THE HETEROTROPHIC NUTRITION OF *CHLORELLA VULGARIS* (BRANNON No. 1 STRAIN)

II. 'Adaptation' to Galactose as Carbon Source

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INTRODUCTION

THE formation of 'adaptive' enzymes in response to specific substrates has been reported from numerous studies with bacteria and fungi (Yudkin, 1938; Doudroff, 1940; Hinshelwood, 1946; Pollock, 1959). There are, however, very few examples of such adaptation in organisms which are undoubted plants. Tolbert and Cohan (1953) reported that the glycolic acid oxidase activity of cereal leaves can be enhanced by spraying with a solution of glycolic acid. Galston and Dalberg (1954), in their studies of the indole acetic acid oxidase in pea seedlings claimed a fivefold enhancement of activity following application of indole acetic acid (0·02 μg/ml.) to the tissues. Tang and Wu (1957) claimed that development of nitrate reductase activity in rice seedlings only occurs when nitrate is supplied.

Unicellular algæ which can be cultivated under heterotrophic conditions would seem to be particularly suitable for studies of enzymic adaptation in plants. Bristol-Roach (1927) reported a long lag-phase prior to active growth of *Chlorella* on maltose, suggesting the necessity for activation of maltase before assimilation of the disaccharide. Neish (1951) noted that while his strain of *Chlorella* utilised β-glucosides, a lag-phase preceded utilisation of α-glucosides and illumination of the cultures, by promoting endogenous carbohydrate production, delayed this induction of an ability to assimilate α-glucosides as carbon sources. Here again it may be suggested that activation of an α-glucosidase is involved.

The present paper arises out of the observation earlier reported (Griffiths, Thresher and Street, 1960) that our stock of the Brannon 1 strain of *Chlorella vulgaris* showed a pronounced lag-phase when cultured in the dark in presence of D-galactose. During the first five days of culture this sugar suppressed cell division as compared with the sugar-omitted control and only towards the end of the 10-day growth period did cell numbers rise appreciably in the galactose medium.

MATERIALS AND METHODS

The general techniques have been previously described (Griffiths Thresher and Street, 1960). The stock cells were maintained at 25° C

in the *dark* in a sterile liquid medium (pH 5·0) containing 1% D-glucose and a modified Craig and Trelease (1937) inorganic solution. These stock cultures were subcultured every 4 days in an aseptic transfer cabinet under a dark green photo-safelight (Ilford filter 907 G), sufficient cells being transferred on each occasion to establish in the new cultures an initial population of 100 cells/mm.^{3} At intervals of 3 months (or sooner if the stock cultures became infected) new stock cultures were initiated from cells growing in diffuse daylight on slants of peptone/salt/agar.

Cells harvested from the 4-day-old liquid stock cultures by low speed centrifuging at 10° C were suspended in sugar-omitted liquid medium and used to inoculate the experimental cultures. Each experimental culture consisted of a 250-ml Erlenmeyer flask containing 100 ml medium and the initial cell density was 100 cells/mm.^{3} Each treatment was represented by 5 replicate cultures. These cultures were incubated at 25° C in the dark, the cells being suspended once daily by hand agitation; except in the experiment depicted in Fig. 2 where the cultures were subjected continuously to 275 $\times \frac{1}{2}$ inch horizontal displacements per min. on a Baird and Tatlock 'Microid' shaking machine.

Cell counts were performed as previously described (Griffiths, Thresher and Street, 1960). Prior to analysis, washed cells were dried at 80° C for 24 hr. Soluble carbohydrates were estimated by extracting 0·1 g. dry cells with 70% ethanol for 12 hr. and applying the anthrone method of Clegg (1956). To determine hydrolysable carbohydrates 0·1 g. dry cells were heated with 50 ml. 6N H_2SO_4 for 1 hr. at 100° C Crude fibre represents the dry matter of the cells which remains insoluble after treatment with H_2SO_4 for 1 hr. at 100° C. Total nitrogen was determined by a modification of the method of Doneen (1932), the digestion period being modified in that before addition of the perchloric acid the reection mixture was slowly heated until fumes no longer appeared at the neck of the tube (15–20 min.), and that, after addition of perchloric acid, the digestion was again carried out slowly until the reaction mixture cleared and, thereafter, vigorously for 2 min.

Oxygen uptake was determined by the direct method of Warburg at 25° C in an apparatus covered by a light-proof canopy so that only a low light intensity reached the flasks when reading the manometers. The shaking speed was 75 oscillations/min. and each flask contained 2 ml. of inorganic medium containing 5,000 cells/mm.^{3} Substrate dissolved in 0·5 ml. inorganic media was contained in the sidearm and added 2 hr. after equilibration (zero time). Oxygen uptake was expressed as μl. $O_2/10^7$ cells, based on the initial population. Prior to introduction into the Warburg flasks the cell suspension was shaken for 24 hr. at 25° C in the dark to yield 'starved' cells.

For determination of hexokinase activity 9 ml. of cell suspension (= 25 mg. dry wt. cells) plus 1 ml. Sorensen's buffer (pH 7·5) was maintained at 4° C in an ice/salt bath and submitted to maximum

cavitation for 30 min using the 19 mm (1 : 1 ratio) probe of the MSE-Mullard Ultrasonic Disintegrator. Microscope examination showed a high degree of cell breakage and 60–62% of the cell nitrogen was recovered in the supernatant clarified by centrifuging. Hexokinase activity in these supernatants was determined by the method of Kunitz and McDonald (1946). The reaction proceeds at 5° C for 30 min. and then the reaction mixture is back-titrated with 0·01 M NaOH to the initial pH of 7·5. One unit of hexokinase activity is defined as producing 1×10^{-8} acid equivalents per minute under the standard conditions of the test.

A test for the ability of single cells to form colonies on agar plates was devised as follows: 9 cm. petri dishes containing 20 ml. of the liquid medium solidified with 1·5% agar and containing either 1% D-glucose (Glucose agar) or 1% D-galactose (Galactose agar) were inoculated with 0·05 ml. of the cell suspension under test (standardised to contain either *ca.* 500 or *ca.* 100 cells). The suspensions were allowed to rest for several hours after collection of cells by centrifuging before being plated. The inoculum was spread evenly over the surface of the medium with a suitably shaped sterile nicrome wire. The petri dishes were incubated for 14 days in darkness at 25° C and then the number of colonies visible to the naked eye counted. The maximum number of colonies counted in such a test was 80% of the number of cells inoculated, and even with favourable medium was frequently 50–60% of the number of cells. Inability to achieve 100% recovery was probably due to 'clumping' and also to the necessity for a cell mass of critical size to be quickly established for growth to proceed to produce a visible colony.

Experimental

Since growth of cells, with D-galactose as carbon source, is at first inhibited but begins to rise in rate towards the end of the culture period, experiments were undertaken which involved a study of the comparative total cell production per culture period with glucose as against galactose as carbon source when cells were carried through a number of successive subcultures. Each passage was initiated by a population of 100 cells/mm.^{3} and involved growth for 7 days in medium containing the test sugar. The results of two such experiments are presented in Figs. 1 and 2. The results presented in Fig. 2 are for cultures subjected to continuous shaking by the technique already described. Figure 1 illustrates that over a series of passages growth rate in presence of galactose rises to reach a steady level close to that immediately established in presence of glucose. The cells can be 'trained' to grow actively with galactose as carbon source. Both 'normal' and 'galactose-trained' cells were used to initiate the cultures whose growth is shown in Fig. 2. Normal cells showed 'training' to growth in galactose reaching, after 6 subcultures, the level of growth achieved immediately in this sugar by 'trained' cells. The enhanced rate of growth of the 'shaken' cultures illustrated that the maximum growth rate achieved in galactose is lower than that in glucose and

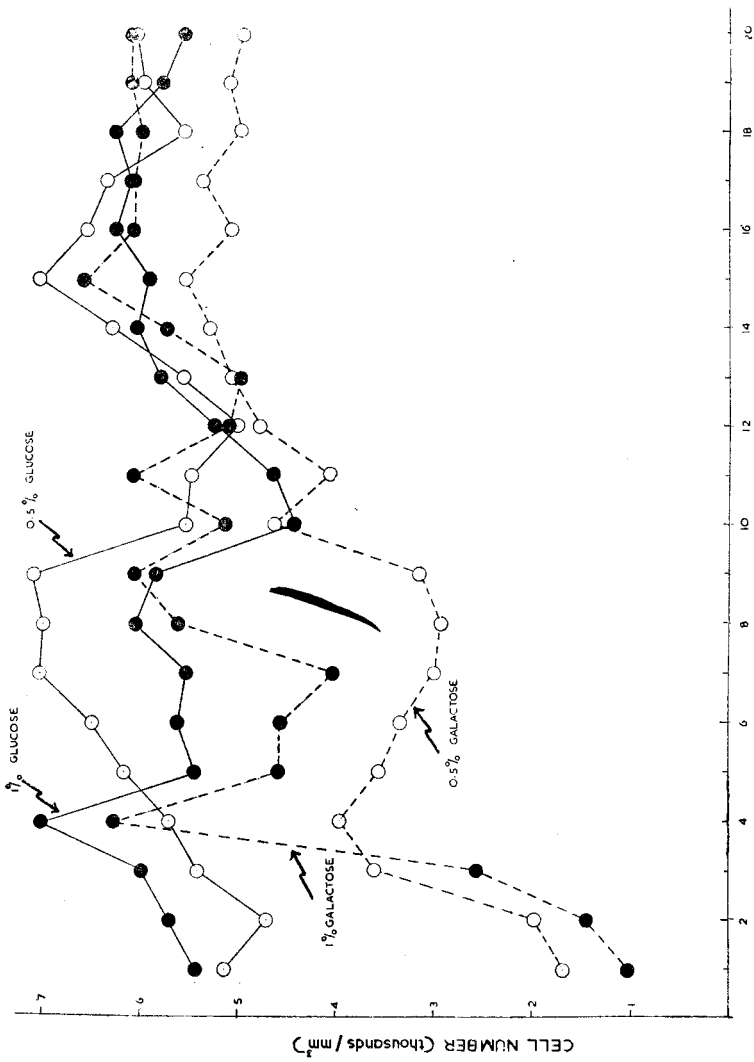

FIG. 1. Total cell counts recorded at the end of each 7-day passage when *Chlorella* cells were repeatedly subcultured in darkness in liquid media containing either D-glucose (1% and 0·5%) or D-galactose (1% and 0·5%). (Initial cell density established at each subculture = 100 cells/mm.³)

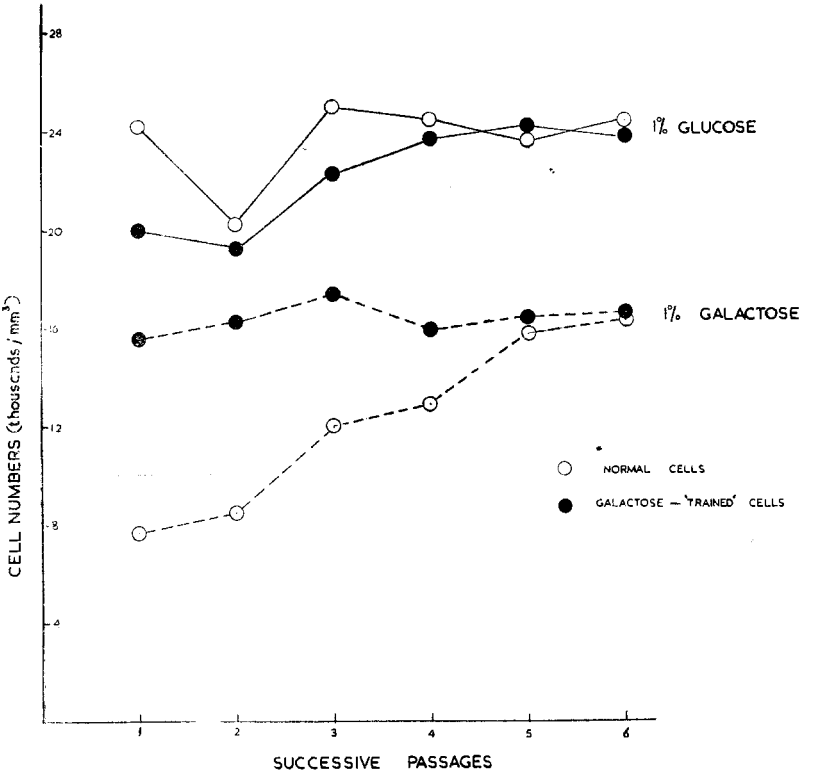

FIG. 2. Growth (as expressed by total cell counts after 7 days incubation) in 'shaken' liquid cultures in darkness of normal and 'galactose-trained' *Chlorella* cells, when repeatedly subcultured in presence of either 1% D-glucose or 1% D-galactose. (Initial cell density established at each subculture = 100 cells/mm.^{3}.)

that 'galactose-trained' cells are not impaired in their ability to utilise glucose as their carbon source.

Results drawn from a series of plating experiments are shown in Tables I–III. Table I shows the number of colonies developed on glucose and galactose agar when inoculated with cells from various *Chlorella* cultures. The cells taken from the slants of peptone/salt/agar maintained in diffuse light had no ability to form colonies on galactose but always yielded colonies on glucose. Cells maintained on glucose liquid medium gave on galactose agar no colonies when plated at the 100 cells per plate level and only 12 small colonies at the 500 cell level. Cells 'trained' on liquid media to utilise galactose gave a good yield of colonies on both galactose and glucose agar.

Table II shows that 'galactose-trained' cells rapidly lose their ability to grow actively on this sugar if transferred to glucose medium,

TABLE I

Number of colonies developed on plates of glucose (1%) *and galactose* (1%) *agar when inoculated with suspensions of* Chlorella *cells*

Inoculum origin — cell No. per plate →	Galactose agar		Glucose agar	
	500 cells	100 cells	500 cells	100 cells
Slant B (on peptone/salt/agar)	0	0	119	41
Slant C ,, ..	0	0	98	18
Slant E ,, ..	0	0	243	68
Culture in glucose (1%) liquid medium	12	0	164	39
'Galactose-trained' cells growing in galactose (1%) liquid medium	196	58	202	32

TABLE II

De-adaptation of galactose-trained Chlorella *cells by culture in glucose* (1%) *liquid medium* (*results of two experiments*)

Growth in liquid medium immediately prior to the agar plate—colony test		Mean number of colonies per plate developed following inoculation of 500 cells per plate, on glucose (1%) and galactose (1%) agar	
Sugar at 1%	Duration of culture period(s) (days)	Sugar	Mean colony count ± standard error
Glucose ..	4	Glucose	214 ± 12 (8)*
		Galactose	184 ± 5 (10)
..	3×4	Glucose	221 ± 14 (9)
		Galactose	*74* ± *5* (10)
Galactose ..	4	Glucose	206 ± 11 (9)
		Galactose	163 ± 12 (8)
..	3×4	Glucose	226 ± 10 (8)
		Galactose	176 ± 8 (10)
Glucose ..	7	Glucose	184 ± 6 (10)
		Galactose	168 ± 11 (10)
..	14	Glucose	198 ± 11 (7)
		Galactose	*53* ± *8* (8)
Galactose ..	7	Glucose	181 ± 5 (10)
		Galactose	178 ± 7 (10)
..	14	Glucose	187 ± 8 (8)
		Galactose	170 ± 6 (7)

* Number of plates counted.

TABLE III

Adaptation and De-adaptation of Chlorella *cells following* 21 *days continuous culture on liquid medium*

Cells taken from	Experimental liquid medium containing 1% of the sugar indicated	Mean number of colonies (± standard error) on glucose (1%) and galactose (1%) agar (500 cells inoculated per plate)			
		Duration of prior culture period in liquid medium			
		2 days		21 days	
		Glucose agar	Galactose agar	Glucose agar	Galactose agar
Glucose (1%) medium ..	Glucose	297±25(9)*	0	280±18 (9)	0
	Galactose	205±13(8)	0	231± 4 (9)	207±9 (10)
Galactose (%) medium ..	Glucose	..	..	370± 8 (8)	123± 8 (8)
	Galactose	..	..	250±20 (9)	295±20 (9)

Three subcultures at 4-day intervals or 14 days continuous culture on glucose medium effect a marked decline in the ability of the cells to grow on galactose. Table III shows that glucose-grown cells regularly show evidence of training when maintained for 21 days on galactose medium, and that galactose-trained cells always show a decline in their ability to utilise this sugar after 21 days continuous growth without subculture on glucose medium. Attention is particularly directed to the relatively low standard errors of the mean numbers of colonies based on 5 replicate test cultures per treatment and duplicate plating from each replicate (if plates became infected with foreign micro-organisms they were rejected: hence the replicate numbers below 10 in Tables II and III).

Cells grown on glucose liquid medium and galactose-trained cells were harvested, washed, dried and submitted to analysis. The analytical data (Table IV) show that the galactose-trained cells differed markedly from the glucose-grown cells, having lower soluble and hydrolysable carbohydrate contents and higher crude fibre and nitrogen contents per g. dry weight. The supernatants obtained following ultrasonic disintegration of fresh cells of the two types had similar hexokinase activities with glucose as substrate but the supernatant from the galactose-trained cells had twice the hexokinase activity of that prepared from glucose-grown cells when galactose was used as substrate (Table IV).

Both types of cell showed equal high rates of O_2 uptake with glucose as substrate, but the galactose-trained cells respired at twice

TABLE IV

Analysis of dried normal and 'galactose-trained' Chlorella cells

	Fractions as per cent. of the dry weight of the material	
	Normal cells	'Galactose-trained' cells
70% ethanol-soluble carbohydrates expressed as $C_6H_{12}O_6$	14	3
Total carbohydrates extracted following hydrolysis with $6N.H_2SO_4$ expressed as $C_6H_{12}O_6$	21	12
Crude fibre (residue after treatment of dry matter with H_2SO_4)	75	86
Total nitrogen ..	7	10
	Hexokinase units per 25 mg. dry cell material	
Hexokinase activity:		
(*a*) with glucose as substrate ..	43·3	32·5
(*b*) with galactose as substrate ..	15·3	31·0

TABLE V

Oxygen uptake of starved Chlorella *cells supplied with glucose* (1%) *or galactose* (1%)

Pretreatment of cells	Experimental substrate	Total O_2 uptake μl./10^7 cells in	
		4 hr.	18 hr.
Glucose-grown ..	glucose	32	384
	galactose	15	107
	no substrate	8	43
'Galactose-trained' ..	glucose	36	302
	galactose	24	21
	no substrate	11	

the rate of glucose-grown cells with galactose as respiratory substrate (Table V).

Discussion

The predictability of the 'training' or adaptation process described in this paper, its rapid loss in absence of galactose and our ability to 'adapt' or 'deadapt' the cells at will seem to eliminate the possibility that the process involves selection of randomly occurring mutations. The uniformity of behaviour of replicate cultures must also be regarded as overwhelmingly against a mutation hypothesis. The training certainly seems to involve induction of higher activity of the galactokinase system (Trucco *et al.* 1948; Wilkinson, 1949; Cardini and Leloir, 1953), although other enzymes may also be involved. Only in the presence of galactose as sole carbon source is this high level of activity maintained. Enhancement of the galactose level of normal cells is inhibitory to their growth even as compared with that of cells transferred from glucose to inorganic medium (Griffiths, Thresher and Street, 1960). Even fully adapted cells are unable to attain, in 'shaken' cultures, the high levels of growth which occur when glucose is supplied. The galactose-training does not impede the capacity of the cells to utilise glucose.

Slow adaptation proceeding over a number of successive subcultures and similar instability in absence of the specific substrate is reported by Hinshelwood (1946) for the response of *B. lactis aerogenes* to glycerol. There was, however, no evidence in our experiments of any stabilisation of the adaptation from prolonged subculture on the substrate.

Shrift (1954) has described an adaptation of a strain of *Chlorella vulgaris* to the antimetabolite, selenomethionine. This adaptation manifested itself as a resistance to the growth uncoupling effect of the antimetabolite. Whereas initial exposure to the antimetabolite inhibits division but allows cell growth to proceed, adapted populations will divide in the presence of the analogue without the extended period of uncoupled growth. The more detailed study of this phenomenon by Shrift, Nevyas and Turndorf (1961 *a*) produced convincing evidence that the adaptation involved a transformation of all cells rather than a selection of mutants. Full adaptation to selenomethionine was a slow process involving several subcultures; at an intermediate stage all cells show some but variable degrees of adaptation. Studies of the reversibility and stability of the adaptation phenomenon by these authors (1961 *b*) show the adaptation to involve induction of greater activity of the enzymes responsible for the reduction of sulphate to methionine, to be stable in absence of the antimetabolite but to be reversed by sulphur starvation or methionine feeding.

Studies of such adaptive enzyme induction in yeasts and bacteria (Gale and Davies, 1953) have opened up interesting problems in microbial biochemistry. The phenomenon of adaptation in plant cells may be more widespread and diverse than has hitherto been realised. For

those interested to explore this possibility it should perhaps be emphasised that unpublished results in our laboratory also indicate that strains within *Chlorella vulgaris* differ in their capacities to adapt to particular substrates.

SUMMARY

Cells of the Brannon No. 1 strain of *Chlorella vulgaris* can be trained to utilise D-galactose as sole carbon source for their growth in darkness. The cells lose this ability when transferred back to D-glucose medium. The evidence indicates that all the cells can be so trained and that selection of mutants is not involved.

The galactose-trained cells differ from glucose cultured cells in cellular composition, in galactokinase activity and in their respiration rate with galactose as substrate.

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