An Enzymically Catalysed Reaction Between D-Glucose and the Protein Cyst Coat of the Ciliate *Colpoda steinii*

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1. The isolated protein cyst coat of *Colpoda steinii* reacted with [^{14}C]glucose to bind ^{14}C label in a reaction that was not an artifact of bacterial contamination or of adsorption. 2. The reaction was enzymically catalysed, had optimum pH 7.0-7.4 and a temperature optimum of 36-38°C, was non-competitively and reversibly inhibited by 10 μM -*p*-hydroxymercuribenzoate and was specific for D-glucose. It had K_m $8.2 \times 10^{-6}\text{M}$. 3. With the exception of C-1, all the carbon atoms of glucose were bound to the same extent and, measured relative to C-2 or C-6, the binding of C-1 varied between 0.45 and 0.82. The lost C-1 was not accounted for as carbon dioxide. 4. On prolonged incubation a coat preparation reacted with 3.6% of its own weight of glucose. 5. The label was tightly bound, but after acid treatment a variable proportion was recovered as glucose and there was no evidence for the release of any other ^{14}C -containing compound. 6. Even after dissolution of the coat protein, bound label was not removed by treatment with periodate or lead tetra-acetate.

Starvation of *Colpoda steinii* causes the cell to encyst, it thus becoming an enclosed metabolic system that presumably depends on its intracellular reserves for survival. As part of the encystment process a coat, comprising mainly protein with a high glutamic acid content, is secreted (Tibbs, 1966). Unlike the situation in the cysts of some amoebae (Griffiths & Hughes, 1969; Tomlinson & Jones, 1962) this coat contains little, if any, carbohydrate. During experiments on the incorporation of [$\text{U-}^{14}\text{C}$]glucose into encysting cells it was found that isolated preparations of *C. steinii* cyst coat appeared to bind or incorporate substantial amounts of the label. This interaction, short accounts of some aspects of which have been published (Tibbs & Marshall, 1969a; Marshall & Tibbs, 1969), is enzymically catalysed and is the subject of this paper.

METHODS

Preparation of C. steinii cyst coat. Cysts, obtained by the method now preferred (Tibbs & Marshall, 1970) and present to the extent of about 10^6 cells/ml, were stored at 4°C and used between 1 and 7 days after encystment. Cells from 50 ml of suspension were concentrated by centrifugation and washed several times with water, and the sludge was shaken at 2°C with an equal volume of 1 mm-diam. glass beads in a Mickle high-speed shaker until the cells were all disrupted as seen under a phase-contrast microscope (2-3 min). Probably because of the presence of less cell debris in the sludge, this time was shorter than that used in earlier work (Tibbs, 1966), but

the subsequent technique for isolating the coat remained unchanged. The final preparation, consisting essentially of the outer layers of the cysts after removal of their cytoplasm, was suspended in about 3 ml of water, and 0.5 ml portions, containing about 1 mg dry wt. of coat, were used in the incubations with glucose.

Incubation of glucose with cyst-coat suspensions and measurement of radioactivity. Coat suspension (0.5 ml), glucose solution of known specific radioactivity and concentration (0.1 ml, usually about 30 μg , 16.7 $\mu\text{Ci/mg}$) and 5 ml of 0.1 M-tris-HCl buffer, pH 7.4, containing 100 i.u. each of benzylpenicillin, neomycin and streptomycin/ml, were incubated at 35°C in a tube that had been treated with dichlorodimethylsilane in carbon tetrachloride to prevent the wall sticking. Incubation time was 1 h unless otherwise stated. About 1 ml of 40% (w/v) trichloroacetic acid was then added, and the coat was filtered and collected quantitatively on a 25 mm glass-fibre filter supported in a Millipore holder. The filter was washed with 10% (w/v) trichloroacetic acid, water and finally ethanol-water (3:1, v/v), and then dried under an infra-red lamp before its radioactivity was counted. Solid counting of radioactivity in a Geiger counter with an efficiency of about 5% was used. Corrections were made for background, but for self-absorption were unnecessary; standard deviations were not greater than 2%. Counts were the same for cyst coat dried on 25 mm flat aluminium planchets and for cyst coat collected on glass-fibre filters. Planchets were used for measuring the radioactivity of glucose solutions.

Analyses. Glucose was determined by using glucose oxidase (Bergmeyer & Bernt, 1963). For a few assays in which it was necessary to know the amount of cyst coat collected on a filter the latter, after its radioactivity had been counted, was boiled for 18 h in 5.7 M-HCl, glass fibre was removed and the hydrolysate was evaporated to

dryness on a rotary evaporator in a partial vacuum. The amino N was then determined (Moore & Stein, 1948). Although somewhat lengthy, this procedure also provided hydrolysates suitable for chromatography. Periodate was measured by the Müller-Friedberger method (Guthrie, 1962).

Chromatographic procedures. To study sugars present in acid digests of coat labelled by reaction with [$U-^{14}C$]glucose the digests were deionized by passing down 1.5 cm-diam. mixed-bed columns made up of Amberlite IR-120 (H^+ form) and Amberlite IR-45 (OH^- form) (analytical grades, 10 g of moist beads of each resin) and the effluents were evaporated to dryness under reduced pressure. The weakly basic Amberlite IR-45 was used in preference to strongly basic exchangers which can interact with sugars (Roseman, Abeles & Dorfmann, 1952; Phillips & Pollard, 1953). After chromatography on silica gel G t.l.c. plates made up with 0.2 M-sodium acetate with ethyl acetate-propan-2-ol-acetic acid-water (20:12:7:6, by vol.) (Lato, Brunelli, Ciuffini & Mezzetti, 1969) and butan-1-ol-acetic acid-water (12:3:5, by vol.) as solvents, sugars were detected visually in the effluents with aniline and diphenylamine (Smith, 1960) and radioactivity was detected with a Panax scanner.

RESULTS

Possible incorporation into contaminating bacteria. The following experiments showed that the uptake of label by cyst-coat preparations was not due to incorporation into contaminating bacteria. Incubations with glucose were carried out in unbuffered solutions at 30°C for, at this stage of the work, the effect of neither pH nor temperature had been studied.

Coat, after incubation with [$U-^{14}C$]glucose for 2h in the absence of antibiotic, was washed, and the radioactivity of a sample was counted at this stage; that of a further sample was counted after overnight incubation in nutrient broth at 37°C followed by the removal of the bulk of the subsequent bacterial population by spinning down the coat and washing. In two such experiments 55% and 80% of the radioactivity incorporated into the coat preparations were still present in the coat after incubation in broth. The rather low and erratic recoveries were probably due to difficulties in handling the coat quantitatively after this treatment, but showed that the original label was associated with the coat and not bacteria. Had the latter been involved the label would have been diluted out into the bacteria, which grew on the overnight incubation.

Two samples of coat (about 2mg) in 5ml of water with 100 i.u. each of the three antibiotics/ml were incubated with 50mg of fructose for 18h at 30°C to induce fructose utilization by any bacteria present. The samples were then washed with water and reincubated with [$U-^{14}C$]glucose (1mg, 0.5 μ Ci) and antibiotics in a total volume of 5ml, 20mg of

fructose being included with the second sample. After 24h at 30°C the coat preparations were washed and suspended in water, and the radioactivities of portions of the suspensions were counted. Label equivalent to 77 μ g of glucose was incorporated into the whole sample in the first case and an amount equivalent to 59 μ g in the second. Had the incubation of glucose really been incorporation into contaminating bacteria, the presence of large amounts of fructose in the second sample should have drastically decreased the utilization of glucose. The labelled coat from this experiment was also assayed for viable bacteria by plating on 3.5% tryptose-blood agar (Difco). Although the complete samples contained label from 77 μ g of glucose and 59 μ g of glucose, they contained only about 2.5×10^6 bacterial cells or about 1 μ g of viable bacteria (Proper & Garver, 1966).

Possible influence of adsorption phenomena. Replicate samples of coat, after incubation with [$U-^{14}C$]glucose, were washed by centrifugation and filtered and their radioactivities counted either at this stage or after further incubation at 35°C with 1mg of unlabelled glucose in 5ml of buffered antibiotic solution. After further incubation for 18h 70% of the original radioactivity remained in the coat. Similarly after labelled coat had been boiled with 2.5mg of glucose in 5ml of water for 10min 78% of the radioactivity was still attached. Labelled coat of known specific radioactivity was dried and treated for 16h at 0°C with performic acid before being freeze-dried and extracted with 0.1 M-sodium hydrogen carbonate (Tibbs, 1966). The extract, containing the bulk of the coat protein, after being dialysed and freeze-dried, was dissolved in a little water and its specific radioactivity determined by counting the radioactivity of a portion dried on a

Fig. 1. Effect of pH on the uptake of label of [$U-^{14}C$]glucose by two coat preparations. O and Δ , Measured in 0.1 M-tris-maleate; $\bullet$ and $\blacktriangle$, measured in 0.1 M-glycine. Experimental details are given in the text.

Fig. 2. Effect of incubation temperature on the uptake of label of $[U-^{14}C]$ glucose. ○ and ● Indicate results from two different preparations. Experimental details are given in the text.

Fig. 3. Effect of treating coat for 10 min at the temperatures indicated on the subsequent incorporation of label of $[U-^{14}C]$ glucose taking place under standard conditions. Experimental details are given in the text.

planchet and measuring the amino N present in a portion after hydrolysis. The specific radioactivity of the extract was 88% of that of the starting material. This experiment also showed that the incorporation was not into a minor cellulose fraction, for cellulose itself was not made soluble by performic acid treatment.

Enzymic nature of reaction. From Fig. 1 the

quantity of $[U-^{14}C]$ glucose bound to the coat in 1 h was maximal at pH 7.0–7.4, and from Fig. 2 the optimum temperature at this pH and for this incubation time was 36–38°C.

Coat washed with 10% (w/v) trichloroacetic acid or maintained at pH 4.0 for 1 h at 35°C would no longer incorporate glucose. Fig. 3 shows that heating the coat for 10 min at 50°C destroyed its ability to react. Sodium fluoride at 0.1 M inhibited the reaction by 65%; at 0.01 M it had no effect. Potassium chloride at 0.5 M inhibited the reaction by 95% and at 0.25 M by 50%; this was not an effect of the cation, since sodium chloride and magnesium chloride gave similar results. Isonicotinylhydrazide, an inhibitor of pyridoxal phosphate enzymes after pre-incubation (Hoare, 1956), did not affect the rate at which glucose became bound when the coat had been treated for 1 h at 35°C in the reagent at 17.5 mM concentration.

The reaction was inhibited by *p*-hydroxymercuribenzoate and the kinetics in the absence and presence of this compound were studied. Binding of a glucose molecule to the insoluble protein may block and thus inactivate an enzymic site, but up to the chosen incubation time of 1 h the uptake of label was linear with time at all substrate concentrations used. Consequently initial conditions of substrate and enzyme concentrations were considered to apply throughout this period. The results obtained, plotted by the Lineweaver–Burk method, are shown in Fig. 4; plots of s/v against s and of v against v/s gave good straight lines but are not shown. The appropriate intercepts and slopes showed that inhibition was non-competitive with a K_m value for the reaction of 8.2×10^{-6} M. The value for the constant equivalent to K_i was 20.7×10^{-6} M. The inhibition caused by *p*-hydroxymercuribenzoate was reversed by cysteine. Thus the 90% inhibition caused by adding inhibitor to a final concentration of $6 \mu\text{M}$ 30 min before the reaction with glucose was started was decreased to 50% when cysteine to 0.6 mM concentration was added 15 min after the inhibitor. This concentration of cysteine itself inhibited the reaction 30%.

Magnitude of uptake on extended incubations. After incubation in the usual way for 24 h, and with $360 \mu\text{g}$ ($1.5 \mu\text{Ci}$) of $[6-^{14}C]$ glucose, a coat preparation bound label to the extent of 3.6% of its own weight in terms of glucose. This value, which shows that the amount of glucose label bound may be appreciable, is not necessarily a maximum, since, for one thing, coat loses some of its reactivity on aging.

Specificity of reaction. The effect of a number of sugars and related compounds on the amount of glucose label bound is shown in Table 1.

Uptake of radioactivity from specifically labelled glucose. Table 2 shows that C-2 and C-6 of glucose combined with cyst coat to the same extent. This

was not so for C-1, the binding of which varied between 0.45 and 0.82 g-atom for every g-atom of C-2 or C-6. The fact that between 5.10 and 6.12 g-atoms of carbon were taken up from $[U-^{14}C]$ -glucose for every g-atom of C-6 implies that all glucose carbon atoms, with the exception of C-1, are taken up to the same extent. Relative to these carbon atoms the proportion of C-1 taken up on longer incubations appears to be somewhat less (Table 3). The ratio of C-1 to C-2 bound was

Fig. 4. Lineweaver-Burk plot showing effect of glucose concentration (s) in mol/l on the radioactivity (in c.p.m.) taken up from $[U-^{14}C]$ glucose ($5.6 \mu\text{Ci/mg}$) in 1 h (v) under standard conditions. $\circ$, With p -hydroxymercuribenzoate ($13.4 \mu\text{M}$); $\bullet$, without p -hydroxymercuribenzoate. Experimental details are given in the text.

unaffected by the glucose concentration. C-1 was not lost during the drying down of the washed coat before counting of radioactivity, for wet samples whose radioactivity was counted in a scintillation counter with a gel scintillant gave the same result.

Balance experiment with $[1-^{14}C]$ glucose and $[6-^{14}C]$ glucose. Table 4 shows the results of an experiment to find out whether C-1, lost on the binding of glucose to coat, is released as carbon dioxide or as some other volatile compound. For unambiguous results it was necessary that no appreciable carbon dioxide be produced by contaminating bacteria, that the coat should bind a large proportion of the substrate and that much of the glucose that had reacted should have lost its C-1. The results showed that the C-1 released was volatile and was probably not carbon dioxide.

Attempts to remove radioactivity from labelled coat. A pH of 10 for 1 h at 35°C removed 30% of the label and the inclusion of m -hydroxylamine to give conditions claimed (Blumenfeld, Rojkind & Gallop, 1965) as specific for ester-bond fission in collagen proved no more effective. Perchloric acid (1 M) and 10% (w/v) trichloroacetic acid at 70°C for 10 min also released about 30% of the label.

To find the effect of more vigorous acid treatment 1–4 mg of coat labelled with $[U-^{14}C]$ glucose and containing radioactivity equivalent to 4–40 μg of glucose was dissolved in 2 M-hydrochloric acid (2 ml)

Table 1. *Inhibition of incorporation of $[U-^{14}C]$ glucose label into coat preparations by some sugars and related compounds*

Incorporation was measured in the normal way with a 2 h incubation, the ratio of inhibitor to glucose concentration being 16:1 (w/w). The experiment was performed twice with most of the inhibitors.

	Inhibitor	Inhibition (%)
Aldehydic group of glucose modified or replaced	D-Glucose 1-phosphate	15, 22
	D-Sorbitol	22, 26
	D-Arabitol	9, 0
	D-Gluconic acid	22, 20
	D-(1→5)-Gluconolactone	17
C-2 grouping of glucose modified	2-Deoxy-D-glucose	24, 19
C-6 grouping of glucose modified or replaced	D-Glucose 6-phosphate	20, 24
	D-Glucuronic acid	13, 9
	D-Xylose	36, 22
Isomers of D-glucose	D-Fructose	5, 12
	D-Galactose	36, 31
	D-Mannose	27, 37
	Lactose	4, 0
Disaccharides containing D-glucose residues	Maltose	13, 3
	Sucrose	8, 0
	Trehalose	12, 4
	D-Ribose	4
	2-Deoxy-D-ribose	2
Other compounds	Glycerol	12
	D-Glyceraldehyde	11
	Pyruvate	12, 6

Table 2. *Relative uptake of specific carbon atoms of D-glucose by coat preparations from ten different batches of cells*

For each experiment equal amounts of cyst coat (approx. 1 mg) were incubated with [1-¹⁴C]-, [2-¹⁴C]-, [6-¹⁴C]- or [U-¹⁴C]-glucose (30 µg, 0.5 µCi) of accurately known specific radioactivity for 2 h at 35°C in buffered antibiotic.

Carbon atom bound	Uptake relative to C-6 uptake							Uptake relative to C-2 uptake		
	Expt....1	2	3	4	5	7	8	6	9	10
C-1	0.49	0.51	0.66	0.75	0.74	0.82	0.52	0.67	0.45	0.54
C-2	—	—	—	1.03	1.00	1.10	1.02	1.00	1.00	1.00
C-U	5.55	5.60	—	5.10	5.58	6.12	5.75	—	—	—

Table 3. *Uptake of C-1 atoms of D-glucose relative to C-2 and C-6 by coat preparations as a function of time*

Details were as in Table 2 but the time of incubation was varied.

Incubation time (h)	Uptake relative to C-2 uptake	Uptake relative to C-6 uptake
	Expt. 1	Expt. 2
2	0.67	0.66
5	0.54	0.51
7	0.57	0.47
20	0.57	0.46

by heating at 100°C for 2h, and the digests were deionized before being evaporated to dryness. They were then taken up in 2ml of water and assayed for ¹⁴C content by solid counting of radioactivity. As estimated by radioactivity [U-¹⁴C]glucose taken through the whole procedure was recovered quantitatively. Chromatography of the effluents showed that the ¹⁴C label ran as one spot, which ran as glucose, and when these effluents were treated each with a crystal of sodium periodate at room temperature for 1h in the dark before chromatography the radioactive peaks had disappeared from the t.l.c. plates. When the effluent was treated with glucose oxidase under similar conditions, the radioactivity now ran with added gluconolactone. With two different batches of coat labelled with [U-¹⁴C]-glucose, 45% and 49% of the label was recovered as glucose. Similar experiments were carried out with coat preparations that had been divided and allowed to react with either [1-¹⁴C]glucose or [2-¹⁴C]glucose under identical conditions. Table 5 shows that only some of the bound C-1 was recovered in the deionized effluent, and the recovery of C-2 was accounted for as material in which the C-2 remained attached to C-1 of the original glucose. Chromatography of these effluents before and after treatment with glucose oxidase showed no radioactivity in any compound other than glucose. If any radioactivity

had been released as a charged molecule, it would have been retained on the mixed-bed column. Equal amounts of coat that had reacted with [¹⁴C]glucose and dissolved in 2M-hydrochloric acid at 100°C for 2h were passed down a mixed-bed column, a column of Amberlite IR-120 (H⁺ form) alone and one of Amberlite IR-45 (OH⁻ form) alone: 49.4, 60.0 and 56.5% of the added activity was recovered respectively from the columns when coat that had reacted with [U-¹⁴C]-glucose was used. The very similar recoveries from the mixed-bed, cationic and anionic columns provided no evidence for the release, from the labelled coat, of molecules other than glucose and those of a zwitterionic nature.

The stability towards alkali of the bonds joining the label to the coat was shown when coat (2mg) labelled with [U-¹⁴C]glucose was dissolved in 0.5M-sodium hydroxide at room temperature for 18h and equal portions were passed down mixed-bed, Amberlite IR-120 (H⁺ form) and Amberlite IR-45 (OH⁻ form) columns: 14.6, 35.5 and 14.6% of the added radioactivity was recovered in the effluents.

Conversion of incorporated radioactive material into laevulinic acid. After hydrolysis of coat labelled with [U-¹⁴C]glucose for 18h in boiling 5.7M-hydrochloric acid, removal of acid by distillation in partial vacuum and chromatography on silica gel G t.l.c. plates with butan-1-ol-acetic acid-water (4:1:1, by vol.), the bulk of the radioactivity ran as one peak faster than the amino acids. Radioactivity could not be found in the latter. Both laevulinic acid and the main radioactive component obtained from [U-¹⁴C]glucose itself after heating with 5.7M-hydrochloric acid under the same conditions behaved in this way. The unknown reacted to spraying with 0.5% 2,4-dinitrophenylhydrazine in 2M-hydrochloric acid. Some 50% of the radioactivity and the unknown were removed by extraction of the hydrolysate with ether; 63% of the label was lost when [1-¹⁴C]glucose-labelled coat was hydrolysed and the radioactivity counted on

Table 4. *Recovery of radioactivity after incubating coat with [1-¹⁴C]glucose or [6-¹⁴C]glucose*

Equal amounts of cyst-coat preparation (approx. 1 mg) showing a glucose C-1 uptake relative to C-6 of 0.60 were incubated with 6 μ g (0.1 μ Ci) of either [1-¹⁴C]glucose or [6-¹⁴C]glucose in antibiotic buffer for 2 h at 35°C. Carbon dioxide evolved in incubation with [6-¹⁴C]glucose was not measured but that formed in the [1-¹⁴C]-glucose experiment was absorbed in KOH and determined as Ba¹⁴CO₃. Samples of the complete suspensions were evaporated to dryness before and after incubation and their radioactivities counted, and the radioactivity bound to the coat was assayed in other samples of the suspension after incubation by collection on glass-fibre filters.

Position of label	Radioactivity (% of that added)			
	Recovered in supernatant after incubation	Taken up by coat	Recovered as CO ₂	Unaccounted for after incubation
C-1	32.4	26.5	5.0	36.1
C-6	37.9	45.4	—	16.7

Table 5. *Recovery of uncharged material on hydrochloric acid treatment of coat labelled with [1-¹⁴C]glucose or [2-¹⁴C]glucose*

Results are from four different coat preparations, each of which had been divided into two, incubated with either [1-¹⁴C]glucose or [2-¹⁴C]glucose under the standard conditions for 2 h and their radioactivities counted on filters. Filters were treated for 2 h in 2M-HCl at 100°C and digests passed down mixed-bed ion-exchange columns. Effluents were evaporated to dryness *in vacuo* before their radioactivities were counted.

C-1 uptake relative to C-2 uptake	Radioactivity (% of that added)		
	Observed recovery from C-1 labelled coat	Calc. recovery from C-2 labelled coat if only molecules containing six C atoms were recovered	Observed recovery from C-2 labelled coat
0.54	31.8	17.2	21.9
0.53	90.9	48.2	52.0
0.65	63.2	41.1	48.5
0.60	56.6	34.0	30.0

planchets after removal of the hydrochloric acid and 41% when [6-¹⁴C]glucose was used.

Periodate and lead tetra-acetate oxidation. Labelled coat preparations were suspended in 0.01 M-sodium periodate, pH 4.5, for 20 h at 21°C in the dark, collected on filters, washed and their radioactivities counted. Of the initial radioactivity 85, 79 and 93% was recovered attached to the coat of three different preparations that had been allowed to react with [1-¹⁴C]-, [6-¹⁴C]- and [U-¹⁴C]-glucose respectively for 1 h under the standard conditions. A more concentrated (0.03 M) solution of periodate buffered at pH 4.5 with 0.1 M-sodium acetate was not more effective. A freeze-dried extract of known specific radioactivity, prepared from coat that had reacted with [U-¹⁴C]glucose and had then been oxidized with performic acid, was dissolved in water and treated with 0.01 M-periodate at pH 4.5 for 1 h at 21°C. Periodate was dialysed away, and the specific radioactivity of the protein measured again and found to be unaltered. When glucose was caused to

react with 0.01 M-periodate in a threefold theoretical excess for 1 h at 21°C, the expected 5 equiv. of periodate were used. Glucose, after treatment with performic acid under the conditions used to produce the soluble coat extract, reduced 3.8 equiv. of periodate, 90% of the glucose being oxidized when measured by glucose oxidase.

Filters on which [U-¹⁴C]glucose-labelled coat had been collected were immersed in 0.1 M-lead tetra-acetate in acetic acid for 1 h at 21°C (Perlin, 1962). No radioactivity was lost compared with a control immersed in acetic acid alone.

Decrease in coat reactivity with increasing age of cell. This effect was pronounced (Fig. 5).

DISCUSSION

The binding of glucose label to cyst coat was not the result of incorporation into contaminating bacteria. The presence of fructose, even after pre-incubation of the coat preparation with fructose,

Fig. 5. Uptake of label of [U- 14 C]glucose by coat preparations isolated at different times after settling from the same batch of cells. The quantity of coat on the filters was measured by the micro-Kjeldahl method. Experimental details are given in the text.

did not affect the amount of glucose label bound and this far exceeded the mass of bacteria present. Labelled coat after overnight incubation in broth was still labelled and chromatography of hydrolysates showed no radioactivity in the amino acids. The stoichiometric incorporation of all carbon atoms from glucose except C-1 suggests that no substantial degradation of the glucose had occurred, as would have been the case had the uptake been incorporation into bacteria. Conventional adsorption processes may be eliminated as the cause of the uptake, which was irreversible, and the label was even attached to coat protein after dissolution of the latter after performic acid treatment. It is also reasonable to expect that heavy bacterial growth resulting from incubation in nutrient broth would remove adsorbed glucose. Moreover, the reaction was found to be specific and affected in a very minor way by a variety of molecules structurally related to glucose.

The temperature optimum of 36–38°C and the loss of ability to react on warming the coat at 50°C suggests a dependence of the reaction on protein conformation. The recovery of radioactivity after holding at pH 9.7, but the slow speed of the reaction at this pH, suggests the involvement of a group or groups with pK values on the alkaline side of the pH optimum. One such group may be the thiol group, for *p*-hydroxymercuribenzoate at low concentrations (10 μ M or thereabouts) inhibited non-competitively and reversibly. The value of 8.2×10^{-6} M as the constant equivalent to K_m emphasizes the high affinity of the coat for glucose.

Cellulose is a major component of the cyst coats of some amoebae (Griffiths & Hughes, 1969;

Tomlinson & Jones, 1962), but in *C. steinii* no components other than protein are present in any quantity (Tibbs, 1966). The encystment process in amoebae takes much longer than in *Colpoda* and, unlike the case in the latter (Tibbs, 1968; Tibbs & Marshall, 1969b), is accompanied by considerable modification of intracellular structure and respiratory capacity (Griffiths, Lloyd, Roach & Hughes, 1967). Although these two classes of organism encyst for the same reason, the biochemical differences are substantial and glucose polymer formation is of no quantitative importance in *C. steinii* cyst-coat synthesis. The glucose incorporated into coat *in vitro* represents at least a doubling in the carbohydrate already there and, if incorporated into glucose polymer, would also require appropriate activation. Glucose 1-phosphate, the immediate precursor of UDP-glucose, did not affect significantly the incorporation of label, as would have been the case had incorporation into polysaccharide been involved. Problems of activation also exist for binding to protein, but reactivity here could be enhanced by conformation changes. At a more practical level the incorporated label was made extractable along with the coat protein by treatment with performic acid. This treatment did not make cellulose soluble, and treatments such as hot perchloric acid or trichloroacetic acid, which removed only a small proportion of the incorporated label, would have removed polymers such as glycogen. It is concluded that the glucose label must be bonded to protein rather than carbohydrate.

After labelled coat had been boiled for 18h in 5.7M-hydrochloric acid radioactivity was readily detectable in laevulinic acid but not in amino acids. In agreement with the loss of C-1 when glucose is converted by acid into laevulinic acid, hydrolysis of coat labelled with [1- 14 C]glucose caused the loss of more radioactivity than did hydrolysis of coat labelled with [6- 14 C]glucose. Some of the bound label in fact could be recovered as glucose after treatment of the coat with 2M-hydrochloric acid for 2h at 100°C. No other uncharged molecule could be identified in these digests, and some of the label still appeared attached to peptide since it was retained on both cation-exchange and anion-exchange columns.

To complicate the situation, some C-1, and probably only C-1, was lost during the reaction *in vitro*. It did not seem to be carbon dioxide and may have been a volatile acid. The proportion lost, which varied from preparation to preparation, was independent of glucose concentration but tended to increase on prolonged incubations. The radioactive C-1 that was bound presumably traced unchanged glucose molecules, and a higher proportion of this label was recovered as glucose than from coat that had reacted with [2- 14 C]glucose. In the latter case,

the label bound would sum unchanged glucose molecules and any modified glucose with a lost or exchanged C-1. These different recoveries suggest that the modified molecule was not glucose, i.e. that the C-1 had not been exchanged by some unrelated metabolic process but had been removed and that the reaction with the coat consists of more than one step.

Several attempts were made to study the type of linkage joining the sugar to the coat, but, no doubt because of the insoluble nature of the material, the unsolved problem of the C-1 loss and the possibility of complex side reactions occurring between sugar and protein during chemical manipulation of the labelled coat, results were not very informative. Aldehyde groups are involved in interchain cross-linking in collagen through aldol (Bornstein & Piez, 1966) and probably Schiff-base condensations (Tanzer, 1967). The label is not easily removed by acid. This suggests that neither Schiff-base compounds nor glycosidic links are involved, although the latter remain a possibility for, in *N*-substituted glycosylamines, the N-C link may be stabilized towards acid by an acid-catalysed Amadori rearrangement (Hodge, 1955). The situation is further complicated by the possibility of a Maillard or 'browning' reaction (Ellis, 1959). Treatment with alkali or *m*-hydroxylamine at pH 10 (Blumenfeld *et al.* 1965) did not remove label, indicating that 'ester-like' bonds are not involved. Alkali treatment should also have split the N-C bond of an *N*-substituted 1-amino-1-deoxy-D-fructose resulting from an Amadori rearrangement (Gottschalk, 1952). Periodate and tetra-acetate had no obvious effect, although the loss of C-3 or 16.7% of the radioactivity from glycosidically bonded [^{14}C]glucose could possibly have escaped detection. In any case inertness towards these reagents does not necessarily mean the absence of vicinal hydroxyl groups, for steric effects may be involved and groups in the *trans* position may be extremely resistant if the molecule is held in a rigid conformation such as exists in 1,6-anhydro- β -D-glucofuranose (Dimler, Davis & Hilbert, 1946). An understanding of the type of linkage is proving elusive and a direct chemical approach made on the products of the reaction *in vitro* may not be the best way to study the problem. Studies on the metabolic fate of the incorporated label during excystment may be more revealing.

Two possibilities are obvious for the function of the reaction *in vivo*. The reaction may be part of a cross-linking mechanism, conceivably of the type suggested by Bensusan (1965) for glucose interaction with collagen. The high reactivity of freshly formed coat favours this idea and certainly mature coat protein is unusual in a number of ways. Far more of the coat becomes soluble after performic

acid treatment than dissolves in 8M-urea with the reducing conditions of Crestfield, Moore & Stein (1963) and, even in the soluble form, it is very resistant to the attack of a number of proteolytic enzymes (J. Tibbs & P. M. Whyte, unpublished work). A second possibility is that the reaction reflects part of a cyst-reactivation mechanism in which glucose is part of the excystment signal. During the initial stages of excystment the binding of glucose to the cyst coat presumably occurs, for the hay infusion used to trigger this process contains glucose in adequate concentrations. The bound sugar may even be used as a metabolite by the excysting cells.

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REFERENCES

- Bensusan, H. B. (1965). In *Structure and Function of Connective and Skeletal Tissue*, p. 42. Ed. by Jackson, S. F., Harkness, R. D., Partridge, S. M. & Tristram, G. R. London: Butterworths Scientific Publications.
- Bergmeyer, H. U. & Bernt, E. (1963). In *Methods of Enzymatic Analysis*, p. 123. Ed. by Bergmeyer, H. U. New York: Academic Press Inc.
- Blumenfeld, O. O., Rojkind, M. & Gallop, P. M. (1965). *Biochemistry, Easton*, **4**, 1780.
- Bornstein, P. & Piez, K. A. (1966). *Biochemistry, Easton*, **5**, 3460.
- Crestfield, A. M., Moore, S. & Stein, W. H. (1963). *J. biol. Chem.* **238**, 622.
- Dimler, R. J., Davis, H. A. & Hilbert, G. E. (1946). *J. Am. chem. Soc.* **68**, 1377.
- Ellis, G. P. (1959). *Adv. Carbohydr. Chem.* **14**, 63.
- Gottschalk, A. (1952). *Biochem. J.* **52**, 455.
- Griffiths, A. J. & Hughes, D. E. (1969). *J. Protozool.* **16**, 93.
- Griffiths, A. J., Lloyd, D., Roach, G. I. & Hughes, D. E. (1967). *Biochem. J.* **103**, 21 p.
- Guthrie, R. D. (1962). In *Methods in Carbohydrate Chemistry*, vol. 1, p. 439. Ed. by Whistler, R. L. & Wolfrom, M. L. New York and London: Academic Press.
- Hoare, D. S. (1956). *Biochim. biophys. Acta*, **19**, 141.
- Hodge, J. E. (1955). *Adv. Carbohydr. Chem.* **10**, 169.
- Lato, M., Brunelli, B., Ciuffini, G. & Mezzetti, T. (1969). *J. Chromat.* **39**, 407.
- Marshall, B. J. & Tibbs, J. (1969). *Biochem. J.* **115**, 10 p.
- Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
- Perlin, A. S. (1962). In *Methods in Carbohydrate Chemistry*, vol. 1, p. 427. Ed. by Whistler, R. L. & Wolfrom, M. L. New York and London: Academic Press.
- Phillips, J. D. & Pollard, A. (1953). *Nature, Lond.*, **171**, 41.
- Proper, G. & Garver, J. C. (1966). *Biotechnol. & Bioengng*, **8**, 287.
- Roseman, S., Abeles, R. H. & Dorfmann, A. (1952). *Archs Biochem. Biophys.* **36**, 232.

- Smith, I. (1960). In *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 251. Ed. by Smith, I. London: W. Heinemann (Medical Books) Ltd.
- Tanzer, M. L. (1967). *Biochim. biophys. Acta*, **133**, 584.
- Tibbs, J. (1966). *Biochem. J.* **98**, 645.
- Tibbs, J. (1968). *J. Protozool.* **15**, 725.
- Tibbs, J. & Marshall, B. J. (1969a). *Abstr. FEBS. 6th Meet., Madrid*, p. 63.
- Tibbs, J. & Marshall, B. J. (1969b). *Biochim. biophys. Acta*, **172**, 382.
- Tibbs, J. & Marshall, B. J. (1970). *J. Protozool.* **17**, 125.
- Tomlinson, G. & Jones, E. A. (1962). *Biochim. biophys. Acta*, **63**, 194.