Cell-Wall Synthesis in *Dictyostelium discoideum*. I. *In Vitro* Synthesis from Uridine Diphosphoglucose*

C. Ward and B. E. Wright

ABSTRACT: Uridine diphospho[^{14}C]glucose ([^{14}C]-UDPG) is incorporated into a 30% NaOH insoluble product from cell husk extracts prepared at the culmination stages of differentiation. The particulate enzyme preparation is activated by EDTA and is specific for [^{14}C]UDPG as substrate for cell-wall synthesis. Adenosine diphospho[^{14}C]glucose and guanosine diphospho-[^{14}C]glucose are incorporated at about one-tenth the rate of UDPG. The apparent K_M for UDPG in the synthesis of cell-wall polysaccharide is 1.3×10^{-3}. Glucose 6-phosphate and Mg^{2+} stimulate cell-wall polymer synthesis. The alkali-insoluble cell-wall material has been separated into two fractions (A and B) by solution in Schweizer's reagent. Chemical, enzymatic, and chromatographic analyses have identified fraction B as an α-D-1,4-linked polymer, and fraction A as cellulose. Analyses of radioactive material revealed that most of the radioactivity is incorporated into fraction B, and that fraction A is contaminated with the α-D-1,4-linked polymer.

The slime mold, *Dictyostelium discoideum*, undergoes a differentiation process which terminates in a period of rapid cell-wall formation during construction of a fruiting body or sorocarp. Cellulose has been identified as one component of the stalk and spore cells (Raper and Fennell, 1952; Mühlethaler, 1956; Gezelius and Rånby, 1957). We have found that an α-D-1,4-linked polymer is also present in the cell wall, in intimate association with the cellulose. The ultimate purpose of the present investigation is to determine why these cell-wall polysaccharides accumulate only during sorocarp construction, *i.e.*, what factor(s) control the initiation of this process. This paper describes the synthesis *in vitro* of cell-wall material from [^{14}C]UDPG.[1] UDPG has been shown to be a precursor of cellulose in *Acetobacter xylinium* (Glaser, 1958), and serves as a substrate for α-1,4-linked polymers in a number of systems (for a review, see Leloir and Cardini, 1962). Previous studies have shown that UDPG accumulates maximally in the cells at culmination, just prior to cell-wall construction (Wright, 1964).

Materials and Methods

Preparation of [^{14}C]*UDPG.* [^{14}C]UDPG was prepared by modifications of the method used by Glaser (1958); α-D-[U-^{14}C]glucose (New England Nuclear Corp., 200 mc/mmole), ATP (Pabst Laboratories), hexokinase, and mutase (Sigma Chemical Co.) were incubated to yield [^{14}C]glucose phosphates (G-1-P and G-6-P). Following heat inactivation, further incubation of the [^{14}C]G-1-P with UTP (Pabst Laboratories) and UDPG yeast pyrophosphorylase (Munch-Peterson *et al.*, 1953) added to the same reaction vessel yielded [^{14}C]UDPG labeled in the glucose moiety. A 59% over-all yield from [^{14}C]glucose was obtained. The [^{14}C]UDPG was separated by fractionation on a Dowex 1 (formate) column with gradient elution. The reservoir contained 4 N formic acid, which fed into a mixing flask containing water. Following elution of [^{14}C]G-6-P, the reservoir was made 4 N with respect to both ammonium formate and formic acid. The formic acid was extracted five times with an equal volume of ether, and the solution was chromatographed in ethanol–ammonium acetate, pH 7.5; 2.4:1. No charcoal absorption step was used, owing to losses in yield of UDPG.

[^{14}C]G-6-P was obtained during synthesis of [^{14}C]-UDPG; UDP-[^{14}C]Gal and [^{14}C]ADPG were obtained through the courtesy of Dr. H. Nikaido; [^{14}C]GDPG was a gift from Dr. W. Z. Hassid (Elbein *et al.*, 1964). Cellulose powder (ashless) was obtained from W. and R. Balston, Ltd., D-cellobiose from Sigma Chemical Co., glycogen from Calbiochem, preparations of α-amylase (highest purity from hog pancreas) from Sigma Chemical Co., maltase from Koch-Light Laboratories, Ltd., and α-glucan phosphorylase from Worthington Biochemical Corp. A cellulase preparation from *Streptomyces* sp. QM B814 was a gift from Dr. E. T. Reese, and a preparation of amylo-1,6-glucosidase was kindly provided by Dr. E. Bueding.

α-Amylase was assayed according to the conditions described by Worthington (1963), cellulase according

* From the John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University, at the Massachusetts General Hospital, Boston. *Received May 28, 1965; revised August 4, 1965.* This work was supported by a grant (GM 08958–04) from the U. S. Public Health Service. This is publication No. 1224 of the Cancer Commission of Harvard University. Preliminary reports of this work have been presented (Wright and Ward, 1965).

[1] Abbreviations used in this work: UDPG, ADPG, and GDPG, the diphosphoglucoses of uridine, adenosine, and guanosine, respectively; ATP, UTP, the 5′-triphosphates of adenosine and uridine, respectively; UDP, uridine 5′-diphosphate; G-1-P, G-6-P, glucose 1- and 6-phosphate, respectively; UDP-Gal, uridine diphosphogalactose.

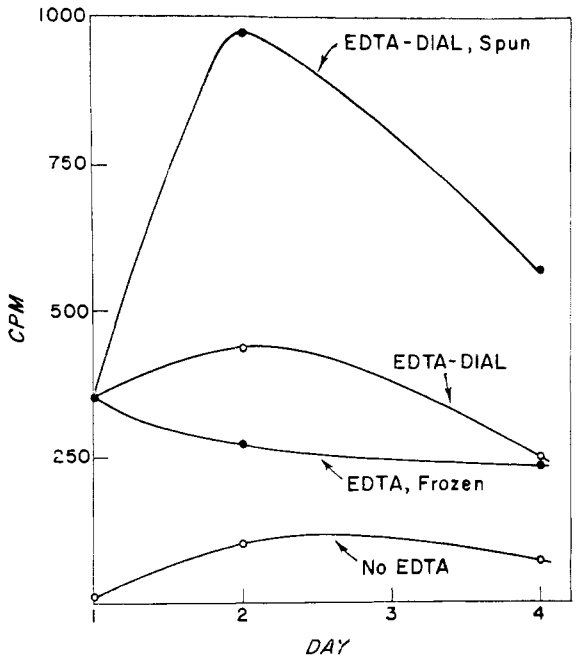

FIGURE 1: Effect of EDTA on enzyme activation. Enzyme (2.6 mg) was incubated with [^{14}C]UDPG (2×10^4 cpm), 0.1 M Tris, pH 8.4, and EDTA as indicated in the text. The solution used for dialysis was 0.1 M EDTA and 0.2 M Tris, pH 8.4. For further details, see text.

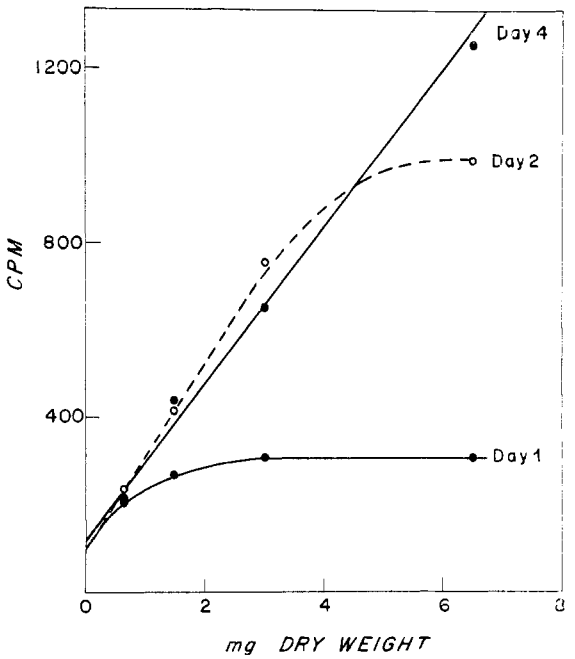

FIGURE 2: Effect of enzyme concentration on enzyme activation. The enzyme was prepared and assayed on day 1, then dialyzed overnight against a solution 0.2 M in Tris, pH 8.5, and 0.1 M in EDTA; it was then spun and resuspended in the same buffer prior to assay on day 2. The enzyme was aged at 5° until day 4. For assay conditions, see Figure 1.

to Reese *et al.* (1959), and amylo-1,6-glucosidase according to Bueding and Hawkins (1964). Phosphorylase was assayed according to the method of Hestrin (1949) with the modification that residual polysaccharide was precipitated by two volumes of methanol and G-1-P in the supernatant fluid was determined by the phenol–H_2SO_4 reagent (Dubois *et al.*, 1956). Contamination by unprecipitated polysaccharide was consistently less than 1%.

Reducing sugar on paper chromatograms was detected by an alkaline $AgNO_3$ reagent (Trevelyan *et al.*, 1950).

Separation of Two Polysaccharide Fractions. When a separation of the two polysaccharides in the alkali-insoluble husk fraction was desired, the following procedure was used. The alkali-insoluble material was dissolved in 1 ml of a cuprammonium hydroxide solution ("Schweizer's reagent") with 13.5 g of copper per liter final concentration (Whistler, 1963). Fraction A was regenerated by the addition of 3 ml of cold water and glacial acetic acid to pH 6.0, and the precipitate was washed extensively with cold distilled water. To the supernatant fluid was added 95% ethanol (1.2 volumes EtOH/volume). Following a brief heating at 100° fraction B was allowed to flocculate out of solution in the cold. This fraction was purified by repeated dissolution in water and reprecipitation by ethanol. The iodine test for glycogen or starchlike materials was performed using a solution of 0.2% I_2 and 0.4% KI (Larner, 1955).

Preparation of Enzyme. D. discoideum was grown on a rich medium in the presence of *Escherichia coli* and then replated on 2% agar plates (Liddel and Wright, 1961) containing potassium phosphate buffer, pH 6.5, 0.01 M, and EDTA, 0.001 M. Unless specified otherwise, the cells were harvested in 0.1 M EDTA–0.2 M Tris, pH 8.5, buffer and ruptured by passage through a French pressure cell (30,000 psi). It was necessary to wash the cells with EDTA–Tris buffer (two to four times), prior to rupture, and to wash the husk fractions (two to four times) as well, in order to remove an inhibitor. Both cells and husk fractions were centrifuged at $1500 \times g$ for 10 minutes. Prior to enzyme assay, an aliquot (usually 0.5 ml) was transferred to a small tube, centrifuged, and washed with water in order to obtain a dry-weight determination.

Assay of Enzyme Activity. A typical reaction mixture contained in 0.2 ml total volume: 10–20 μmoles EDTA, 20 μmoles Tris, pH 8.5, 0.01 μmole [^{14}C]UDPG (2×10^6 cpm/μmole), and enzyme (approximately 1 mg). Incubation was at 37° for 90 minutes. The reaction was stopped by the addition of 1 ml of a suspension of cellulose (10 mg/ml) and boiling for 5 minutes. The insoluble material was routinely washed three times with 10 ml of water, boiled 10 minutes in 3 ml of 1% NaOH (see Table IV), and washed two or more times, and radioactivity was determined in a Tri-Carb liquid scintillation spectrometer (Gordon and Wolfe, 1960). The pH optimum was broad, centering around 8.5. No incorpora-

tion has ever been observed in the presence of boiled enzyme.

Results

Characteristics of Enzyme Prepared at Culminating Stages of Differentiation

Activation and Stabilization by EDTA. EDTA at a concentration of 10^{-4} M has a slight stabilizing effect on the enzyme, but is virtually ineffective compared to 0.1 M EDTA; at the latter concentration the enzyme is not only stabilized but often is activated in a striking manner. Figure 1 illustrates the activity over a 4-day period of a husk preparation from culminating cells in the presence and absence of 0.1 M EDTA under various conditions. By dialyzing the preparation overnight against 0.1 M EDTA and removing by centrifugation the dialyzed supernatant, the activity is 10-fold higher than in an undialyzed preparation containing no EDTA. This enzyme preparation was maximally activated on day 2 and had lost considerable activity by day 4 at 5°. Comparable activation can also be achieved by aging diluted enzyme overnight in the presence of 0.1 M EDTA. Figure 2 demonstrates that enzyme activity is linear with enzyme concentration after a preparation has become fully activated. The husk preparation used in this study was not fully activated until the fourth day of aging at 5°. The time of maximal activation is unpredictable from one preparation to the next, and varies from the first to the fourth day. EDTA (0.1 M) stabilizes as well as activates the enzyme, whether or not glass-distilled water is used in preparing all solutions with which the enzyme comes in contact. The extent of activation is somewhat less, however, with the double-distilled water. Glycylglycine, citrate, and 8-hydroxyquinoline are not effective in replacing EDTA. Cu^{2+} is somewhat inhibitory at 10^{-6} M to the synthesis of cell-wall polysaccharides.

Substrate Specificity. Possible precursors other than UDPG were examined for their ability to be incorporated into the alkali-insoluble fraction. As Table I indicates, the reaction is specific for UDPG.

Dependence of Reaction on UDPG Concentration. Prior to the determination of an apparent K_M, an experiment was carried out with a well-washed particulate preparation to determine how much UDPG was left at the end of the incubation period. At two concentrations of [^{14}C]UDPG, the amount remaining after incubation was measured by chromatography of an aliquot of the supernatant followed by elution of the area at the R_F of UDPG. The μmoles of [^{14}C]glucose incorporated into the alkali-insoluble fraction during incubation were also determined. As Table II indicates, the UDPG level did not decrease significantly during the incubation period. At 0.4 μmole of [^{14}C]UDPG there appears to be a small fraction of the substrate used for reactions other than alkali-insoluble polysaccharides.

The dependence of cell-wall polysaccharide synthesis on UDPG concentration was then determined for cell-husk preparations as well as for intact cells (Figure 3). A lower UDPG level is required for comparable activity of cell-wall preparations than of intact cells. Half-maximal activity for the husk preparations is at 1.3×10^{-3} M, and for the intact cells is at 4.0×10^{-3} M UDPG. Apparently, either the enzyme is on the surface of the intact cell or UDPG (or breakdown product) can readily permeate the cell. Based on dry weight, intact cells are about as active as ruptured cells in catalyzing the incorporation of glucose (as [^{14}C]UDPG) into alkali-insoluble polysaccharides.

Stimulation of Reaction by G-6-P and Mg^{2+}. G-6-P was found consistently to stimulate incorporation into the cell-wall fraction (Table III). Mg^{2+} also can stimu-

TABLE I: Substrate Specificity.[a]

Experiment	Substrate	μMole	Glucose Incorporated into Product (μmoles × 10^3)
I	UDPG	1.0	39.0
	G-6-P	1.0	0
	Glucose	1.0	0
	UDP-Gal	1.0	0
II	UDPG	0.2	5.6
	GDPG	0.2	0.5
	ADPG	0.2	0.3

[a] Each reaction mixture contained in 0.2 ml total volume: 10 μmoles EDTA, 20 μmoles Tris, pH 8.5, radioactive substrate as indicated, and enzyme (approximately 1 mg). The specific radioactivity of each substrate was approximately 10,000 cpm/μmole in experiment I and 200,000 cpm/μmole in experiment II.

TABLE II: UDPG Levels during Polymer Synthesis *in Vitro*.[a]

[^{14}C]UDPG (μmoles)		[^{14}C]Glucose in Alkali-Insoluble Material (μmole)
Before Incubation	After Incubation	
0.20	0.188	0.008
0.40	0.364	0.017

[a] UDPG was identified by descending chromatography in ethanol–ammonium acetate, pH 7.5, 2.4:1. For further details see text.

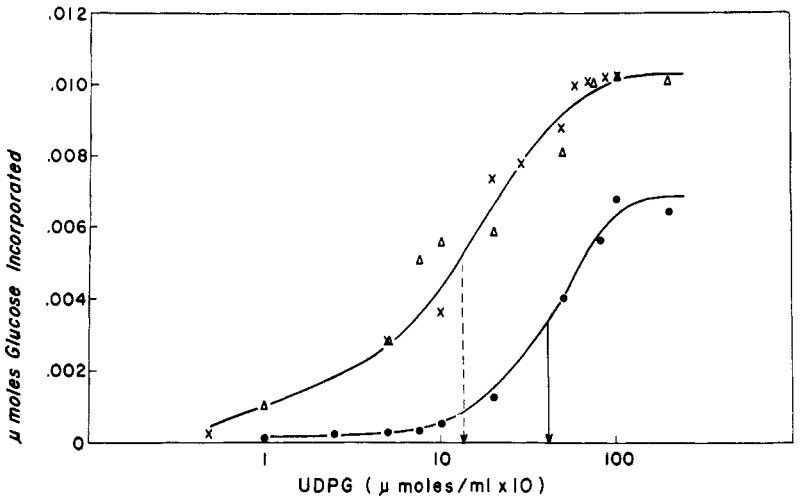

FIGURE 3: Enzyme activity as a function of UDPG concentration in intact cells (circles) and in husk preparations obtained by freezing (triangles) or by passage through a French pressure cell (crosses). For assay conditions, see Methods.

TABLE III: Stimulation of Polymer Synthesis by G-6-P and Mg^{2+}.[a]

Additions	Alkali-Insoluble Material (cpm)	
	Day 1	Day 2
None	261	485
G-6-P	973	2158
$MgCl_2$	309	603
G-6-P + $MgCl_2$	1508	2317

[a] The usual incubation conditions were used. G-6-P was present in a final concentration of 2.5×10^{-3} M and $MgCl_2$ at 10^{-3} M.

late, but the effect was not consistent and the extent of the stimulation was variable. In this enzyme preparation, the stimulating effect of these two substances was similar before and after "activation" of the enzyme.

Characteristics of Cell-Wall Material

Solubility and Stability of Enzymatic Product. The enzymatic product was subjected to various treatments with hot water, alkali, and acid. Substituting hot water for the hot 1% NaOH step gave somewhat higher counts; boiling in 5% or 30% NaOH for 10 minutes or in 1% NaOH for 20 hours did not reduce the total counts (Table IV). This latter treatment was the most vigorous used by Gezelius and Rånby (1957) in purifying *D. discoideum* stalk material prior to X-ray diffraction analysis. Complete acid hydrolysis (6 N HCl, 4 hours at 23°) of the radioactive, alkali-insoluble husk fraction gave only [^{14}C]glucose, in confirmation of the results of Gezelius and Rånby (1957) in analyzing nonradioactive material.

Fractionation of Nonradioactive Cell-Wall Material. Significant amounts of alkali-insoluble material began to accumulate only at the terminal stages of differentiation. At this time, slime mold material was harvested in gram quantities, treated three times with hot 30% NaOH, and washed extensively between each treatment. Solubilization of cell-wall material in Schweizer's reagent usually leaves a small residue; the combined yield (fraction A + B, see Methods) is about 50%, with equal amounts in each fraction. Fraction A is water- and alkali-insoluble, and does not stain with iodine. Treatment of this fraction and cotton cellulose with *Streptomyces* cellulase revealed cellobiose and glucose as major hydrolysis products. Cellobiose was identified chromatographically under conditions in which it separates from maltose (descending, 44 hours at 23° in butanol–pyridine–water, 6:4:3). Trehalose, laminarobiose, gentiobiose, and isomaltose separate well in this system. Treatment of fraction A with crystalline α-amylase and phosphorylase *a* gave no release of soluble reducing sugar.

Fraction B is water soluble, stains reddish-brown to iodine, and exhibits a similar absorption spectrum to the glycogen–iodine complex. The extent of hydrolysis of this fraction (as compared to oyster glycogen) by phosphorylase *a* and amylo-1,6-glucosidase is shown in Table VI. A detailed study with phosphorylase *a* revealed that the kinetics of hydrolysis of known glycogen and of fraction B were very similar, with the maximum rate occurring in the first hour of incubation. On obtaining a limit dextrin (38–41% hydrolysis) from phosphorylase *a* treatment of known glycogen and fraction B, the addition of fresh enzyme gave less than a 10% increase in hydrolysis, whereas addition of the amylo-1,6-glucosidase gave complete hydrolysis. Treat-

TABLE IV: Solubility and Stability of Enzymatic Product.[a]

Expt		Total cpm
I	Boiled 10 min, water, no NaOH	505
	Boiled 10 min, 1% NaOH	354
	Boiled 10 min, 5% NaOH	410
	Boiled 10 min, 1% NaOH + 2 hr, N H_2SO_4	6
	Boiled 10 min, 5% NaOH + 2 hr, 2.5 N H_2SO_4	10
II	Boiled 10 min, 5% NaOH	900
	Boiled 20 hr, 1% NaOH	947
	Boiled 20 hr, 1% NaOH + 2 hr, 2.5 N H_2SO_4	55
III	Boiled 10 min, water, no NaOH	1584
	Boiled 10 min, 30% NaOH	1494

[a] Following the usual conditions of incubation and assay (see Methods), the radioactive, alkali-insoluble material was treated as indicated and washed with water to neutral pH, and radioactivity was determined.

ment of fraction B and known glycogen by α-amylase, followed by chromatography, revealed the presence of maltose and maltotriose as the major hydrolysis products. Maltose was identified by its conversion to glucose by maltase, and by its R_F compared to authentic maltose, under the conditions described above for the identification of cellobiose.

Analysis of Radioactive Cell-Wall Material. In order to compare the radioactive, enzymatically synthesized product to the cell-wall polysaccharides just described, a similar fractionation in Schweizer's reagent was carried out using alkali-insoluble material synthesized *in vitro* from [^{14}C]UDPG (Table V). The over-all yield as measured in cpm is usually higher than in nonradioactive fractionations, measured by weight, suggesting that the addition of carrier cellulose and glycogen in the former case improves the quantitative precipitation of these fractions. Fraction B contains significantly more radioactivity than fraction A, in spite of the fact that the cell-wall material contains approximately equal amounts of each fraction by weight.

Phosphohydrolysis of radioactive fraction B by phosphorylase *a* shows similar kinetics to known glycogen and nonradioactive fraction B, but 100% solubilization of radioactivity (following precipitation of unhydrolyzed sugar) was obtained (see Table VI). Ninety-six per cent solubilization of radioactivity from this fraction is also obtained following α-amylase treatment. These results suggest no *in vitro* synthesis of α-1,6-linkages. α-Amylase treatment of radioactive fraction B produces maltose and maltotriose. Radioactive maltose was identified by paper chromatography and by its quantitative conversion to [^{14}C]glucose by maltase (which is not active on cellobiose).

Radioactive fraction A was degraded by *Streptomyces* cellulase, and in addition was hydrolyzed about 70% by phosphorylase *a* and α-amylase (in contrast to the results with nonradioactive fraction A; *vide supra*). Treatment of this fraction with a mixture of cellulase and amylase gave disaccharide 80% of which was converted to [^{14}C]glucose by maltase. The remaining 20% is presumably cellobiose, which is not attacked by maltase. A sufficient amount of this material is not yet available for further analysis.

Discussion

The data presented suggest that systems in which alkali-insoluble glycogen fractions have been reported (Trevelyan and Harrison, 1956) may involve a complex structure partially composed of an insoluble cellulose-

TABLE V: Fractionation of Radioactive Cell-Wall Material with Schweizer's Reagent.[a]

Experiment	Original	Undissolved Residue	Total cpm Fracn A	Total cpm Fracn B (soluble)	Total cpm Fracn C	Per Cent Total Recovery
I	2,140	132	400	1,267		85
II	138,000	200	2,770	70,000	10,000	60
III	128,900		19,790	68,620	[b]	68
IV	130,345	8,310	8,074	77,850	3,770	82

[a] The fractionation procedure is described under Methods; approximately 5 mg of carrier cellulose and glycogen was added prior to the precipitation of fractions A and B, respectively. [b] Not determined.

TABLE VI: Hydrolysis of Fractions A and B by α-1,4-Phosphorylase and Amylo-1,6-glucosidase.[a]

Sample	Enzyme Treatment: α-1,4-Phosphorylase	Enzyme Treatment: Amylo-1,6-glucosidase	Per Cent Hydrolysis
Oyster glycogen[b]	Present	Absent	38
Oyster glycogen[b]	Present	Present	100
Nonradioactive fracn B[c]	Present	Absent	41
Nonradioactive fracn B[c]	Present	Present	90–100
Radioactive fracn B[d]	Present	Absent	100
Radioactive fracn A[d]	Present	Absent	60–80

[a] See Methods for enzyme assays. [b] 2.0 mg/ml; hydrolysis based on G-1-P formation. [c] 1.2 mg/ml; hydrolysis based on G-1-P formation. [d] Hydrolysis based on release of soluble counts after precipitation of unhydrolyzed sugar after 24-hr incubation.

like material. In one other organism, the mollusk, a fraction containing a sulfated complex of cellulose and amylose, has been reported (Egami *et al.*, 1955). Here, also, the fractions were separated by an initial solubilization in Schweizer's reagent; one was characterized as a type of cellulose and the other as an amyloselike polymer. A small amount of cellobiose was also present in the amylose fraction.

Attempts to stimulate incorporation into the cell-husk fraction by "primer" materials such as cellodextrins, glycogen, and cell-wall preparations have revealed occasional but not reproducible effects. Although cell-wall material contains approximately equal amounts of the cellulose and glycogenlike polymers, synthesis of the latter from [^{14}C]UDPG occurs preferentially *in vitro*. This discrepancy could be due to a number of factors, such as the relatively low concentration or activity of some enzyme involved in cellulose synthesis.

The effect of EDTA in activating the enzyme is not yet understood. It is of interest that a UDP-glucuronyl transferase is also stabilized and activated by EDTA (Halac and Bonevardi, 1963). It has not yet been possible to solubilize and further purify the cell wall–synthesizing enzyme system. However, since the UDPG level does not change significantly during polysaccharide synthesis in the presence of a well-washed particulate fraction, it appears justified to consider 1.3×10^{-3} to be the approximate K_m for UDPG in the synthesis of the α-1,4-linked polymer. During differentiation in the slime mold, the intracellular concentration of UDPG is well below 10^{-3} M except in culminating cells, which are rapidly accumulating cell-wall polysaccharides (Wright *et al.*, 1964). Assuming the UDPG values determined approximate the concentration available to the enzyme *in vivo*, it would appear that the UDPG level may be one limiting factor for the initiation of cell-wall synthesis in the differentiating cell. Conversely, the depletion of UDPG, which occurs very rapidly during sorocarp construction, could of course be an important causal factor in the termination of polysaccharide synthesis.

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Nature of the Insoluble Pigmented Structures (Chromatophores) in Extracts and Lysates of *Rhodopseudomonas spheroides**

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ABSTRACT: When extracts of anaerobically grown *Rhodopseudomonas spheroides*, made with a French press, were centrifuged in a linear gradient of cesium chloride the great majority of the pigment was found in a single narrow band. Four other protein-containing bands could be identified. Extracts of aerobic cells contained the same bands apart from the pigmented one. Examination of anaerobic extracts by moving boundary sedimentation showed two types of pigmented particle whose $s_{20,w}$ values were 160 S and 55 S and whose densities were 1.17–1.18. The larger particle contained 85% or more of the pigment. An estimate of the polydispersity of the 160 S particles showed that the standard deviation of the distribution of their diameters was equal to 9–12% of its mean. From these results and others [K. D. Gibson, 1965c, *J. Bacteriol.* (in press)] the particles were calculated to consist of about 50% water. Anaerobic cells were converted to spheroplasts and lysed by various methods. Most of the pigment remained attached to large fragments, but a small amount was released in the form of 160 S particles. Application of shearing forces liberated more pigmented particles. The results were consistent with the view that the pigmented structure is particulate *in vivo*.

Schachman *et al.* (1952) found that extracts of anaerobically grown *Rhodospirillum rubrum*, when observed in the analytical ultracentrifuge, contained particles whose $s_{20,w}$ value was 155 S, with which nearly all the photosynthetic pigment was associated. They named these particles chromatophores. The remainder of the pigment was apparently contained in smaller particles whose $s_{20,w}$ value was 55 S. The two fractions seemed to be fairly homogeneous, and it was possible to isolate some material corresponding to the larger particles by differential centrifugation. In the next few years a number of reports appeared describing the isolation of pigmented particles from extracts of *Chromatium* (Newton and Newton, 1957; Bergeron, 1958), *Chlorobium thiosulfatophilum* (Bergeron and Fuller, 1961), *R. rubrum* (Frenkel and Hickman, 1959; Cohen-Bazire and Kunisawa, 1960), and *Rhodopseudomonas spheroides* (Bull and Lascelles, 1963; Worden and Sistrom, 1964). In these studies the cells were ruptured by sonication, grinding with abrasives, or by passage through the Hughes press or French press. The isolated particles were for the most part active in catalyzing photophosphorylation (Frenkel, 1954) and certain photooxidations and reductions (Vernon and Ash, 1959), and their composition was fairly similar in all these species. The structures in the cell from which the pigmented particles arose were identified in electron micrographs of thin sections of whole cells of *Chromatium*, *R. rubrum*, and *Rps. spheroides* as rather uniform electron-lucent vesicles bounded by electron-dense membranes, which are distributed in clumps throughout the interior of the cell, almost filling it in extreme cases (Vatter and Wolfe, 1958; Bergeron, 1958; Hickman and Frenkel, 1959; Cohen-Bazire and Kunisawa, 1963; Cohen-Bazire, 1963).

However, there were some observations which suggest that the pigmented structure is not particulate *in vivo*, but consists of a reticulum which becomes comminuted during mechanical rupture of the cell in a manner analogous to the conversion of the reticular endothelium to microsomes. The pigment did not seem to be in a single subcellular fraction but could be found in at least two types of particle (Newton and Newton, 1957; Cohen-Bazire and Kunisawa, 1960). These were named "heavy" and "light" chromatophores. It appeared that the "heavy" fraction consisted of "light" particles attached to other components of the cell, indicating that *in vivo* the particles formed part of a larger structure. More recently it was observed that the membrane-bound vesicles which are seen in electron micro-

* From the Department of Chemical Pathology, St. Mary's Hospital Medical School, London W. 2. *Received May 18, 1965.* Part of this work was presented at the Sixth International Congress of Biochemistry at New York (Gibson, 1964a).

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