase *b*. The data at low UDPG concentrations is presented with complete data points in Figure 3. When the data of Figure 3 are plotted in double reciprocal plots, complete linearity is seen in the presence of UDPTG, indicating that UDPTG gives rise to truly hyperbolic binding reaction kinetics for UDPG. The apparent K_m for UDPG in the presence of UDPTG is decreased from approximately 2.0 to 0.62 mM. Activation by UDPTG is cancelled at concentrations of UDPG which are saturating for synthetase *a* (Figures 2 and 3), suggesting that binding of UDPTG may occur at the UDPG sites. In summary, the data of Figures 2 and 3 suggest that activation of glycogen synthesis by low concentrations of UDPTG may be the result of activation of glycogen synthetase *a*. The data are consistent with an interaction of UDPTG at the cooperative UDPG sites of glycogen synthetase *a*.

To confirm that UDPTG, at the concentrations examined in this work, is not acting on glycogen synthetase *b*, rat liver glycogen synthetase *b* was purified by the method of McVerry and Kim (1974). The glycogen synthetase *b* preparation was highly dependent on D-glucose 6-phosphate; at 1 mM UDPG, specific activity was 12.5×10^3 units/mg of protein in the presence of 20 mM D-glucose 6-phosphate and 0.16×10^3 units/mg of protein in the absence of D-glucose 6-phosphate. The activity of the enzyme preparation, however, was the same in the presence or absence of 0.05–5.0 mM UDPTG. These observations support those obtained from Figure 2 supporting the suggestion that UDPTG does not significantly affect glycogen synthetase *b* activity at low concentrations. Higher concentrations of UDPTG were not examined due to the limited quantities of the analogue available.

Rabinowitz and Goldberg (1963) reported that, although UDPG inhibited the reaction of pseudo-UDPG with glycogen synthetase, the reverse inhibition could not be observed and, in fact, a stimulatory effect by pseudo-UDPG was suggested in several experiments. The results reported here with UDPTG suggest that the stimulatory effect these workers observed was real, and that the lack of inhibition of the reaction of UDPG by pseudo-UDPG was possibly due to a compensatory stimulatory effect. Stimulation of glycogen synthetase by other UDPG analogues has not been reported; even though a great number of analogues have been tested for substrate activity, few have been examined as effector molecules. The analogues studied with mammalian liver glycogen synthetase include pseudo-UDPG (Rabinowitz and Goldberg, 1963), UDP-galactose, GDP-mannose, and UDP-*N*-acetylglucosamine (Leloir and Cardini, 1962), UDP-3-deoxy-α-D-*ribo*-hexopyranose (Gabrieljan et al., 1971), and 5,6-dihydro-UDPG and 5-hydroxy-UDPG (Roy-Burman et al., 1968). If UDPTG causes its effects by direct interaction at the UDPG sites of glycogen synthetase *a*, it may prove to be valuable in examination of the nature of cooperative interactions of UDPG with glycogen synthetase in much the same way as substrate analogues of aspartate have proved useful in the understanding of aspartate transcarbamylase regulation (Jacobson and Stark, 1973).

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The Vitelline Envelope of Eggs from the Giant Keyhole Limpet *Megathura crenulata.* I: Chemical Composition and Structural Studies†

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ABSTRACT: The egg vitelline envelope of the marine invertebrate *Megathura crenulata* is a glycoprotein composed of 37.3 mol % protein and 62.7 mol % carbohydrate. Of the total amino acid content, 61 mol % consists of a single amino acid, threonine. The carbohydrate content includes galactosamine, galactose, and fucose. The molar ratio of threonine to galactosamine is about 1:1. Most of the threonine residues are linked to galactosamine residues via O-glycosidic bonds. A single peptide that was purified following alkaline borohydride treatment of the vitelline envelope had the structure: Abu-Pro-Abu-(Abu_6, Pro_1, Thr_1), where Abu is 2-aminobutyric acid. Several sugar residues have been isolated following the alkaline hydrolysis of the vitelline envelope that include an octasaccharide Gal_4Fu_4, an hexasaccharide Gal_3Fu_3, a trisaccharide Gal_3, fucose, and galactose. It is proposed that the vitelline envelope of *Megathura crenulata* eggs is composed of polypeptide chains built to a large extent of closely spaced threonine residues. Almost every threonine residue is linked to a saccharide moiety.

The egg of the marine invertebrate *Megathura crenulata* is surrounded by a thick jelly coat underneath of which lies the vitelline envelope. Both of these structures have to be penetrated by the spermatozoon to start the process of fertilization. Similar structures are present in eggs of other animals such as sea urchins (Runnstrom, 1966; Austin, 1968), amphibians (Wyrick et al., 1974), and mammals (Piko, 1969). The vitelline envelope and the mammalian zona pellucida contain sperm binding sites essential for fertilization since treatment of the envelopes with lectins or antibodies prevents sperm binding and blocks fertilization (Lallier, 1972; Aketa and Onitake, 1969; Oikawa et al., 1974; Shivers et al., 1972). Further, the vitelline envelope and the zona pellucida have been implicated in the block of polyspermy (Vacquier et al., 1973; Barros and Yanagimachi, 1971).

Although there is a wealth of information concerning the morphology of the vitelline envelope of various animals (Austin, 1968), little is known about the chemical composition and structure of this entity. Chemical studies have been conducted on the vitelline envelope of hen's egg (Bellairs et al., 1963) and the marine gastropod *Tegula pfeifferi* (Haino and Kigawa, 1966), both of which have been found to contain glycoproteins. The mammalian zona pellucida has also been characterized as glycoprotein (Lowenstein and Cohen, 1964), although the low abundance of mammalian eggs renders a detailed chemical study difficult.

This paper describes studies of the chemical composition of the vitelline envelope of the *Megathura crenulata* egg, its protein and carbohydrate composition, and the nature of the chemical link between these two components.

Experimental Section

Materials. Giant keyhole limpets (*Megathura crenulata*) were purchased from Pacific Bio-Marine, Venice, Calif., Dowex 50-X2 and Bio-Gel P-2 were from Bio-Rad Lab., Richmond, Calif., Sephadex G-10 was from Pharmacia, Piscataway, N.J., tetrazolium blue was from Sigma Chemical Co., St. Louis, Mo. *N*-Allyl-*N,N*-dimethylamine, 1-propanol, and trifluoroacetic acid used in the Edman degradation were sequanal grade from Pierce Chemical Co., Rockford, Ill. Phenyl isothiocyanate and benzene (Spectrar)

† Contribution No. 5128 from the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received July 28, 1975.* This research was supported by U.S. Public Health Service Grants No. NS 10294, GM 16424, and NS 12018 and a National Institutes of Health Career Development Award (M.A.R.).