TRANSIENT INHIBITION BY CYCLOHEXIMIDE OF PROTEIN SYNTHESIS IN CULTURED PLANT CELL SUSPENSIONS : A DOSE RESPONSE PARADOX

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SUMMARY

Rosa cell suspension cultures supplied with high concentrations (>10^{-5}M) of cycloheximide (CH) rapidly develop a resistance to the drug under conditions in which protein synthesis is initially severely impaired. CH at 10^{-6}M, while equally effective in inhibiting protein synthesis, is markedly less efficient in inducing resistance. Development of resistance is not due to selection, or to induction of the capacity to metabolize the drug. Cells washed free of CH retain their resistance over several generations before reverting to the CH-sensitive condition.

Numerous studies on metabolic regulation in eukaryotes rely heavily on the use of antibiotic inhibitors of translation, of which cycloheximide (CH) is one of the more important. The validity of interpretations based on such studies is therefore limited by the precision with which these inhibitors interfere with cell metabolism. Cycloheximide is reported to be a specific inhibitor of protein synthesis on 80s ribosomes, where it appears to bind to the 60s subunit and inhibits initiation and peptide chain elongation (1). Other important, and possibly secondary, effects of the drug include inhibition of RNA and DNA synthesis (2).

The reaction of higher plant tissues is somewhat anomalous since, although almost universally inhibitory to *in vivo* protein synthesis, *in vitro* syntheses mediated by certain plant ribosomal preparations are insensitive to CH (3, 4, 5). Furthermore, for some plant tissues CH seems primarily to disrupt energy transfer in respiration (6). The present communication reports on a further phenomenon, in which cultured plant cells develop a resistance to the drug, and describes the paradoxical effect of CH concentration on the acquisition of this resistance.

MATERIALS AND METHODS

The techniques for the culture of Paul's Scarlet rose and for the estimation of growth parameters have been previously described (7). Details of the ^{14}C-leucine incorporation assay are given in Fig. 1. Protein contents of cell extracts were determined by the method of Lowry et al (9).

The CH contents of culture media and chloroform extracts of cells were determined by bioassay with Saccharomyces cerevisiae (N.C.Y.C. 1073) on large agar diffusion plates, essentially as described by Whiffen (10), but with a single, seeded layer and employing cut wells for test solutions in place of the filter paper discs. The lower limit of detection was about 1 µg CH/ml. Cycloheximide was obtained from the Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Previous results using CH at 10^{-4} M indicated that although initially a potent inhibitor of the development of phenylalanine ammonia-lyase in rose cell suspensions, the effect was only temporary, and synthesis of the enzyme was resumed at original rates within 48 h (11). The same pattern of response is shown by the cells with respect to ^{14}C-leucine incorporation. At CH levels in excess of 10^{-5} M some recovery from the initially severe inhibition of protein synthesis is obvious 4 - 10 h after addition of the drug, with virtually complete recovery after 24 h (Fig. 1). In contrast, CH at 10^{-6} M shows a more persistent inhibition of ^{14}C-leucine incorporation, although considerable recovery does occur over the second day of treatment (Fig. 2).

Fig. 2 also shows that all cultures which recover from CH inhibition reveal a markedly diminished sensitivity when challenged with a second, large dose of the drug. Two significant features of this development of CH resistance should be noted. Firstly, the response occurs at all stages of the growth cycle, including stationary phase cultures in which cell divisions are almost completely absent. Secondly, CH does not appear to be accumulated or degraded by the cells to any appreciable extent. In cultures treated with 2×10^{-4} M CH (Fig. 3A) a decrease in the CH concentration of the medium was barely detect-

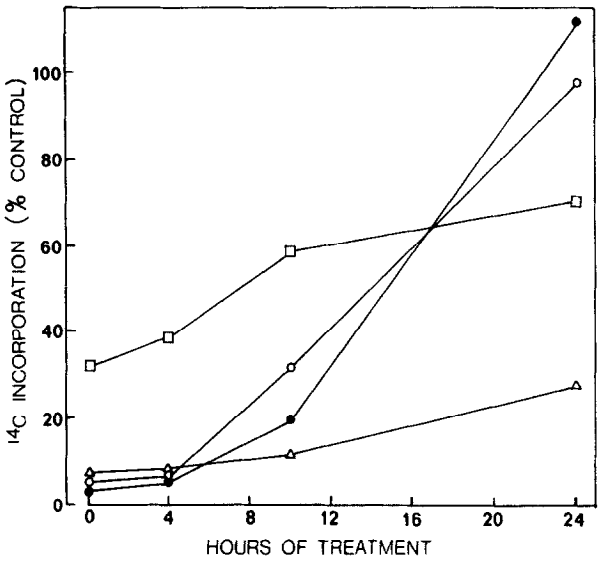

Figure 1. Effect of CH concentration on ^{14}C-leucine incorporation. CH pre-treatments and incorporation assays were performed in 25 ml flasks containing 3 ml of a 3-day-old cell suspension (c. 0.3 g F.Wt.) and CH at the following concentrations:- 10^{-3} M (—●—); 10^{-5} M (—○—); 10^{-6} M (—△—) and 10^{-7} M (—□—). At each sampling period, duplicate flasks of each treatment were incubated with 0.5 µCi L-leucine-U-^{14}C (10 µCi/umole) at 28° for 1 h. Cells harvested in plastic filtration tubes were then subjected to a preliminary extraction with successive, 7 ml portions of 10% trichloracetic acid (x2), 80% ethanol (x3), 100% ethanol (x2) and acetone, using the apparatus previously described (8). Dried cell residues were digested with 0.3 N KOH and aliquots transferred to glass fibre discs for liquid scintillation counting, using a toluene based PPO/POPOP phosphor. The incorporation rate (cpm/mg protein/h) in supplemented cultures is expressed as a percentage of untreated controls.

able after 4 days of treatment, and washed cells used for inoculation of the first transfer (Fig. 3B), while showing a high degree of CH-resistance, were also shown to contain approximately 2 µg CH/g F.Wt.

The induced resistance is itself only temporary, sensitivity to the drug being re-established within a few generations following withdrawal of CH (Fig. 3C). It is noteworthy that again the fairly rapid transition from one condition to the other can occur in an essentially non-dividing stage of the growth cycle, in the present case the lag phase.

DISCUSSION

The paradoxical effects of CH concentration on protein synthesis in rose cell suspensions, where low levels of the drug show a more persistent inhibition

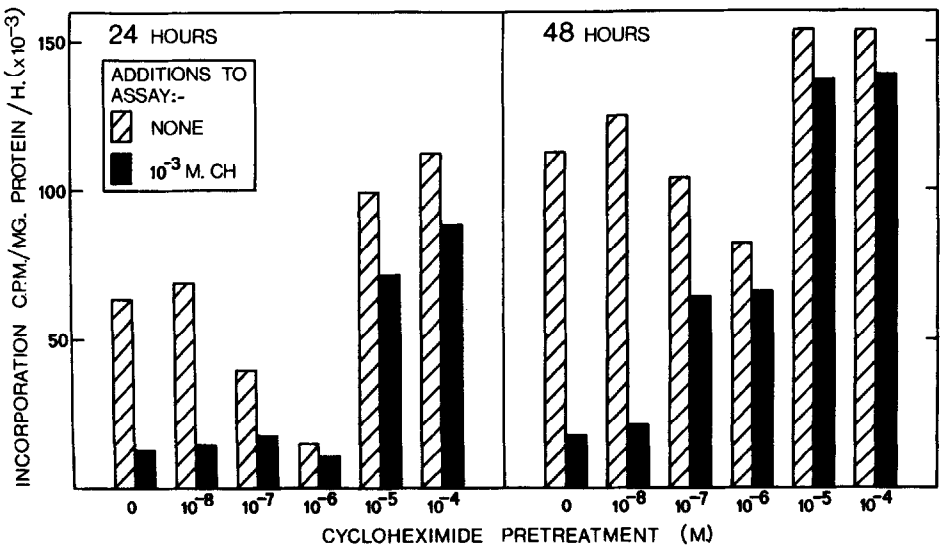

Figure 2. Time course of development of resistance to high CH concentrations. Four-day-old cultures, treated with the indicated concentration of CH for 24 h and 48 h, were tested for ability to incorporate ^{14}C-leucine, with and without further supplementation with 10^{-3} M CH. Sampling and assay procedures as described in Fig. 1.

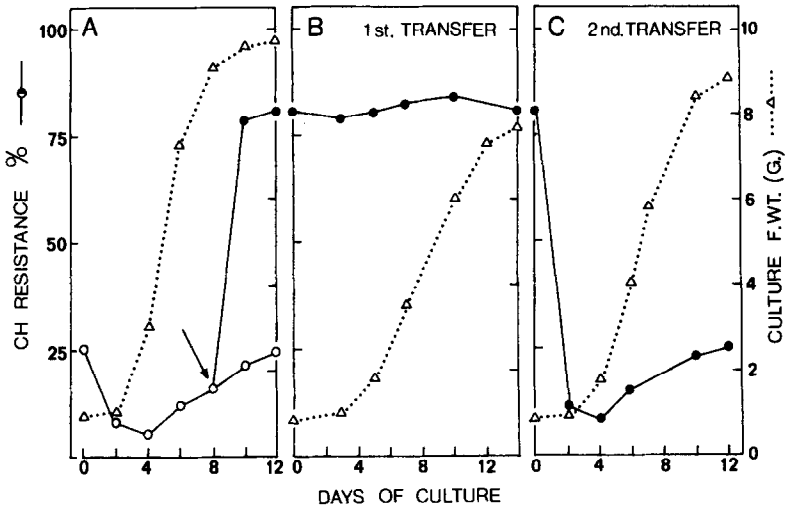

Figure 3. Effect of removal of CH on the duration of resistance. Cultures in the eighth day of the treatment cycle (A) were supplemented with 2 x 10^{-4} M CH. Four days later the treated cells were harvested and washed with five 200 ml portions of sterile H_2O under aseptic conditions. Washed cells were resuspended in sterile growth medium and used as inoculum for the 1st. transfer passage (B). Fourteen-day-old cells of this passage were then used directly for inoculation of the second transfer (C). CH resistance, defined as the incorporation rate observed in assay mixtures containing 10^{-3} M CH expressed as a percentage of that observed in its absence, was determined at the indicated times, using the assay procedures given in Fig. 1. Cultures pretreated with 2 x 10^{-4} M CH at the point indicated (—●—); cultures not given CH pretreatment (—○—). Periodic sterility checks were used to establish the absence of microbial contamination.

suggests the involvement of two partly related processes. Firstly, there is the initial inhibition of protein synthesis, presumably a primary response since CH has little immediate effect on respiration (12). The second response, slower than the first and requiring a higher CH threshold, leads to the release of the protein-synthesis machinery from this initial inhibition. Of the likely explanations for this latter development, the obvious possibility of selection of a CH-resistant cell line can be discounted. Development of resistance occurs in non-dividing populations and, moreover, the time course of recovery in exponential phase cells is much shorter than the minimum mean generation time of such cultures (7). Equally untenable is the hypothesis that the cells, in the presence of high CH levels, develop the ability to degrade or sequester the drug, for in such a case the rate of recovery would be expected to be inversely proportional to the drug concentration. In addition, the results indicate that CH is not extensively accumulated or degraded by the cells. Other possible explanations for the phenomenon include : (a) a modification of the permeability characteristics of the cell membrane leading to drug exclusion, and (b), an induced change in the sensitive component of the synthetic apparatus, which, on the basis of current views on the site of CH inhibition, is possibly located on the heavy ribosomal subunit (1). The first suggestion cannot be completely excluded on the basis of the present information, but seems unlikely in view of the demonstrated presence of CH in resistant cells. The second hypothesis remains a distinct possibility, although the nature of the modification is open to speculation. Desensitization of the system by de novo synthesis of a resistant ribosomal component would require the participation of protein synthesis, and this seems unlikely to be accomplished in a situation in which up to 97% of the synthetic potential is inhibited. As an alternative, it is suggested that development of resistance could arise from a rearrangement of existing protein(s), possibly through an allosteric interaction resulting in a reduced affinity for CH at the inhibitory site.

The ability of micro-organisms to develop a resistance to a variety of

antibiotics is well documented, and has largely been shown to be due to selection of resistant mutants. But in at least one case, namely the development of erythromycin resistance in Staphylococcus aureus, there is evidence for the rapid conversion of the majority of cells within the population to the resistant condition (13). In some respects, this non-selective development of erythromycin resistance is similar to the phenomenon reported here, but with one important difference; the induction of erythromycin resistance occurs only at low and essentially non-inhibitory levels of the antibiotic, and its development can be prevented by other protein synthesis inhibitors.

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