ratio of dichloropyridazine and the sodium thiophenolate. The procedure was otherwise much as described in the case of thiosalicylic acid. A waxy, pale yellow solid was obtained as the crude product which separated during the reflux period. It was triturated well with ether and the creamy solid collected (A). The aqueous material and ether were retained (B). Solid A (9.4 g. from 7.5 g. of 3,6-dichloropyridazine; m.p. 138–140°) was crystallized from cyclohexane (800 cc.) to give feathery needles, m.p. 150–150.5° (5.6 g.). The liquors were retained for work-up. Analyses indicated that A was the bis compound (61% yield, based on 4-chlorothiophenol consumed, as evaluated).

Anal. Calcd. for $C_{16}H_{10}Cl_2N_2S_2$: Cl, 19.41; N, 7.67. Found: Cl, 19.51; N, 7.41.

The aqueous liquors (B) from the crude product were extracted well with ether, and these combined with the ether washings. Extraction of the ether (retained as C) with 10% caustic ultimately led to recovery of *ca.* 4% of 4-chlorothiophenol; C was washed with sodium chloride solution, dried and stripped of solvent, leaving a white solid which melted at 94–96° (6.0 g. from 7.5 g. of dichloropyridazine). Evaporation of the cyclohexane mother liquors from A left a white residue, m.p. 94.5–96° (3.6 g. from above run). The crude materials were combined and crystallized from pentane to yield fine white needles, m.p. 96.5–97.5°. This was 3-chloro-6-(4-chlorophenylthio)-pyridazine, a 38% yield being obtained.

Anal. Calcd. for $C_{10}H_6Cl_2N_2S$: N, 10.90; S, 12.47. Found: N, 10.73; S, 12.63.

When two equivalents of sodium 4-chlorothiophenolate were employed, the bis type was formed in 99% yield.

3,6-Bis-(3-diethylaminopropylthio)-pyridazine Bis-(4-nitrobenzobromide).—This quaternary salt was made by mixing the requisite base[1c] with 2.1 equivalents of 4-nitrobenzyl bromide and heating on the steam-bath (without solvent) for an hour. The crude product was pulverized, triturated with acetone, boiled in acetone–ethanol, and then crystallized twice from ethanol. A 62% yield of powder having an orange cast was obtained, m.p. 205–206.5° dec.

Anal. Calcd. for $C_{28}H_{46}Br_2N_6O_4S_2$: Br, 19.91; S, 7.99. Found: Br, 19.89; S, 8.20.

Acknowledgments.—It is a pleasure for the authors to make recognition of the friendly interest which Dr. C. M. Suter and (the late) Dr. J. S. Buck have shown in these researches, in addition to the generous support, given graciously.

RENSSELAER, N. Y.

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Enzymatic Syntheses of C^{14}-Labeled Uridine Diphosphoglucose, Galactose 1-Phosphate, and Uridine Diphosphogalactose[1]

BY E. P. ANDERSON,[2] ELIZABETH S. MAXWELL AND ROBERT MAIN BURTON

RECEIVED MAY 1, 1959

Methods are described in detail for the enzymatic syntheses of C^{14}-labeled uridine diphosphoglucose from glucose-C^{14}, and of labeled galactose 1-phosphate and uridine diphosphogalactose from galactose-C^{14}. The syntheses are feasible on a preparative scale, and essentially pure samples of the labeled uridine diphosphoglycosyl compounds can be isolated in good yield. In the synthesis from galactose, the intermediate galactose 1-phosphate can be isolated as the barium salt, or, in a somewhat different procedure, the over-all synthesis of uridine diphosphogalactose from free galactose can be carried out in a single incubation. Methods are also defined for the enzymatic synthesis, on a preparative scale, of C^{14}-labeled uridine diphosphoglucuronic acid.

Uridine diphosphoglucose (UDPG[3]), first discovered by Leloir and co-workers[4] as a coenzyme for the transformation of Gal-1-P to G-1-P,[3] has been shown to act as a glucosyl donor in a variety of enzymatic reactions for the biosynthesis of di- and polysaccharides.[5–9] UDPG also undergoes enzymatic conversions to UDPGal[10,11] and to UDPGA,[12] both of which have likewise been implicated as glycosyl donors in biosynthetic reactions.[13–16]

Because of the interest in having radioisotope-labeled uridine diphosphoglycosyl compounds for use in the exploration of such pathways of glycosyl transfer and as tools to assay for interconversions of the nucleotide compounds themselves,[17] enzymatic syntheses were undertaken to label these compounds with C^{14} in the carbohydrate portion of the molecule. Such syntheses have been developed for UDPG and UDPGal, starting in each case with a readily available radioactive substrate, and achieving synthesis on a preparative scale in good yield and with a high degree of purity. In

(1) A preliminary report on part of this work has already appeared; E. P. Anderson and H. M. Kalckar, Absts. Am. Chem. Soc. 5C, Minneapolis, September, 1955.

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(3) The following abbreviations are used: UDPG, uridine diphosphoglucose; UDPGal, uridine diphosphogalactose; UDPGA, uridine diphosphoglucuronic acid; Gal-1-P, α-D-galactose 1-phosphate; G-1-P, α-D-glucose 1-phosphate; G-6-P, glucose 6-phosphate; 6-PG, 6-phosphogluconate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; PP, inorganic pyrophosphate; P_i, inorganic orthophosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; *, C^{14}-labeled.

(4) R. Caputto, L. F. Leloir, C. E. Cardini and A. C. Paladini, *J. Biol. Chem.*, **184**, 333 (1950).

(5) E. Cabib and L. F. Leloir, *ibid.*, **231**, 259 (1958).

(6) C. E. Cardini, L. F. Leloir and J. Chiriboga, *ibid.*, **214**, 149 (1955).

(7) L. F. Leloir and C. E. Cardini, *ibid.*, **214**, 157 (1955).

(8) L. F. Leloir and C. E. Cardini, THIS JOURNAL, **79**, 6340 (1957).

(9) L. Glaser, *J. Biol. Chem.*, **232**, 627 (1958).

(10) L. F. Leloir, *Arch. Biochem. Biophys.*, **33**, 186 (1951).

(11) H. M. Kalckar, B. Braganca and A. Munch-Petersen, *Nature*, **172**, 1039 (1953).

(12) J. L. Strominger, E. S. Maxwell, J. Axelrod and H. M. Kalckar, *J. Biol. Chem.*, **224**, 79 (1957).

(13) J. E. Gander, W. E. Petersen and P. D. Boyer, *Arch. Biochem. Biophys.*, **60**, 259 (1956).

(14) R. M. Burton, M. A. Sodd and R. O. Brady, *J. Biol. Chem.*, **233**, 1053 (1958).

(15) L. Glaser and D. H. Brown, *Proc. Natl. Acad. Sci.*, **41**, 253 (1955).

(16) A. Markovitz, J. A. Cifonelli and A. Dorfman, *Biochim. et Biophys. Acta*, **28**, 453 (1958).

(17) H. M. Kalckar, E. P. Anderson and K. J. Isselbacher, *ibid.*, **20**, 262 (1956).

addition, methods are available for the enzymatic synthesis, on a preparative scale, of UDPGA-C.[14]

Experimental

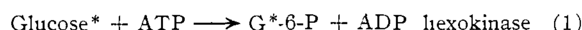
Materials.—Uniformly labeled glucose-C^{14} (0.473 μc./mg.) was obtained from Tracerlab, Inc. Glucose-6-C^{14} (2.22 μc./mg.) and galactose-1-C^{14} (2.13 μc./mg.) were prepared by the National Bureau of Standards. Crystalline ATP (disodium salt), DPN, TPN and UDPG were obtained from either Sigma Chemical Company or Pabst Laboratories. Crystalline hexokinase was also purchased from Pabst Laboratories. Crystalline phosphoglucomutase[18] was generously provided by Dr. Victor Najjar. Phosphopyruvate was a gift of Mr. William Pricer. Pyruvic phosphokinase[19] was kindly donated by Dr. Jerard Hurwitz and inorganic pyrophosphatase[20] by Dr. Russel Hilmoe.

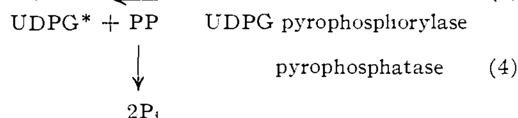
Yeast UDPG pyrophosphorylase was prepared by the method of Munch-Peterson, *et al.*,[21]; the cut representing 0.45–0.6 saturation in the second ammonium sulfate fractionation was stored in 0.6 saturated ammonium sulfate, which improved the stability of the enzyme. UDPG dehydrogenase was purified according to the procedure of Strominger, *et al.*,[12] and glucose-6-phosphate dehydrogenase according to the method of Kornberg and Horecker.[22]

Galactokinase was prepared by the method of Leloir and Trucco[23] from galactose-adapted *Saccharomyces fragilis*. The organism was adapted to galactose by several transfers through galactose-containing liquid media.[24,25] The galactose-adapted yeast was then grown in 10-liter batches using 2% galactose medium, a 10% inoculum, and constant vigorous aeration. The cells were harvested, after two days growth at 30°, with a Sharples continuous flow centrifuge, washed with water and air dried.

Liver Gal-1-P uridyl transferase was purified by the procedure of Kurahashi and Anderson[26]; preparation of the yeast fraction used in the alternative synthesis of UDPGal is described below.

UDPG and UDPGA.—UDPG-C^{14} was synthesized from free glucose-C^{14} by the following series of enzymatic reactions[3]





Free glucose was phosphorylated by ATP in the presence of hexokinase (reaction 1), the G-6-P formed was converted to G-1-P by phosphoglucomutase (reaction 2), and G-1-P was reacted with UTP in the presence of UDPG pyrophosphorylase to form UDPG and inorganic pyrophosphate (reaction 3[27]). The limiting step was apparently reaction 2, in which the equilibrium is distinctly unfavorable for the formation of G-1-P, and inorganic pyrophosphatase was used to pull the over-all reaction in the direction of UDPG synthesis by cleavage of the PP formed simultaneously (reaction 4).

Glucose-C^{14}, labeled either uniformly or specifically in carbon atom 6, was incubated together with hexokinase, ATP, an ATP-regenerating system consisting of phosphopyruvate and pyruvic phosphokinase, phosphoglucomutase and its cofactors, UTP, UDPG pyrophosphorylase and inorganic pyrophosphatase. UDPG formation was measured by assaying DPN reduction in the presence of UDPG dehydrogenase,[28] a highly specific and essentially quantitative method; yields of 80–95% UDPG from glucose could be achieved.

The UDPG-C^{14} formed was isolated by conventional methods, involving separation from non-nucleotide contaminants by adsorption on and elution from charcoal, followed by further purification with paper chromatography. The product so isolated was then assayed for radioactivity and, enzymatically, for UDPG content. The glucose-C^{14} was found to be incorporated without isotopic dilution. Yields up to 80% could be obtained with this isolation procedure (giving a very satisfactory over-all yield from glucose-C^{14} of 65–75%).

This method has been convenient for laboratory synthesis and has also proved feasible for the commercial preparation of UDPG.[29] Other satisfactory procedures for the biosynthesis of UDPG have also been described. The methods of Burma and Mortimer,[30] of Ganguli,[31] of Murthy and Hansen[32] and of Glaser[9] all also depend upon UDPG pyrophosphorylase; the first two utilize leaf homogenates of plant tissues (sugar beet and *Impatiens holstii*, respectively), while the last two procedures, like ours, use the yeast enzyme. In addition, an excellent chemical method[33] and an isolative procedure[34] are also available. Of these various workers, Burma and Mortimer,[30] Ganguli,[31] Murthy and Hansen[32] and Glaser[9] have also prepared the C^{14}-labeled compound. A mixture of UDPG-C^{14} and UDPGal-C^{14} has also been prepared enzymatically by crude extracts of *Saccharomyces fragilis*.[35]

In a separate reaction UDPG could be converted nearly quantitatively to UDPGA with the UDPG dehydrogenase. Details of this procedure have been described previously[12] for the preparative synthesis of unlabeled material and are fully applicable to the preparation of labeled UDPGA from UDPG-C^{14}.

Procedure for the Preparation of UDPG-C^{14}.—Various procedures were tried for carrying out the different enzymatic steps of the synthesis separately, but the simpler method of using a single incubation gave highly satisfactory results and was routinely used. Incubation of the reagents and enzymes on a small scale gave best yields; the reaction mixtures from several small vessels could then be pooled for product isolation on a preparative scale. A total volume of 10 ml. incubation mixture contained 5 μmoles of glucose-C^{14} (labeled uniformly or in carbon atom 6), 7.5 μmoles of ATP, 30 μmoles of phosphopyruvate and 10 μmoles of UTP. The reaction was run in 0.1 *M* tris-(hydroxymethyl)-aminomethane buffer, *p*H 7.5. Trace amounts of $MgCl_2$ (4 μmoles) and of glucose 1,6-diphosphate (0.008 μmole[36]) were added, together with an excess of cysteine (275 μmoles) and of each of the necessary enzymes, hexokinase, pyruvic phos-

(18) V. A. Najjar, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 294.

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(20) L. A. Heppel and R. J. Hilmoe, *ibid.*, **192**, 87 (1951).

(21) A. Munch-Petersen, H. M. Kalckar and E. E. B. Smith, *Kgl. Danske Videnskab. Selskab. Biol. Med.*, **22**, 3 (1955).

(22) A. Kornberg and B. L. Horecker, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 323.

(23) L. F. Leloir and R. E. Trucco, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 290.

(24) R. E. Trucco, R. Caputto, L. F. Leloir and N. Mittelman, *Arch. Biochem.*, **18**, 137 (1948).

(25) J. F. Wilkinson, *Biochem. J.*, **44**, 460 (1949).

(26) K. Kurahashi and E. P. Anderson, *Biochim. et Biophys. Acta*, **29**, 498 (1958).

(27) This pyrophosphorolytic cleavage of UDPG has been described and certain characteristics of the enzyme studied[21]; use of the reaction for the formation of UDPG from G-1-P has also been reported (E. P. Anderson, H. M. Kalckar and A. Munch-Petersen, *Pubbl. Staz. Zool. Napoli*, **29**, 119 (1957)). The reaction is specific for UTP, as distinguished from ATP or CTP,[1] and can also be used for the assay of UTP (H. M. Kalckar and E. P. Anderson, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Vol. III, Academic Press, Inc., New York, N. Y., 1957, p. 976).

(28) J. L. Strominger, E. S. Maxwell and H. M. Kalckar, in S. P. Colowick and N. O. Kaplan, "Methods in Enzymology," Vol. III, Academic Press, Inc., New York, N. Y., 1957, p. 974.

(29) By Pabst Laboratories, Milwaukee, Wisconsin.

(30) D. P. Burma and D. C. Mortimer, *Arch. Biochem. Biophys.*, **62**, 16 (1956).

(31) N. C. Ganguli, *J. Biol. Chem.*, **232**, 337 (1958).

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(33) J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **80**, 3756 (1958).

(34) H. G. Pontis, E. Cabib and L. F. Leloir, *Biochim. et Biophys. Acta*, **26**, 146 (1957).

(35) R. E. Trucco, *Nature*, **174**, 1103 (1954).

(36) The diphosphate in particular is needed in only minute amounts and may not be a necessary addition if larger quantities of the phosphoglucomutase are used.[18] The diphosphate used here was a generous gift from Dr. Hans Klenow.

phokinase, phosphoglucomutase, UDPG pyrophosphorylase and inorganic pyrophosphatase.

It was found necessary to free commercial hexokinase of the glucose in which it is prepared and supplied. To accomplish this the commercial preparation was fractionated with ammonium sulfate; the fractionation was carried out in 0.1 *M* phosphate buffer, *p*H 6.2, containing 1% glycine to stabilize the enzyme. The fraction precipitating between 0.5 and 0.75 saturation was dissolved in 0.1 *M* glycylglycine buffer, *p*H 7.5, and used in the preparative synthesis. With this preparation, combined with the other enzymes used, there was no synthesis of UDPG in the absence of added glucose. This ammonium sulfate cut from 8 mg. of the crystalline commercial preparation was used in the incubate.

Pyruvic phosphokinase was assayed by DPNH oxidation in the presence of lactic dehydrogenase and ADP; the quantity added to the incubate was capable of converting 28 μmoles of phosphopyruvate per hour. Phosphoglucomutase was assayed by TPN reduction with G-1-P and an excess of G-6-P dehydrogenase; the quantity used could convert 400 μmoles of G-1-P per hour. A quantity of the UDPG pyrophosphorylase preparation equivalent to 8.5 mg. of protein was used, and the amount of highly purified inorganic pyrophosphatase added was equivalent to 0.9 μg. of protein.

No particular attempt was made to modify these experimental conditions by adding more of the various enzymatic activities, and the reaction rate might have been increased by such modifications. The concentration of ATP was found to be quite critical, and yields (apparently in the hexokinase step) were somewhat decreased with higher concentrations of this nucleotide. A large excess of phosphopyruvate was crucial, but an increase beyond the level used here did not materially improve the yield. An excess of UTP was also necessary and could perhaps have been higher, although large quantities of the nucleotides seemed to inhibit the phosphoglucomutase (*cf.* 37). Phosphoglycerate and phosphocreatine were also tried as regenerating systems for ATP, but, under the conditions used, did not give yields comparable to those obtained with phosphopyruvate.

The reaction mixtures were all incubated at room temperature. Aliquots were removed at intervals and assayed for UDPG with UDPG dehydrogenase. When a satisfactory yield of UDPG had been formed (in these preparations yields were 80–95% after 8 hr. of incubation time). the reaction was stopped by deproteinization of the mixture with heat (80° for 2 min.), and the deproteinized incubate was cooled in ice and centrifuged. The supernatant fluids from two or more incubates (representing 10–15 μmoles UDPG) were pooled and treated batch-wise with acid-washed Darco KB[38] to adsorb nucleotides.[39] Adsorption was carried out at *p*H 2 and 0° for 20 min., with intermittent stirring of the Darco suspension; 12–15 mg. of Darco was added per μmole total nucleotide in the mixture. The Darco adsorption was found to be the most critical step of the isolation with respect to yield but if a *p*H of 2 was carefully observed, good yields could be achieved. After adsorption of the nucleotides, the Darco was washed twice with a volume of 0.001 *N* HCl equal to the volume of the adsorption mixture, and the nucleotides were eluted with twice this volume of ammoniacal 50% ethanol (1 ml. concentrated ammonia per liter). Elution was carried out at 0° for 30 min., again with intermittent stirring of the suspension. The Darco was then removed by high-speed centrifugation, and the eluate was evaporated with a stream of air and subjected to descending paper chromatography on Whatman #3 paper, using a solvent system of neutral ammonium acetate–ethanol.[40] The UDPG band was eluted with water and the eluate was again concentrated.

The content of UDPG in the final preparation was specifically determined by measuring the DPN reduction at 340 mμ in the presence of UDPG dehydrogenase.[28] Radioactivity was measured in a gas-flow proportional counter, and specific activity was calculated. In each case the specific activity of the UDPG-C^{14} was the same as that of the original glucose-C^{14}.

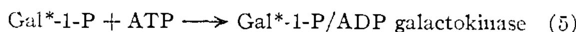
(37) H. Klenow, *Arch. Biochem. and Biophys.*, **58**, 288 (1955).

(38) Darco KB from Atlas Powder Company was treated batchwise with 1 *N* HCl in a single washing.

(39) R. K. Crane and F. Lipmann, *J. Biol. Chem.*, **201**, 235 (1953).

(40) A. C. Paladini and L. F. Leloir, *Biochem. J.*, **51**, 426 (1952).

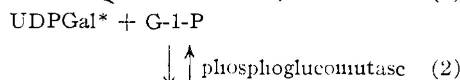
Gal-1-P and UDPGal.—Galactose-1-C^{14} 1-phosphate and UDPGal-C^{14} were prepared enzymatically from free galactose-1-C^{14} according to the reactions



Gal*-1-P + ATP ⟶ Gal*-1-P/ADP galactokinase (5)

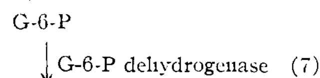
Gal*-1-P + UDPG ⇄ Gal-1-P uridyl transferase (6)

UDPGal* + G-1-P



↓↑ phosphoglucomutase (2)

G-6-P



↓ G-6-P dehydrogenase (7)

6-PG

Galactose was phosphorylated by ATP in the presence of galactokinase (reaction 5), and Gal-1-P was converted to the uridine nucleotide form with Gal-1-P uridyl transferase (reaction 6). Reaction 6 was pulled toward completion by conversion of G-1-P to G-6-P with phosphoglucomutase (reaction 2) and the further complete transformation of G-6-P to 6-PG with G-6-P dehydrogenase (reaction 7).

Galactose-1-C^{14} was incubated with ATP in the presence of appropriate cofactors and the labeled Gal-1-P formed could be isolated in 95% yield, as the alcohol-insoluble barium salt, or the crude deproteinized mixture could be used without purification for the preparation of UDPGal according to reaction 6. For this the mixture was incubated with UDPG, Gal-1-P uridyl transferase,[26] G-6-P dehydrogenase and TPN.

Since methods are not available for the separation of UDPG and UDPGal, the small amount of UDPG remaining after incubation was converted to UDPGA by the addition of DPN and UDPG dehydrogenase.[12] The nucleotides were then isolated by adsorption on charcoal, and UDPGal was separated from other nucleotides by paper chromatography. UDPGal was determined spectrophotometrically using UDPGal-4-epimerase, UDPG dehydrogenase, and DPN.[41] The over-all yield based on galactose was about 50%.

An alternative procedure for UDPGal formation has been used successfully with unlabeled galactose, and should be equally applicable to labeled material. This method employs the same reactions as that described above but can be done in a single step. An ammonium sulfate fraction of galactose-adapted yeast has been obtained which contains little, if any, of the interfering UDPGal-4-epimerase but large amounts of the necessary galactokinase, Gal-1-P uridyl transferase and phosphoglucomutase. It is therefore necessary to add only ATP, UDPG, TPN and G-6-P dehydrogenase for a complete system which will convert free galactose into UDPGal.

Procedure for the Preparation of Gal-1-P.—A mixture composed of 2000 μmoles of potassium phosphate buffer, *p*H 7, 400 μmoles of $MgCl_2$, 240 μmoles of ATP and 140 μmoles of galactose-1-C^{14} in a final volume of 15 ml. was adjusted to *p*H 7 with 2 *N* KOH. To this was added 0.6 ml. of the galactokinase preparation; with the assay procedure of Leloir and Trucco,[23] this quantity of enzyme was able to catalyze the esterification of 295 μmoles of galactose per hour. After 60 min. incubation at 30°, the mixture was heated in a boiling water bath for 1 min., cooled in ice, and centrifuged at 0° until the supernatant liquid was clear.

Gal-1-P was isolated as the barium salt. The supernatant liquid was adjusted to *p*H 8.3 with KOH, 2 ml. of saturated $Ba(OH)_2$ was added, and the precipitate which formed was removed by centrifugation. The barium salt of Gal-1-P was then precipitated from the supernatant liquid by the addition of four volumes (65 ml.) of cold absolute ethanol followed by chilling overnight at −4°. Most of the supernatant fluid was decanted, and the remainder was removed by centrifugation. The precipitate of Ba-Gal-1-P was washed with 15 ml. of cold 20% diethyl ether–80% ethanol and then with 15 ml. of cold anhydrous diethyl ether. The salt was dried over P_2O_5 under vacuum. The yield of Ba-Gal-1-P was 133 μmoles (95% of theory). This material was somewhat contaminated with adenine nucleotides.

Preparation of UDPGal from Gal-1-P.—The preparation of unlabeled UDPGal from Gal-1-P with liver Gal-1-P uridyl transferase has been reported[41]; in the method previously described a two to threefold excess of Gal-1-P was used in order

(41) E. Maxwell, *J. Biol. Chem.*, **229**, 139 (1957).

to obtain UDPGal essentially free of UDPG. The same method was used for preparation of labeled UDPGal from Gal(-1-C^{14})-1-P, but in this case the ratio of Gal-1-P to UDPG was reduced to nearly one, and residual UDPG was removed from the product. A typical reaction mixture consisted of the following ingredients, in a final volume of 40 ml. 0.1 *M* glycine, *p*H 8.9: 80 μmoles of UDPG, 200 μmoles of $MgCl_2$, 40 μmoles of cysteine, 100 μmoles of TPN, 5 mg. of lyophilized G-6-P dehydrogenase,[22] 150 mg. of lyophilized liver Gal-1-P uridyl transferase[26] and 12 ml. of the crude, heat-inactivated solution from reaction 5 containing 95 μmoles of Gal(-1-C^{14})-1-P. It was essential to inactivate the Gal-1-P incubation mixture before proceeding with the conversion to UDPGal, since the galactokinase preparation contained UDPGal-4-epimerase, which prevented the accumulation of UDPGal by conversion of most of this product to UDPG. It was not necessary to add phosphoglucomutase since the uridyl transferase preparation contained relatively large amounts of this enzyme. The course of the reaction could be followed by observing the increase in optical density at 340 mμ due to TPN reduction. Incubation of the reaction mixture was carried out at 37° for 1 hr. after which the mixture was assayed for UDPG and UDPGal with UDPG dehydrogenase and UDPGal-4-epimerase[42] and found to contain 10 μmoles of UDPG and 62 μmoles of UDPGal. After heating the reaction mixture at 100° for 2 min. 30 μmoles of DPN and a quantity of purified UDPG dehydrogenase[12] equivalent to 7 mg. of protein were added and the mixture was incubated for 30 min. at room temperature to convert UDPG to UDPGA. UDPGal was isolated as described for UDPG and assayed according to Maxwell.[41]

Alternative Procedure for the Preparation of UDPGal from Galactose.—This procedure used yeast as the source of Gal-1-P uridyl transferase and of phosphoglucomutase as well as of galactokinase. When the intermediate Gal-1-P is not desired, it has two advantages in that the necessary enzymes are easier to prepare and the reactions can be carried out in a single incubation. The procedure could be made practical for the preparation of UDPGal-C^{14} by decreasing the ratio of galactose to UDPG and removing residual UDPG from the product as described above.

The yeast fraction used was prepared from galactose-adapted *Saccharomyces fragilis* grown and harvested essentially as described above for the galactokinase preparation. In this case the medium consisted of 50 g. of galactose, 1.5 g. of yeast extract (Difco), 1.8 g. of $(NH_4)_2SO_4$ and 1.5 g. of KH_2PO_4 per liter of solution. The cells were grown at room temperature for 24 hr.; a yield of 250 g. of wet washed cells was obtained from 18 liters of medium. An extract was prepared by the procedure of Lamanna and Mallette.[43] The wet cell mass was placed in a Waring blendor containing 400 g. of Alconox-washed Superbrite glass beads (100–500 mμ diameter, #p-1091, from Minnesota Mining and Manufacturing Company) and 150 ml. of 0.1 *M* phosphate buffer, *p*H 7.0 and 0.005 *M* with respect to cysteine. Water (350 ml.) was added to fill the blendor, and homogenization was carried out at −10° for two successive 5 min. periods with intermediate cooling. The mixture was then centrifuged and 380 ml. of clear supernatant was obtained. Nucleic acid was removed by autolytic digestion[44]; 25 ml. of 1 *M* phosphate buffer, *p*H 7.0 was added to 320 ml. of the extract and the mixture was incubated at 37° for 2.5 hr. Ammonium sulfate fractionation was then carr[illegible] out at 2–5° by the addition of solid ammonium sulfate; fractions precipitating between 0 and 40%, 40 and 50%, 50 and 60%, and 60 and 70% saturation (at 0°) were collected. The last fraction (60–70%) contained the bulk of Gal-1-P uridyl transferase as well as sufficient galactokinase and phosphoglucomutase for the preparation of UDPGal; UDPGal-4-epimerase was located in the 40–50% fraction. The precipitate from 60–70% saturation was dissolved in 10 ml. water and lyophilized. The dry powder stored at −20° was stable for at least three months.

For the preparation of UDPGal, a typical reaction mixture consisted of the following materials in a final volume of 80 ml. of 0.1 *M* glycine, *p*H 8.9: 400 μmoles of galactose, 80 μmoles of UDPG, 100 μmoles of TPN, 160 μmoles of ATP, 400 μmoles of $MgCl_2$, 80 μmoles of cysteine, 10 mg. of lyophilized G-6-P dehydrogenase[22] and 100 mg. of the lyophilized yeast fraction described above. Incubation of the reaction mixture was carried out at 37° for 30 min. UDPGal was then isolated as already described; about 60 μmoles of UDPGal (75% of theory from UDPG), containing less than 5% UDPG, was obtained.

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Structural Influences on the Stability of Dipeptide–Metal Ion Complexes[1]

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Dissociation constants of glycylglycine, glycyl-L-alanine, glycyl-L-phenylalanine, glycyl-D-phenylalanine and L-phenylalanylglycine and stability constants of the complexes of these peptides with Co(II) and Cu(II) ions have been determined by potentiometric titration. Variations in the stability constants are discussed in relation to the structural differences in the peptides. Substitution on the carbon adjacent to the peptide nitrogen produces small changes, whereas substitution on the carbon adjacent to the terminal amino group leads to more pronounced changes in stability constants. Both electronic and steric effects are produced by the structural changes studied. The sensitivity of stability constant values to the dissociation constants employed in their calculation is pointed out.

Introduction

Many proteolytic enzymes are activated by metal ions,[3–5] but the mechanism of this activation is still obscure. Smith's suggestion[6,7] that an enzyme–metal ion–substrate complex is formed has been criticized[8–10] as inconsistent with the complex

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