Induction of Cyst Coat Protein Synthesis by Starvation in the Ciliate *Colpoda steinii*

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1. At 28°C, synthesis of protein cyst coat in ciliates of *Colpoda steinii* is induced by washing with water and, as judged by glutamic acid assays and incorporation studies with L-[U-¹⁴C]leucine, starts about 30 min after the cells have stopped swimming and is largely complete 90 min later. During this time up to 70% of the protein synthesized by the cell is coat protein. 2. When cells were placed in L-[U-¹⁴C]-leucine at low concentrations (0.25–0.76 mM) during the period of coat synthesis there was no lag in uptake. Only a small proportion of the leucine incorporated into the coat was from the external substrate, implying that the rate of radioactive isotope incorporation measured the rate of transport of amino acid into the cell. Transport of L-[U-¹⁴C]leucine into the cell was markedly stimulated by L-glutamic acid and L-lysine. 3. When cells were placed in L-[U-¹⁴C]leucine at high concentrations (38 mM) the rate of incorporation was considered to measure the rate of protein synthesis, but because the latter may have been affected by substrate it is concluded that such measurements are of doubtful value.

About 2 h after washing away of nutrient from motile cells of *Colpoda steinii* the ciliates stop swimming and secrete a protein coat rich in glutamic acid residues (Tibbs, 1966). In cells encysting spontaneously from culture the coat nitrogen eventually accounts for about 18% of the total cell nitrogen (Tibbs & Marshall, 1970a). The stimulation of specific protein synthesis by simple laboratory manipulation of eukaryotic cells which are readily grown and cultured is of obvious interest. The work described here has verified that the coat is synthesized during encystment and is not merely secreted from a soluble protein pool present in the cell before starvation. The work has also given some information about the extent to which the protein-synthetic machinery of the starving cell is concerned with cyst coat synthesis. A short account of part of the work has been published (Tibbs, 1970).

METHODS

Culture, purification and encystment of ciliates. The ciliates were grown on a suspension of *Escherichia coli* strain B as food. The cells encysted about 48 h after inoculation. Ciliates were obtained from these cysts by shaking with hay infusion and were purified by differential centrifugation and washing, a process that removed any cell debris, unexcysted cells and hay infusion. The method has been described in detail (Tibbs & Marshall, 1970a), the only modification being that the purified

ciliates were subsequently allowed to encyst in conical flasks shaken at 28°C, instead of on petri dishes at room temperature. Under these conditions cells, at a typical concentration of 10⁶ cells/ml, settled synchronously within 5 min of each other and about 90 min after washing away of nutrient. Encysting cells tend to adhere to glass surfaces and this tendency was decreased by treating glassware with dimethyldichlorosilane in carbon tetrachloride.

Analyses. Cell counts were made with a haemocytometer. Cells and cell fractions were hydrolysed by boiling in 5.7 M-HCl for 18 h and the hydrolysates were evaporated to dryness on a rotary evaporator under reduced pressure. As a routine L-glutamic acid was assayed manometrically by using L-glutamate 1-carboxy-lyase (glutamate decarboxylase, EC 4.1.1.15) (Gale, 1963), the enzyme preparation being an acetone-dried *E. coli* powder from Sigma Chemical Co., St Louis, Mo., U.S.A. Amino N was determined by the Moore & Stein (1948) procedure with L-leucine as standard. Protein on glass-fibre filters was assayed after scintillation counting by washing the filters with acetone and then boiling in 5.7 M-HCl for 18 h. Glass fibre was filtered away, then the filtrate was dried in a partial vacuum and the amino N content determined. The amino acid analyses of hydrolysed cyst coat and cytoplasmic protein were carried out by Dr G. N. Graham of the Astbury Department of Biophysics, University of Leeds, by using a Technicon autoanalyser with the single column of Piez & Morris (1960) and a standard 20 h run. Norleucine was used as internal standard.

Cyst disruption and preparation of fractions for radioactivity counting. Cysts were disrupted as described by Tibbs & Marshall (1970a) and, after removal of glass

beads, the coat was sedimented from the homogenate by centrifugation at 500g for 5 min. As seen under a phase-contrast microscope the sediment consisted almost entirely of the outer layers of the cyst (Tibbs, 1966, 1968). It was judged free from significant contamination by whole cells and debris. Before measurement of the total radioactivity incorporated into this coat fraction, the supernatant was removed as completely as possible, then the sediment was transferred in 5% (w/v) trichloroacetic acid on to a 2.1 cm glass-fibre filter where it was washed several times with more 5% (w/v) trichloroacetic acid, water, and finally acetone. The sediment was dried under an i.r. lamp before its radioactivity was counted. If the coat fraction was washed by centrifugation before transfer to a filter, the washed coat tended to adhere to the walls of the container and losses occurred. However, where quantitative recovery of the coat was unnecessary this procedure was adopted, since it was considered more efficient at removing small particles of debris, which were a minor contaminant.

Cell protein from the homogenate supernatant was obtained by precipitation with trichloroacetic acid at a final concentration of 5% (w/v). After heating in 5% (w/v) trichloroacetic acid for 15 min at 90°C to hydrolyse any aminoacyl-tRNA a sample was collected on a filter and washed in the normal way. Intact cells, which had been incorporating label into protein, were also collected on filters. Extraction with trichloroacetic acid [5% (w/v), 90°C, 15 min] was carried out either on the cells in suspension or after collection on filters.

Measurement of radioactivity. Radioactivity incorporated into protein fractions that had been collected on filters was measured with a scintillation counter after immersion of the filters in a toluene-based scintillant (Davies & Cocking, 1966). Although this technique avoids problems of quenching the counter was set to monitor the pulse-energy spectrum by the channels-ratio method. The existence of self-absorption or inadequate penetration of the specimen is immediately obvious when radioactivity is counted in this way. Such problems were, in fact, infrequent. The counting efficiency was about 75% and counting rates were many times that of the background radioactivity. Sufficient counts were accumulated to give a standard deviation of 2% or less. L-[U-¹⁴C]Leucine (10 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and was diluted with unlabelled L-leucine before use, the specific radioactivity of the diluted leucine being calculated from its amino N content and the counts obtained from 10 μ l of the solution dried on a filter as described above.

Distribution of ¹⁴C amongst amino acid residues. After one-dimensional t.l.c. of hydrolysates of labelled proteins on Macherey and Nagel 300-Cellulose with butan-1-ol-acetic acid-water (12:3:5, by vol.) as solvent the distribution of ¹⁴C among the amino acids was assessed qualitatively by using a Panax scanner. After scanning, the plates were sprayed with a polychromatic ninhydrin spray (Moffat & Lytle, 1959).

RESULTS

Amino acid composition of coat and cytoplasmic protein. This is given in Table 1. Several minor peaks were present in the coat hydrolysate, in

addition to the amino acids listed, and also one pronounced peak (approx. 30 residues/1000) occurred between phenylalanine and ammonia. Unidentified ninhydrin-reacting substances, apparently not α -amino acids, were detected in earlier studies (Tibbs, 1966). Since 100 μ g of ash-free coat give 0.95 μ mol of amino N on hydrolysis (Tibbs, 1966) the results show that 100 μ mol of coat hydrolysate amino N contain 3.80 μ mol of leucine.

L-Glutamic acid content of hydrolysates of encysting cells. Hydrolysates of cells settling at the start of encystment contain about 22pg of glutamic acid per cell less than hydrolysates of mature cysts (Tibbs & Marshall, 1970a). This amount of glutamic acid is about the same as that bound in the coat protein. Cells were induced to encyst at 28°C and sampled at intervals. The results show that the increase in glutamic acid content occurs in a 2h period starting about $\frac{1}{2}$ h after settling (Fig. 1). At the end of this time the coat is well formed and is readily isolated.

Proportion of cell protein present as coat material at various times after settling. Equal numbers of ciliates (25ml, about 10⁷ cells) were shaken in a series of flasks, then at various times after settling were cooled in an ice-water mixture, rapidly harvested and disrupted. In each case the cyst coat was isolated by centrifugation as quantitatively as possible and washed only once with water to minimize losses in processing. The coat preparations and also whole cells at the same stages of encystment were hydrolysed after washing with 5% (w/v) trichloroacetic acid and the amino N contents of the hydrolysates measured. At 2, 3, 4 and 20h after settling, the percentage of whole-cell-hydrolysate amino N from the coat was 26.4, 21.5, 27.5 and 25.6 respectively. The coat isolated in this way from four different batches of cells obtained 2h after settling represented $26.0 \pm 1.6\%$ (S.E.M.) of the whole-cell-hydrolysate amino N.

Incorporation of L-[U-¹⁴C]leucine into whole cells. Incorporation commenced immediately without any lag period when L-[U-¹⁴C]leucine (final concentration 0.25 and 0.30mM) was added to either motile ciliates or settled encysting cells and the addition of unlabelled leucine immediately decreased the rate of incorporation of label (Fig. 2).

Period during which added L-[U-¹⁴C]leucine is incorporated into the coat of encysting cells. Determination of the total glutamic acid in encysting cells suggested that coat synthesis started approximately $\frac{1}{2}$ h after settling time and was complete 2h later (Fig. 1).

This starting time for coat synthesis is confirmed by the fact that maximal incorporation of radioactive label into the coat occurred when L-[U-¹⁴C]-leucine was added to the cells at 30min after settling, and incorporation was not increased by

Table 1. Amino acid composition of coat and cytoplasmic protein from *C. steinii* cysts

Coat protein was isolated as described by Tibbs (1966). Other details are given in the text.

Amino acid	Wt. of amino acid residue ($\mu\text{g}/100\ \mu\text{g}$ of ash-free cyst coat)	Coat amino acid (residues/1000 residues)	Cytoplasmic protein amino acid (residues/1000 residues)
Ala	3.92	68.3	70.9
Arg	3.59	28.5	52.2
Asx	5.99*	64.4	106.4
Glx	39.27*	376.8	138.6
Gly	5.46	118.4	84.6
His	1.62	14.5	32.4
Hyp	0.00	0.0	0.0
Ile	2.34	25.7	48.5
Leu	4.08	44.7	85.4
Lys	4.47	43.2	78.4
Met + Cys	2.88†	27.2	20.6
Phe	2.40	20.3	43.0
Pro	0.00	0.0	38.5
Ser	4.52	64.3	50.0
Thr	3.46	42.5	59.3
Tyr	2.41	18.3	34.0
Val	3.43	42.9	57.2
Total	89.84	1000.0	1000.0

* Calc. as the dibasic acid.

† Calc. as methionine.

Fig. 1. L-Glutamic acid content of hydrolysates of *C. steinii*, expressed as pg/cell, after ciliates had been shaken at 28°C for various times after purification. Each flask contained 2.15×10^7 cells in 25 ml and at the times shown the encysting cells were harvested rapidly by using low-speed centrifugation, then hydrolysed in 5.7 M-HCl and assayed for glutamic acid (see the Methods section). Zero time was within 15 min of washing away nutrient from the purified ciliates and the cells settled 90 min later. The settling time is indicated by a vertical dotted line.

mencing 30 min after settling, 55% and 52% of the radioactivity incorporated by the cells had been incorporated into the coat. When label was added to settling cells and incorporation stopped and the coat isolated 90–210 min later, it was found that incorporation was reaching a maximum after 120 min (Fig. 4), confirming that coat synthesis is complete 120 min after settling.

Eleven different cell preparations were incubated for 2 h after settling with L-[U-¹⁴C]leucine (0.30–0.76 mM). Some preparations incorporated label more readily than others and the amount of label incorporated into the coat varied between 0.34 and 1.84 $\mu\text{mol}/100\ \mu\text{mol}$ of hydrolysed coat amino N. Chromatography showed that most, although not all, of the incorporated radioactivity was in the leucine/isoleucine spot. Since hydrolysed coat contains 3.80 μmol of leucine/100 μmol of amino N it is apparent that much of the coat leucine was supplied by the cell itself. Either the added leucine was not equilibrating with the intracellular leucine pool or, less likely, because of the erratic nature of the incorporation, some of the coat was synthesized outside the times studied, e.g. during cell growth.

Effect of concentration of L-leucine on incorporation into coat and whole cells. This was studied in an attempt to increase the incorporation of substrate amino acid (Fig. 5). Neither the cells nor cyst coat preparations were washed with unlabelled L-leucine

adding substrate earlier than this (Fig. 3). It can be calculated that in the two experiments summarized (Fig. 3), during the 90 min period com-

Fig. 2. Incorporation of L-[U- 14 C]leucine, present at low concentration, into *C. steinii* cells at 28°C. Settling time for both cell preparations was 100 min after purification. ○, L-[U- 14 C]Leucine (final concn. 0.25 mM, sp. radioactivity 160 μ Ci/mmol) was added at purification time. The cells from 5 ml samples were collected and counted for radioactivity after incorporation had been stopped, at the times indicated, by trichloroacetic acid [final concentration of 5% (w/v)]. ●, Effect of adding unlabelled L-leucine (final concn. 2.5 mM) at 50 min on the incorporation into the same number of these cells. △, L-[U- 14 C]Leucine (final concn. 0.30 mM, sp. radioactivity 33 μ Ci/mmol) added to a second batch of cells at settling time, the cells being sampled and counted for radioactivity as described above. ▲, Effect of adding unlabelled L-leucine (final concn. 6.0 mM) 60 min after addition of radioactive leucine on incorporation into the same number of these cells. The settling time is indicated by a vertical dotted line.

before counting of radioactivity. After incorporation of radioactivity from both low and high concentrations of L-leucine such preparations, collected on filters, did not show a significant decrease in count rate after immersion for 24 h in unlabelled L-leucine (38 mM). Consequently adsorption was judged to be no problem and washes with unlabelled leucine solution were omitted from the normal procedure.

In 38 mM-leucine, incorporation into the coat reached a value equivalent to 7.0 μ mol/100 μ mol of hydrolysed coat amino N. Up to this concentration of added leucine, the proportion of whole-cell label present in the coat was fairly constant at about 33%, but at higher concentrations general protein turnover in the cell appeared stimulated, as the proportion was less. Since the coat contains 3.8 μ mol of leucine/100 μ mol of hydrolysed coat amino N the value of 7.0 μ mol suggested fairly extensive use of the leucine by the cell to produce other amino acids. This possibility was examined by scanning t.l.c. plates of hydrolysates for radioactivity. The results were assessed semi-quantitatively and indicated that about half of the incorporated radioactivity was present as leucine,

whereas the rest was distributed among a number of amino acids. Because of this, and because incorporation into coat had now become independent of further increases in leucine concentration it seems reasonable to conclude that all the coat leucine residues were supplied by external substrate. This conclusion also confirmed that essentially no coat-protein synthesis occurred outside the period studied. A further experiment similar to the one shown in Fig. 5 also showed that incorporation was greatly increased at high leucine concentrations. Incorporation into coat had not reached a maximum in 38 mM-leucine and was equivalent to 4.6 μ mol of amino N/100 μ mol of hydrolysed coat. In 76 mM-leucine (the highest concentration used) the value was 8.3, and the proportion of whole cell counts contributed by the coat was still independent of leucine concentration and was about 30%.

Influence of pH on incorporation of L-[U- 14 C]-leucine and encystment. At low concentrations of substrate many of the coat leucine residues were provided by the endogenous metabolism of the encysting cell and incorporation of label was limited by the penetration of labelled substrate into the cell. The erratic extent of this incorporation with

Fig. 3. Effect of the time of addition of L-[U- ^{14}C]leucine on the final incorporation into *C. steinii* cells and cyst coats at 28°C. There are two different experiments, shown by ---- and —. Zero time in each case was arbitrary but was during the starvation period before the cells settled, and the settling time in each case was indicated by a vertical line through the graph. Δ , Whole cells: L-[U- ^{14}C]leucine (final concn. 0.76 mM, sp. radioactivity 33 $\mu\text{Ci}/\text{mmol}$) was added to 10 ml samples of ciliate suspension at the times shown. At 150 min (120 min after settling) the cells in each sample were treated with trichloroacetic acid and collected and counted for radioactivity as described in the Methods section. $\bullet$, Cyst coats: L-[U- ^{14}C]leucine was added in the same final concentration to 10 ml samples of the same encysting ciliate suspension and at 150 min unlabelled L-leucine was added to a final concentration of 7.6 mM, and the cells were cooled in ice; then the coat from each sample was isolated quantitatively and counted for radioactivity. $\circ$, Whole cells: L-[U- ^{14}C]leucine (final concn. 0.76 mM, sp. radioactivity 33 $\mu\text{Ci}/\text{mmol}$) was added to 10 ml samples of a different encysting ciliate suspension and at 210 min (130 min after settling) the cells were treated, collected and counted for radioactivity. $\bullet$, Cyst coats: L-[U- ^{14}C]leucine was added to the same final concentration to 10 ml samples of this ciliate suspension and at 210 min unlabelled L-leucine was added to a final concentration of 7.6 mM and the coat isolated from each sample and counted for radioactivity.

different batches of cells was not due to a dependence of the transport system on external pH, since cells normally encyst at pH 5.6–6.0 and only minor fluctuations of pH within this range occurred in encysting cell suspensions. When L-[U- ^{14}C]leucine (0.76 mM and 38 mM) was present for 90 min

Fig. 4. Effect of time of harvesting on incorporation of L-[U- ^{14}C]leucine into *C. steinii* cyst coat at 28°C. L-[U- ^{14}C]leucine (final concn. 0.76 mM, sp. radioactivity 33 $\mu\text{Ci}/\text{mmol}$) was added to settling cells (final volume 10 ml) and uptake was stopped at various times afterwards by adding unlabelled L-leucine to a final concentration of 1.5 mM. The coat material was prepared and its radioactivity counted as described in the Methods section.

after purification, cells buffered in 12.5 mM-sodium phosphate incorporated less than 10% more label at pH 6.0 than at pH 5.6. Thus, factors other than pH were probably involved in the erratic incorporation.

Influence of other amino acids on incorporation of L-[U- ^{14}C]leucine. The effects of other amino acids and of D-glucose are shown in Table 2. That the percentage activation or inhibition of incorporation caused by a particular compound is not the same in all cell preparations is to be expected, since these preparations would have different unstimulated rates of incorporation. L-Norvaline, L-valine and L-isoleucine inhibited incorporation fairly strongly, possibly because of competition for the cell transport mechanism. Other compounds also had some effect, but the most notable one was the activation by L-glutamic acid or L-lysine. These effects were on transport rather than protein synthesis, since when L-glutamic acid increased whole-cell uptake of leucine by 70.4% the quantity of coat material was unaltered, whereas the amount of leucine substrate incorporated into the coat had increased from 0.65 $\mu\text{mol}/100 \mu\text{mol}$ of hydrolysed coat amino N to 1.13 μmol , i.e. by 73.8%. In a similar experiment, which was also intended to check that the glutamic acid solution, even though carefully neutralized, was not influencing incorporation by causing pH changes, the same incubation conditions were used except that the cell suspensions were buffered at pH 6.0 in 5 mM-sodium phosphate. The amount of coat produced remained the same, but the presence of L-glutamic acid raised the incorporation into the coat from 0.92 μmol of leucine substrate/100 μmol of hydrolysed coat amino N to 1.97, i.e.

Fig. 5. Effect of L-[U-¹⁴C]leucine concentration on incorporation into *C. steinii* cells and cyst coat at 28°C. At final concentrations of 0.76–3.8 mM the specific radioactivity of the leucine was 33 μ Ci/mmol; at concentrations of 7.6 mM and above a specific radioactivity of 3.3 μ Ci/mmol was used. O, Cyst coats. Samples (5.0 ml) of purified cell suspension were added at settling time to 5.0 ml of L-[U-¹⁴C]leucine, the final concentrations of leucine being as shown. The cells were shaken for a further 2 h, then rapidly cooled in ice, and the coat from each sample was isolated, counted for radioactivity and its protein content determined as described in the Methods section. The results are expressed as μ mol of substrate incorporated/100 μ mol of hydrolysed coat amino N. ●, Whole cells. Samples (2.5 ml) were added to 2.5 ml of leucine and shaken for 2 h. At the end of this time the cells were treated with trichloroacetic acid, collected and counted and the protein content was determined as described in the Methods section. The results are expressed as μ mol of substrate incorporated/382 μ mol of cyst hydrolysate amino N, since this amount of cyst hydrolysate contained 100 μ mol of amino N from the cyst coat.

by 114%. Incorporation into cytoplasmic protein increased correspondingly from 0.58 μ mol of leucine substrate/100 μ mol of hydrolysed cell protein amino nitrogen to 1.11, i.e. by 91%.

Rate of protein synthesis in starving and encysting cells. At low concentrations of L-[U-¹⁴C]leucine the incorporation rate appeared to be a measure of the rate of transport into the cell rather than of protein synthesis. In concentrations of leucine greater than about 38 mM the specific radioactivity of the leucine incorporated into the coat approached that of the substrate and it seemed probable that at these concentrations the rate of incorporation was approximating to the rate of protein synthesis. Fig. 6 shows that the variation in rate of incorporation with time at high concentrations was quite different from that at low concentrations (Fig. 2), since a rapid initial rate was followed by a slower incorporation. Fig. 6 shows that this phenomenon was independent of the stage of encystment and did not result from an altered rate of protein synthesis as encystment proceeded. The situation is complicated by the fact that high concentrations of leucine, added at purification time, delayed settling and made it rather asynchronous.

DISCUSSION

Calculations from the present results show that about 28 pg/cell of the cyst glutamic acid content is bound in the coat. This is a little higher than the value originally published (21–25 pg/cell; Tibbs & Marshall, 1970a), which was calculated slightly differently and used a value of 18.0% as the proportion of whole cyst N present as coat material. This value was for cells encysted from culture and is too low for cysts obtained from purified ciliates. The latter cells have encysted for the second time, are smaller, and the coat represents a greater proportion of the cell. The increase, of 22 pg, in the glutamic acid content of the cyst compared with the settling cell suggested that the coat was synthesized between about $\frac{1}{2}$ h and $2\frac{1}{2}$ h after settling, but did not rule out that some component proteins were present in the swimming cell. The coat is not homogeneous, since 30% of it may be extracted by formic acid, this fraction having a relatively low glutamic acid content (Tibbs, 1966).

Coat synthesis is complete 120–150 min after settling, as indicated by the glutamic acid content of the cell, quantitative measurement of the coat produced, and the fact that incorporation into the coat stops at about this time. Incorporation of added label into the coat was constant when label was added to cells at all times after purification until 30 min after settling, showing that no detectable amounts of coat protein were synthesized during this time. When label was added later, the final incorporation decreased rapidly, as coat was being synthesized. The purified ciliates used were obtained from freshly excysted cells and, since excystment appears to be accompanied by the loss

Table 2. *Effect of amino acids and D-glucose on the incorporation of L-[U-¹⁴C]leucine into C. steinii cells*

Ciliate suspension (5 ml) was incubated in L-[U-¹⁴C]leucine (0.76 mM and 132 μ Ci/mmol) for 2 h at 28°C in the presence of the compound indicated (7.6 mM). Incorporation was stopped by trichloroacetic acid and the cells were collected and counted for radioactivity, as described in the Methods section. For the first four columns incorporation was started at purification time and for the last two at settling time.

Added compound	Incorporation started at...	Activation (+) or inhibition (-) (%)					
		Purification time				Settling time	
L-Norvaline		69.4 (-)	59.8 (-)				
L-Phenylalanine		2.0 (-)	13.9 (-)				
Glycine		7.3 (-)	1.6 (-)				
L-Serine		25.1 (-)	23.3 (-)				
L-Glutamic acid		271.3 (+)	152.6 (+)	116.7 (+)	45.7 (+)	70.4 (+)	46.1 (+)
L-Lysine		83.6 (+)	133.3 (+)	43.0 (+)			
D-Leucine		32.7 (-)					
L-Valine			42.6 (-)				
L-Isoleucine			51.3 (-)				
D-Glucose			31.0 (+)

Fig. 6. Incorporation of L-[U-¹⁴C]leucine, present at high concentration, into *C. steinii* cells at 28°C. Settling time was 120 min in the absence of leucine but was delayed to about 150 min when leucine was present from purification. O, L-[U-¹⁴C]Leucine (final concn. 38 mM, sp. radioactivity 13.1 μ Ci/mmol) was added immediately after purification and at known times 2 ml samples of the incubate were added to trichloroacetic acid [final concn. 5% (w/v)] and the cells extracted, washed and counted for radioactivity as described in the Methods section. Δ , The same batch of cells was shaken without leucine until settling time, whereupon L-[U-¹⁴C]leucine was added to the same final concentrations and specific radioactivity. At known times, 2 ml samples were treated with trichloroacetic acid and counted for radioactivity as described above. The settling times are indicated by vertical dotted lines.

of coat protein into solution (Marshall & Tibbs, 1970) it is unlikely that there is a store of some coat components in freshly excysted cells. It is concluded that essentially all coat protein is synthesized later than 30 min after settling and that synthesis is practically complete after a further 90 min. By using the amino acid analyses in Table 1 and

assuming that all label is incorporated as leucine it can be calculated that in the two experiments where 55% and 52% of the radioactivity incorporated in this 90 min period were in the coat 70% and 67% respectively of the protein synthesized by the cell was coat protein. Since amino acids other than leucine became labelled during the experiment the true value was probably nearer 60%. Assuming that this value represents 26% of the cyst protein and all of the coat protein, it appears that about 23% of the cytoplasmic protein is renewed in the same period, corresponding to an average turnover time of 6½ h. These calculations are approximate but emphasize the extent to which protein synthesis by the cell is related to coat synthesis. Phenomena of this type may not be uncommon in differentiating eukaryotic cells, but in this case the cells are readily grown and induced to produce their characteristic coat protein. Thus there seems no reason why the cells should not provide a useful experimental system for studying the control of specific protein synthesis.

The experiments just discussed were performed in low concentrations of leucine (0.25 and 0.30 mM), and the endogenous metabolism of the cell provided much of the coat leucine. This fact, and the absence of a lag period in uptake of label (Fig. 2), show that it can be assumed that the rate of incorporation measures the rate of transport of amino acid into the cell. In two experiments using 38 mM-leucine the majority of the leucine residues incorporated into the coat, and presumably into cell protein, were from the substrate. This confirmed that all the coat protein was synthesized after settling. Since the amount of coat produced was not affected by increasing the leucine concentration, and since the proportion of whole cell

label present in the coat remained constant up to a substrate concentration of 38mM no general stimulation of cyst protein synthesis was taking place. At high concentrations of leucine the rate of its entry into the cell was not an important factor in regulating the rate of incorporation of leucine substrate and under these conditions the latter rate is probably a reasonable measure of the rate of protein synthesis.

The erratic incorporation of leucine substrate into different batches of whole cells at low leucine concentrations could have been partially due to pH variations but the pH of encysting suspensions did not vary significantly from the range pH 5.5–6.0, and other factors probably affected transport. One of these could have been the cell metabolites discharged into solution during the starvation period, since several amino acids, in particular L-glutamic acid and L-lysine, had a profound effect on the movement of leucine into the cell.

A reaction between D-glucose and isolated cyst coat has been described (Tibbs & Marshall, 1970b) and unpublished work (J. Tibbs & B. J. Marshall) has shown that cyst coat has a high affinity for a number of metabolites, including some L-amino acids. Even if this association is only the result of strong adsorption, it could imply that not all the measured incorporation *in vivo* of leucine into the coat resulted from coat synthesis. The measurements that have been made *in vitro* indicate that only a very minor fraction of the leucine incorporated into the coat *in vivo* is likely to be bound by a mechanism other than protein synthesis. This is also shown by the fact that the binding *in vitro* of L-leucine to the coat was hardly affected by the inclusion of L-glutamic acid in the incubation mixtures, and yet this amino acid stimulated incorporation into whole cells, cell cytoplasmic protein and cyst coat to the same extent.

An attempt was made to assess the alteration in the rate of cell protein synthesis as cells starved and then proceeded to encyst. This was done by studying incorporation of label in the presence of 38mM substrate, when the endogenous contribution

to the leucine incorporated into the coat was minor, and the overall stimulation of general protein synthesis by substrate for cells which had settled was not detectable. This attempt was not very successful for, unexpectedly, a high initial rate of uptake was soon followed by a very much slower rate (Fig. 6). The curve was not dependent upon the state of encystment, but was a function of the time of addition of label. Since the overall incorporation of radioactivity into the coat was sufficient to account for all the leucine residues present, it is likely that the high initial rate represents a temporary stimulation of protein synthesis by the added leucine. Further, the fact that high concentrations of leucine delayed the settling of motile cells and made their subsequent encystment asynchronous suggests that, for ciliates at any rate, their metabolic condition, and probably protein turnover rate, may have been altered. The general conclusion is that although incorporation may be a measure of the rate of protein synthesis the possibility that this rate is affected by the high concentration of substrate required makes the technique of doubtful value.

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