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NATURE OF THE STABLE ADAPTATION INDUCED BY SELENOMETHIONINE IN *CHLORELLA VULGARIS*

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SUMMARY

A strain of *Chlorella vulgaris*, after having been induced to resist the growth uncoupling effect of selenomethionine (and which maintained that resistance after removal of the analogue), absorbed L-[^{35}S]methionine much more slowly than did unadapted cells. Although uptake was slower, a greater proportion of the absorbed ^{35}S was incorporated into protein of the induced cells. [^{35}S]Sulfate absorption and conversion to protein, however, were the same in both strains. Sulfur starvation, a treatment that causes adapted cells to deadapt and regain their sensitivity to selenomethionine, restored permeability to methionine in the adapted cells. The uptake of methionine was inhibited by dinitrophenol and appears, therefore, to be an active, energy-requiring process. Repression of a methionine-absorbing system, perhaps a "permease", is held responsible for the induced resistance to selenomethionine.

INTRODUCTION

The term "maintenance" was introduced by NOVICK AND WEINER to describe a situation in which *Escherichia coli* could be induced to synthesize the β-galactosidase–permease system under one set of conditions and to maintain the new phenotype in another, non-inducing environment[1]. The term can aptly be applied to a number of other biological systems: the induction of respiratory-deficient Saccharomyces (petites) after exposure to euflavine[2,3] or after panthothenate starvation[4]; the development of L forms in penicillin-treated Salmonella[5]; the synthesis of new antigenic proteins in Paramecium which has been subjected to a variety of agents[6,7]. In each of these examples a high proportion of the exposed cells, if not all, are phenotypically altered, and the new character is transmitted to the progeny despite removal of the inducing conditions.

The *Chlorella vulgaris*–selenomethionine system also displays this type of permanent adaptation. The evidence to show that all or most cells are able to adapt has been presented elsewhere[8]. The permanency of the induced phenotype was established under controlled experimental conditions and lasted through 220 generations in the absence of selenomethionine, at which time the experiments were terminated[9]. Adapted strains have since been carried on inorganic salt–glucose agar slants for over 2 years without loss of resistance to selenomethionine.

Despite the permanency of the new phenotype, it is nevertheless possible to deadapt the cells by such a simple expedient as sulfur starvation. This close relationship to sulfur metabolism made it seem likely that the rate of methionine synthesis from sulfate had been sufficiently accelerated to enable the adapted cells to grow and divide normally in the presence of the otherwise division-inhibitory selenomethionine[9]. Such an hypothesis is amenable to isotopic dilution experiments with ^{35}S-labeled metabolites. The uptake of [^{35}S]sulfate and L-[^{35}S]methionine and their incorporation into protein have been followed in sensitive and resistant cells in order to test the hypothesis and to characterize further the two strains of Chlorella. The results show that resistance is correlated with a decreased permeability to methionine, and that sulfur starvation restores the permeability.

MATERIALS AND METHODS

Cultures of *Chlorella vulgaris* B. (Columbia strain) were maintained on slants of the following composition: KNO_3, 0.025 M; $MgCl_2 \cdot 6H_2O$, 0.020 M; KH_2PO_4, 0.018 M; $FeCl_2 \cdot 4H_2O$, 0.00001 M; potassium citrate$\cdot H_2O$, 0.00001 M; K_2SO_4, 0.0031 M; $ZnCl_2$, 0.07 ppm Zn; H_3BO_3, 0.05 ppm B; $CuCl_2 \cdot 2H_2O$, 0.002 ppm Cu; $MnCl_2 \cdot 4H_2O$, 0.44 ppm Mn; MoO_3, 0.02 ppm Mo; 2% agar; 1% glucose. Slants were incubated under a fluorescent-light intensity of about 350 ft-candles at 18°.

For liquid cultures, stock solutions of each salt, and other substances as required, were dispensed into 200-ml Pyrex culture tubes (300 × 32 mm) calibrated at 100 ml, and were then diluted to a level of about 50 ml. All materials were sterilized by autoclaving for 20 min at 15 lbs pressure.

Adapted cultures were obtained by 2–3 serial passages in the presence of $3.1 \cdot 10^{-5}$ M selenomethionine. The morphological and physiological features of an adapting culture, described previously[8], were checked. Adapted strains were maintained on agar slants without selenomethionine.

Inocula were prepared either from slants 4–5 days old or from liquid cultures. The cells were centrifuged and washed three times with distilled water. Samples were counted with a hemacytometer or with a Coulter electronic counter Model B. $20 \cdot 10^6$ cells were added to each culture tube and diluted to 100 ml to give an initial inoculum size of $2 \cdot 10^5$ cells per ml. An aseptic technique was maintained throughout the procedure.

A gauze-wrapped cotton plug, through which a gas inlet had been fitted, was inserted into each culture tube. A 5% CO_2–95% air mixture provided the carbon source and the bubbling served to stir the cells. The gas mixture was first scrubbed through a cotton-filled tube and then through a flask of sterile distilled water. The tubes were supported in a glass-walled water bath whose temperature was maintained at 25°–26°. Light was provided by 40-W, cool white fluorescent lamps, with a light intensity at the position of the culture tubes of about 750 ft-candles, as measured with a Weston light meter Model 756.

Selenomethionine was synthesized by the method of KLOSTERMANN AND PAINTER[10]. In our earlier work this analogue had been sterilized by sintered glass filtration before it was added to the culture medium. In the present experiments, autoclaving in the growth medium was found not to affect the biological activity of the selenomethionine. L-Methionine was obtained from Nutritional Biochemicals

Corp.; L-[^{35}S]Methionine was obtained from Schwarz Biochemical Research, Inc., or from New England Nuclear Corp.; [^{35}S]sulfate was purchased from Union Carbide Nuclear Co. as $H_2{}^{35}SO_4$, carrier-free in weak HCl.

For the isotopic uptake experiments, cells were harvested either by centrifugation or by filtration through a membrane disc (Schleicher and Schüll, type A, coarse porosity). Care was taken to avoid desiccation during the filtrations. The cells were washed with and then suspended in medium that contained either $3.0 \cdot 10^{-5}$ M carrier sulfate for the radiosulfate uptake experiments, or $3.0 \cdot 10^{-3}$ M sulfate for the radiomethionine uptake experiments. After the cultures were incubated for 1 h under growth conditions, the radioactive compound was added, with the bubbling CO_2–air mixture serving to stir the cell suspension. Aliquots were withdrawn periodically for measurement of rates of absorption and incorporation into protein. During the experimental period there were no significant increases in cell number, size, or weight. Dry weights and packed cell volumes were determined in Bauer and Schenck hematocrit tubes. Rates of uptake and protein incorporation were constant unless otherwise specified.

Radioactivity measurements and cell fractionations were based on techniques developed for *E. coli* by ROBERTS *et al.*[11]. For an estimate of protein sulfur, aliquots of cells were pipetted into an equal volume of cold 10% trichloroacetic acid, and stored at 4° for 0.5–3 h. They were then filtered through membrane discs and washed under filtration with cold 5% trichloroacetic acid. For measurement of total cellular radioactivity, aliquots were filtered directly through a membrane disc and washed under filtration with the non-radioactive medium. The membrane discs were fastened to aluminum planchets with rubber cement. For measurement of total radioactivity in the medium, aliquots of the cell suspension were dispensed into aluminum planchets fitted with a disc of lens paper. Several drops of a mixture of 0.5% sucrose–0.5% detergent uniformly dispersed the suspension. Planchets were dried in air or under heat lamps.

Counts were made with a nuclear model 181A scaler equipped with a D-47 gas-flow detector. Counts were corrected for geometry, self-absorption, and background. Specific activities were used to express all cellular concentrations on a molar basis. Absorption rates were calculated by the method of averages from the slopes of the uptake experiments.

RESULTS

Methionine uptake

The two strains of *Chlorella vulgaris* differed conspicuously in their ability to absorb methionine. Unadapted cells, harvested from young cultures, absorbed the amino acid at a much faster rate than did young, selenomethionine-adapted cells. They also metabolized the sulfur into protein more rapidly (Fig. 1). But of the total radioactivity absorbed, the adapted cell incorporated a larger proportion into its proteins than did the unadapted cell. In all experiments with young cultures, the unadapted cells incorporated about 35% of the ^{35}S into protein by the end of 1-h incubation, in contrast to the adapted cells which utilized about 75%.

A drop in the rate of absorption, particularly sharp for the unadapted cells, occurred with increasing age of the cultures. This rapid drop, coupled with small variations in the duration of the lag phase of simultaneously inoculated cultures,

was responsible for a constantly changing quantitative relationship between the two strains of cells. Measurements, therefore, were made on cells harvested along the entire course of the growth curve, a method that allows a more direct comparison

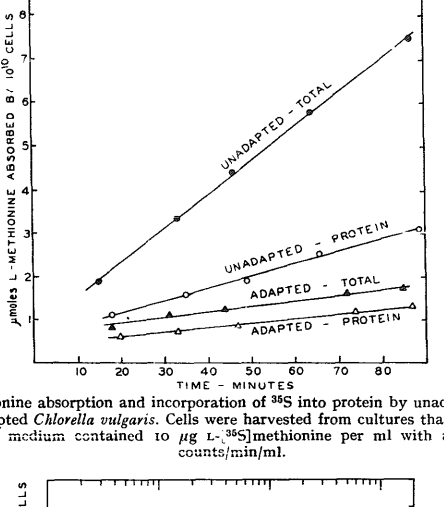

Fig. 1. L-Methionine absorption and incorporation of ^{35}S into protein by unadapted and selenomethionine-adapted *Chlorella vulgaris*. Cells were harvested from cultures that were 3 days old. The incubation medium contained 10 µg L-[^{35}S]methionine per ml with a count of 31000 counts/min/ml.

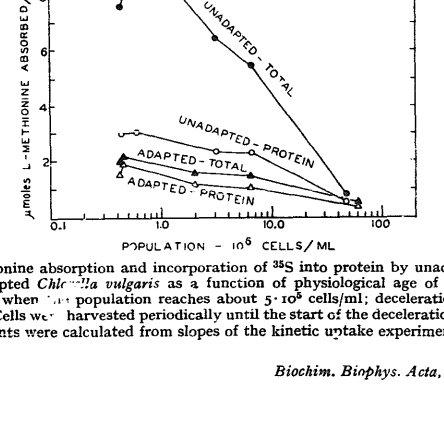

Fig. 2. L-Methionine absorption and incorporation of ^{35}S into protein by unadapted and selenomethionine-adapted *Chlorella vulgaris* as a function of physiological age of cells. Exponential divisions begin when the population reaches about $5 \cdot 10^8$ cells/ml; deceleration starts at about $8 \cdot 10^8$ cells/ml. Cells were harvested periodically until the start of the deceleration phase of growth. Sulfur contents were calculated from slopes of the kinetic uptake experiments (see Fig. 1).

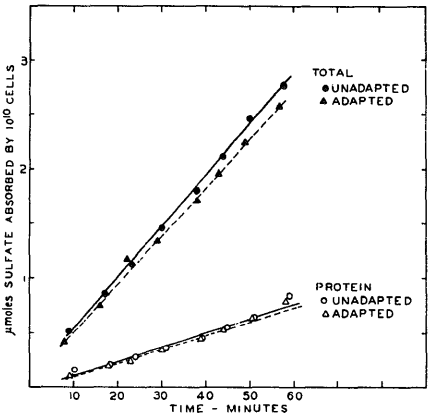

Fig 3. Sulfate absorption and ^{35}S incorporation into protein by unadapted and selenomethionine-adapted *Chlorella vulgaris*. Cells were harvested from cultures that were 4 days old. The incubation medium contained 0.03 μM carrier sulfate per ml and radiosulfate with a count of 11000 counts/min/ml.

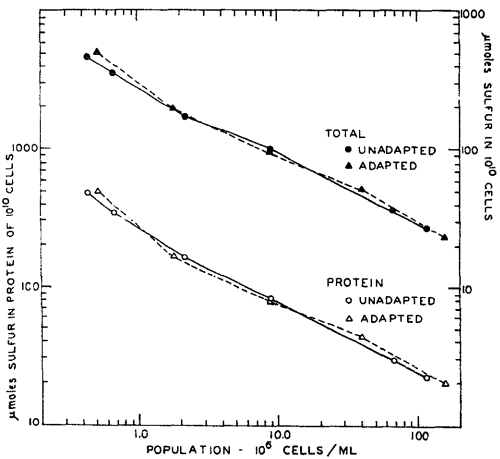

Fig. 4. Total sulfur and protein sulfur contents of unadapted and selenomethionine-adapted *Chlorella vulgaris* as a function of physiological age of cells. Exponential divisions begin when the population reaches about $5 \cdot 10^6$ cells/ml; deceleration starts at about $8 \cdot 10^7$ cells/ml. Cells were grown in medium that contained 3.0 μM carrier sulfate per ml and radiosulfate with a count of 10500 counts/min/ml.

of cells that have not reached identical physiological ages (Fig. 2). The striking differences between the two cell types vanished at the onset of the deceleration phase of growth, about 5 days after inoculation. In addition, the trichloroacetic acid-soluble ^{35}S fraction all but disappeared, with as much as 90% of the absorbed radioactivity being incorporated into protein. These curves point to the need for comparing the two cell lines at a number of physiological ages rather than at some arbitrarily chosen harvest time.

Sulfate uptake

The pattern of sulfate absorption and metabolism into protein by the two types of cells is illustrated in Fig. 3. No significant differences were detected. As with methionine absorption, the rate of sulfate absorption was related to the age of the culture from which cells were taken. The size of the soluble sulfur pool, however, remained relatively unchanged with increasing physiological age of the cells.

These results were confirmed from periodic analyses of cells grown with radiosulfate added at the time of inoculation. A high level of carrier sulfate ($3 \cdot 10^{-3}$ M) was used to preclude exhaustion of the ion. The total sulfur content was the same in both strains, as was the amount of ^{35}S incorporated into protein. Both total sulfur and protein sulfur dropped considerably with increasing age of the cultures (Fig. 4).

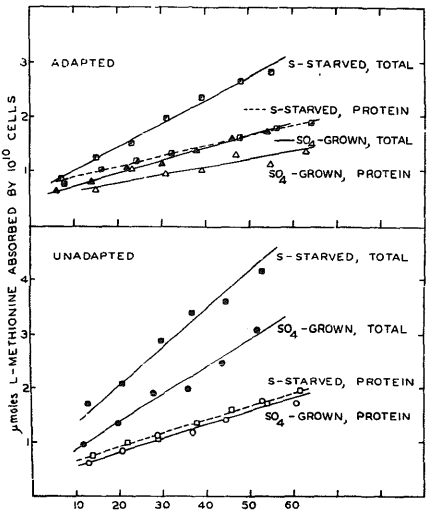

Fig. 5. Effect of sulfur starvation on L-methionine absorption and ^{35}S incorporation into protein by unadapted and selenomethionine-adapted *Chlorella vulgaris*. Cells were harvested after 1 day of sulfur starvation. Population densities had increased from $2.0 \cdot 10^6$ to $2.2 \cdot 10^6$ cells/ml. The incubation media contained 10 μg L-[^{35}S]methionine per ml with a count of 8000 counts/min/ml.

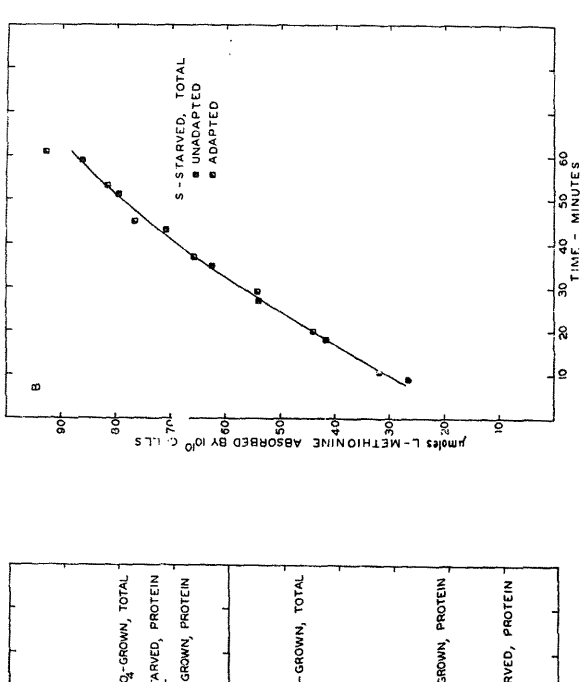

Fig. 6. Effect of sulfur starvation on L-methionine absorption and ^{35}S incorporation into protein by unadapted and selenomethionine-adapted *Chlorella vulgaris*. Cells were harvested after 2 days of sulfur starvation. Population densities had increased to $1.0 \cdot 10^8$ cells/ml in sulfur-starved cultures to $1.4 \cdot 10^8$ cells/ml in sulfate-grown cultures. Note change in ordinate scale for total absorption by sulfur-starved cells (B). The incubation media contained 10 μg L-[^{35}S]methionine per ml with a count of 8000 counts/min/ml.

Effect of sulfur starvation on methionine uptake

Methionine and selenomethionine are practically identical in structure, and they are interchangeable in several biochemical systems[12–15]. Previous studies have indicated that they may also be competitive during absorption into the *Chlorella vulgaris* cell[16]. It can therefore be inferred from the induced loss of permeability to methionine that a decreased permeability to selenomethionine was also incurred in the adapted cell, this loss being the basis of the resistance to the analogue. Since sulfur starvation causes adapted cells to deadapt[9], such treatment should also cause the adapted cells to regain their capacity to absorb methionine. (A more direct test with selenomethionine, though preferable, could not be carried out because radioactive selenomethionine was unavailable.)

Under conditions of sulfur starvation, endogenous sulfur pools and traces of sulfur in the medium are sufficient to allow several divisions during the first 2 days of incubation. After about 1 day of sulfur starvation increases in cell number, size, and weight equaled those in sulfate-containing cultures, and there were significant elevations in the rate of methionine absorption by both strains of cells (Fig. 5). Metabolism of ^{35}S into protein of the sulfur-starved unadapted cell remained unchanged, but in the sulfur-starved, adapted cell the rate had increased. After 48 h of sulfur starvation, cells cease to divide and chlorosis becomes evident. By this time the rate of methionine uptake had risen steeply but was no longer linear (Fig. 6B); in unadapted cells the absorption per h was 17 times greater, and in adapted cells 52 times greater than in the respective sulfate-grown cells. In both cell strains most of the ^{35}S remained soluble in cold trichloroacetic acid, and there was a decrease in the rate of ^{35}S incorporation into protein (Fig. 6A). This decrease became even more marked with longer starvation of the cells (Fig. 7). Two successive events, therefore, developed during the course of sulfur starvation in the adapted cells: (1) methionine uptake was restored and a faster rate of ^{35}S incorporation into protein was observed; (2) during extended starvation an impairment to the protein synthesizing system

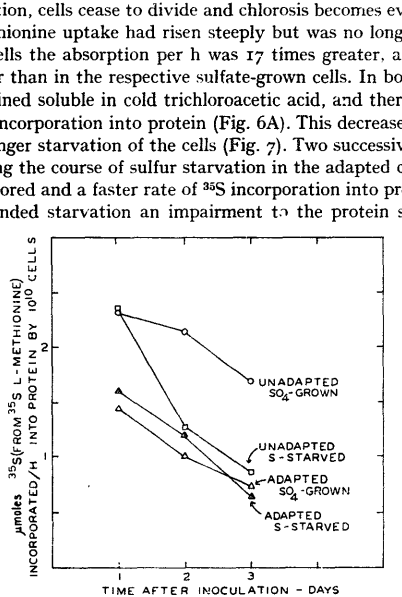

Fig. 7. Changes in ^{35}S incorporation (derived from L-[^{35}S]methionine) into protein of unadapted and selenomethionine-adapted *Chlorella vulgaris* grown with and without sulfate.

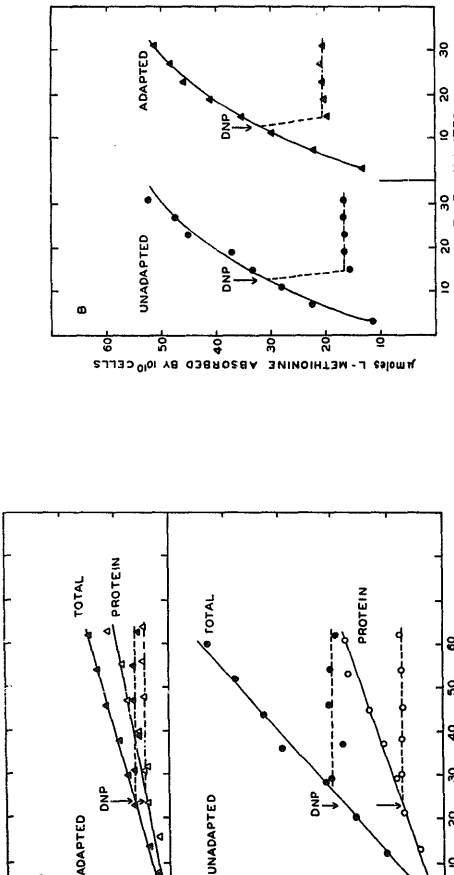

Fig. 8. Effect of 2,4-dinitrophenol on L-methionine absorption by *Chlorella vulgaris*. Cells were harvested from cultures that were 3 days old. The incubation medium contained 10 μg L-[^{35}S]methionine per ml with a count of 8500 counts/min/ml. The concentration of 2,4-dinitrophenol was $2 \cdot 10^{-5}$ M. A, sulfate-grown cells; B, sulfur-starved cells.

developed. The greater absorption could not be attributed to increased cell surface, since abnormal cell enlargement was not seen during the starvation treatment.

Effect of dinitrophenol on L-*methionine*

An active absorption process was indicated by the rapid accumulation of trichloroacetic acid-soluble radioactivity to cellular levels much higher than the initial exogenous methionine concentration (Table I). 2,4-Dinitrophenol, an agent that prevents ATP synthesis[17], and which could be expected to cut off the energy supply for active transport, was therefore tested for its effects on methionine uptake. Almost immediately after addition of DNP, absorption of the amino acid stopped (Fig. 8A).

TABLE I

ACCUMULATION OF COLD TRICHLOROACETIC ACID SOLUBLE ^{35}S BY *Chlorella vulgaris*

External concentration of L-[^{35}S]*methionine*	*μM S per ml of packed cells**			
	Unadapted		*Adapted*	
	Sulfate grown	*Sulfur starved*	*Sulfate grown*	*Sulfur starved*
0.067 μM/ml	0.95	36.3	0.22	31.5

* Calculated from data graphed in Figs. 6A and 6B.

Sulfur-starved cells, in addition, exhibited a noticeable drop in their total radioactivity content (Fig. 8B). Part of the absorbed methionine appears to have been bound loosely enough to be released from the cell after DNP was added, or perhaps during the washing procedure. The possibility that the DNP had been lethal was checked by inoculation of inhibited cells into DNP-free medium; growth continued at the normal rate.

DISCUSSION

The low permeability to methionine shown by the selenomethionine-adapted *Chlorella vulgaris* suggests that a methionine "permease"*, or "carrier"*, responsible for the active uptake of this amino acid, has been repressed or is non-functional. The active nature of the absorption is seen from its inhibition by DNP. Since methionine and selenomethionine are so similar, it can be concluded that inability of the selenomethionine to penetrate fast enough to exert its toxic effect is responsible for the induced resistance. The earlier suggestion[9] that the adapted cells are induced to synthesize methionine from sulfate at a higher rate, and in this way prevent the absorbed selenomethionine from exerting its effect, is not borne out by the present experiments. Had there been a higher rate of endogenous methionine formation, much less of the radioactive methionine should have appeared in the proteins of the adapted cell, owing to endogenous dilution. Instead, 75% or more of the absorbed methionine sulfur was incorporated into protein by the adapted cells, in contrast to the 35% utilized by the unadapted cell.

* The term "permease", generally used by microbiologists[18], and the term "carrier", generally used by plant physiologists[19], are equivalent. Both were introduced to describe a component in the cell membrane responsible for the active absorption of exogenous substances.

The two types of cells do not appear to differ grossly in their metabolism of exogenous [^{35}S]sulfate as measured by the techniques of these experiments. Even the utilization of whatever methionine did penetrate the adapted cell seems to be the same as in the normal cell: almost all of the methionine entering the adapted cell occurred in the proteins; after a brief period of sulfur starvation, the rate at which methionine sulfur was incorporated into protein increased, as would be expected to result from the restored permeability. Crypticity, a condition in which a cell, though it contains the necessary enzymes, is unable to absorb a nutrient[18,20], appears to have been induced in these cells.

Although the overall metabolisim of inorganic sulfate seems the same, subtler differences in the sulfur pathways of the two cell types must exist, because sulfur starvation renders the adapted cell once more sensitive to selenomethionine. Since this treatment also restores permeability to methionine, and undoubtedly to selenomethionine, it is likely that the sulfur pathway controls the synthesis or functioning of the methionine permease. Selenomethionine, therefore, may be responsible only for an initial triggering of a change in the sulfur pathway which in turn is more directly linked to control of the methionine permease. As long as sulfate is present, even in the absence of selenomethionine, the cells maintain the induced phenotype. By analogy with the effects of methionine in bacteria[21–23], selenomethionine might be expected to mimic methionine and repress enzymes that lead to the synthesis of methionine from sulfate. However, all known cases of repression are reversible soon after removal of the metabolite[20].

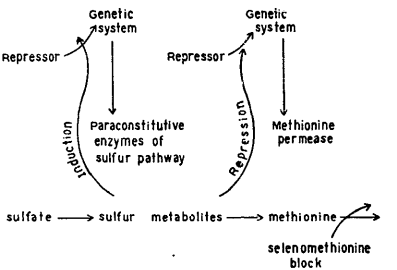

Fig. 9. Model to describe a hypothetical mechanism for the induction and maintenance of resistance to selenomethionine in *Chlorella vulgaris*. For explanation see DISCUSSION.

The mechanism for the induction of the new pherotype, its maintenance, and its reversibility by sulfur starvation is not, of course, fully known, but two hypotheses bear discussion. The first, for which a model is given in Fig. 9, is based on the model of JACOB AND MONOD (ref. 20, p. 344) which describes genic and cytoplasmic controls of repression and induction in bacteria.

In our model, selenomethionine, on being absorbed by the algal cell, is assumed to create a metabolic block which causes sulfur metabolites to accumulate. The endogenous accumulation, in turn, induces a greater synthesis of certain of the

enzymes responsible for the further metabolism of one or more of the sulfur compounds. These enzymes are considered to be paraconstitutive, a state described by SZILARD in which enzymes are neither completely repressed nor at their maximally induced level[24]. As long as sulfate is given to the cells, this new level as well as a larger pool of the sulfur metabolites can be maintained. The methionine permease in this model is also assumed to be under repression control by one or more of the sulfur metabolites. The induced higher steady-state level would then repress the permease indefinitely unless some environmental change such as sulfur starvation caused endogenous sulfur levels to decrease.

An alternative explanation for the maintenance and reversal effects is based on the possibility that contaminating traces of Se are absorbed by the algal cells from the culture medium. Calculations show that by the 27th generation after removal of the analogue, if complete absorption occurs during the initial exposure, each cell would contain 1 molecule of the original selenomethionine. By the sixth subculture all cells which contain the 1 molecule would have been eliminated from the population*. Traces of Se, however, are ubiquitous, and the possibility cannot be overlooked that the growth medium includes contaminating traces of inorganic Se compounds which may have been biosynthesized into selenomethionine. No data are at present available on the Se content of the *C. vulgaris* growth medium. However, OKADA[25], using neutron activation, estimated the Se content of an ammonium sulfate sample as less than 0.05 ppm. If K_2SO_4, probably the major source of Se contamination in the Chlorella medium, contains this trace level it can be estimated that each Chlorella cell at maximum population density would contain 1000 atoms of Se**. In this respect, POLLOCK[26] has calculated that as few as 100 penicillin molecules per cell of *Bacillus cereus* are needed for maximum induction of penicillinase, and that as long as there is only 1 molecule bound per cell some production of the induced enzyme continues. Whether the 1000 Se atoms alone could be responsible for the maintenance effect is unknown, but cannot be ruled out at present.

ACKNOWLEDGEMENT

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* The inducing concentration of selenomethionine (mol.wt. 196) is 6 μg/ml or $1.84 \cdot 10^{16}$ molecules/ml. Inocula are kept at $2.0 \cdot 10^5$ cells/ml. Cultures increase to a maximum population of $2.0 \cdot 10^8$ cells/ml, the equivalent of 10 generations. At the end of the first passage, each cell will contain $9.2 \cdot 10^7$ molecules of the analogue, assuming complete absorption. A second passage would yield cells with essentially the same number of molecules, since the selenomethionine absorbed during the initial passage would have been diluted to $9.0 \cdot 10^4$ molecules/cell after 10 generations. On subculture in the absence of selenomethionine, the $9.2 \cdot 10^7$ molecules/cell will have been diluted to about 1 molecule/cell by the 27th generation. By the 30th generation $2.6 \cdot 10^7$ cells in the population of $2.0 \cdot 10^8$ cells/ml will contain 1 molecule. Each ml of the inoculum for the next subculture will have only $2.6 \cdot 10^4$ cells that contain 1 analogue molecule. By the end of the subculture this number per ml will remain the same. At the fifth passage only 26 cells in the inoculum of $2.0 \cdot 10^5$ cells will contain 1 selenomethionine molecule. By the sixth passage these cells will have been diluted out of the culture.

** The K_2SO_4 concentration of the liquid medium is $3.0 \cdot 10^{-3}$ M or 545 μg/ml. If the Se content of the K_2SO_4 is assumed to be 0.05 ppm, there would be $2.7 \cdot 10^{-5}$ μg of Se or $2.1 \cdot 10^{11}$ atoms/ml. If all the Se is absorbed, each of the $2 \cdot 10^8$ cells would therefore contain 1000 atoms of Se.

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