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Cycloheximide Is Not a Specific Inhibitor of Protein Synthesis *in Vivo*[1]

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ABSTRACT

Cycloheximide is frequently presumed to inhibit specifically the cytoplasmic protein synthesis of eukaryotes. Although previous investigators have shown that it had other effects on the cells of a variety of organisms, these results were frequently presumed to be secondary effects of the inhibition of protein synthesis. This paper shows that a wide range of deleterious effects are produced by cycloheximide on a single organism, *Chlamydomonas reinhardi* Dangeard. If, protein synthesis is inhibited by nonpermissive conditions in temperature-sensitive mutants or with other treatments these "secondary" effects are not produced. Instead, cycloheximide appears to have two or three independent inhibitory effects on the cell. Moreover, in contrast to a number of previous investigations, these results show that protein synthesis is not required for RNA synthesis. Instead the rate of RNA synthesis is actually increased by interference with protein synthesis.

Cycloheximide is widely used as an inhibitor of protein synthesis *in vivo* and *in vitro*. Although it does inhibit synthesis of proteins on cytoplasmic ribosomes *in vitro*, some ambiguity remains regarding its mechanism of action. It has variously been described as an inhibitor of initiation, elongation, and chain termination (1, 23, 30). Cycloheximide has been used in a very large number of studies *in vivo*. These studies have generally attempted to use CH[2] to answer one of two questions. Is a particular protein made on ribosomes of the cytoplasm or on those of an organelle? Is *de novo* synthesis of proteins required for some biological process? Accurate answers to either question depend upon the assumption that CH inhibits only protein synthesis in the living cell. Insofar as the effects of CH deviate from this ideal, corresponding care must be taken in interpreting its inhibitory effects.

Many investigators have shown that CH interferes with processes other than protein synthesis. It inhibits DNA synthesis (2, 4). It does not inhibit RNA synthesis in the alga, *Acetabularia mediterranea* (3), but this organism is an exception for it inhibits the synthesis of RNA in *Neurospora crassa* (34), yeasts (6, 22, 32), *Dictyostelium discoideum* (16), rat (11, 29), monkey (8), and man (17, 36). Cycloheximide interferes with other cellular functions including respiration (9, 21), ion absorption (21), amino acid uptake (7, 15, 33), and the interconversion of pyrimidine nucleotides (31). Although it is often believed to be easily reversible, some studies have led to the opposite conclusion (3, 6, 36) and, in fact, it kills yeast at a rate which is proportional to their metabolic activity (19, 35).

Many investigators (4, 6, 8, 11, 22, 32) have suggested that these processes are inhibited because concurrent protein synthesis is required for their continuation. For example, inhibition of protein synthesis may reduce the rate of respiration by reducing the cell's demand for ATP or labile proteins may be required for RNA synthesis and ion uptake. Because this reasoning may be flawed, I have re-examined this question in *Chlamydomonas reinhardi*. This study has demonstrated that CH can have a very broad array of deleterious effects on *C. reinhardi* but that other treatments which interfere with protein synthesis do not necessarily produce these effects on the cell. Because these results were obtained with concentrations of CH (and times of treatment) quite comparable to those frequently used, inhibitory effects of CH on a cellular process may be uninterpretable and only the lack of inhibition of a process by CH may be meaningful providing that CH enters and inhibits protein synthesis in the cell in question.

MATERIALS AND METHODS

All procedures or materials which are not described here have been presented in previous papers (24–26). Nucleotide pool sizes were determined on cells growing in growth medium (25) containing one-tenth of the normal content of phosphate plus 1 μCi/ml of $H_3{}^{32}PO_4$. Cells grow at normal rates for more than 24 hr in this medium. The nucleotides were extracted with repeated freezing and thawing in 1 N acetic acid and chromatographed on plates of polyethyleneimine cellulose (28). Three strains of *Chlamydomonas reinhardi* Dangeard (mt^+) have been used. One, *arg 2*, is an arginine *requiring* auxotroph defective in arginosuccinate synthetase (14). The others are mutants derived from *arg 2* (24). One of these, *ts 13*, is a mutant conditionally defective in the initiation of protein synthesis. The lesion of the other mutant, *ts 1*, is undefined with the exception that it is defective in some step of polypeptide elongation and that all of the amino acyl tRNA synthetases of this mutant appear to be normal (J. Cross and D. McMahon, unpublished results).

Cycloheximide was obtained from CalBiochem and Sigma Chemical Co. Both preparations had identical properties. Radioactive chemicals were the products of Schwarz/Mann or New England Nuclear Co. Unless otherwise noted, tritium-labeled adenine (3-^{3}H) and arginine (2-^{3}H) were used at spe-

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[2] Abbreviation: CH: cycloheximide.

cific radioactivities of 1.68 Ci/mmole or 7 mCi/mmole, respectively.

RESULTS

Cycloheximide rapidly inhibits protein synthesis in *C. reinhardi* (12, 20) (Fig. 1) at concentrations which are similar to those effective against many other organisms and against ribosomes *in vitro*. The extent of inhibition of protein synthesis by 1.8×10^{-6} M cycloheximide ranged from 80 to 90% in this and other experiments, whereas 7.1×10^{-6} and 3.6×10^{-5} M cycloheximide inhibited 92 to 99% of protein synthesis. The extent of this inhibition indicates that CH is probably also inhibiting protein synthesis in organelles, either directly or indirectly. In contrast to the results of Hoober *et al.* (12), chloramphenicol or erythromycin can inhibit about 30% of protein synthesis in *C. reinhardi* (unpublished results). If we assume that this represents chloroplast and mitochondrial protein synthesis (18), the effect of CH on organelle protein synthesis would be substantial. The two higher concentrations completely inhibited multiplication of cells while the lowest one did not. These concentrations of CH also inhibited the incorporation of ^{3}H-adenine into nucleic acids. A concentration of 1.8×10^{-6} M inhibited incorporation into RNA by more than 50% and into DNA by more than 65% (Fig. 2). The higher concentrations initially inhibited RNA accumulation by more than 65% and completely inhibited accumulation by 2 hr. One-third of the RNA labeled under these conditions is tRNA and the remaining two-thirds are rRNA.

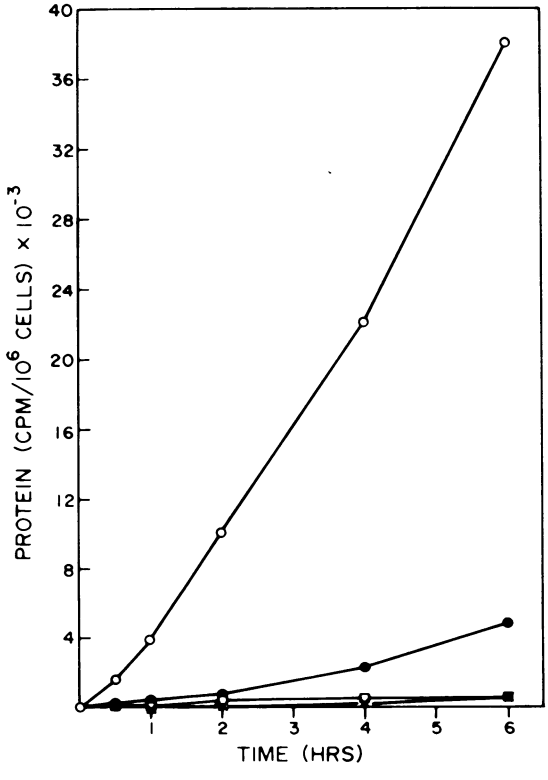

FIG. 1. The effects of different concentrations of cycloheximide on protein synthesis. Cells of *arg 2* (4.3×10^5 cells/ml) were incubated in growth medium containing ^{3}H-arginine (0.2 μCi/ml) with: nothing in addition (○); 1.8×10^{-6} M cycloheximide (●); 7.1×10^{-6} M cycloheximide (□); 3.6×10^{-5} M cycloheximide (×); and 1 ml samples were added to equal volumes of 10% trichloroacetic acid at intervals. These were then washed and prepared for scintillation counting.

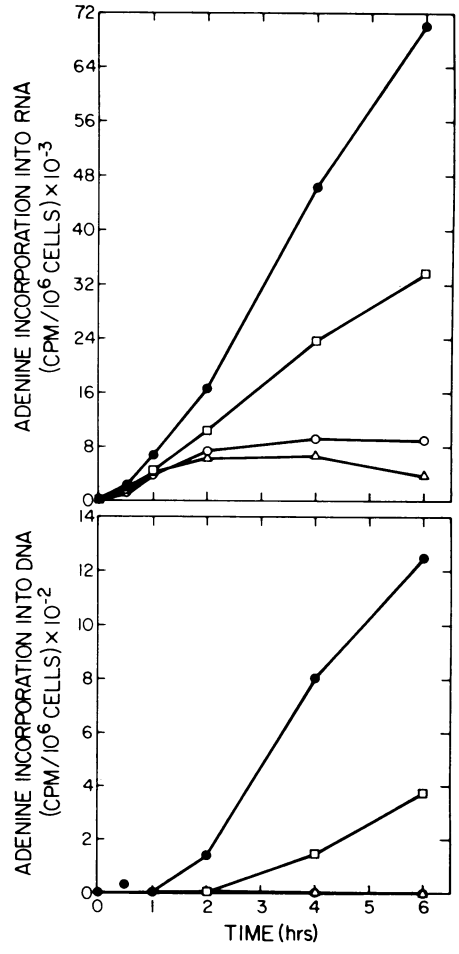

FIG. 2. Cycloheximide inhibits the accumulation of both RNA and DNA. *Arg 2* cells were incubated in a medium which contained arginine and 0.4 μCi/ml of ^{3}H-adenine (22 Ci/mmole) with: nothing in addition (●); 1.8×10^{-6} M cycloheximide (□); 7.1×10^{-6} M cycloheximide (○); 3.6×10^{-5} M cycloheximide (△). At intervals samples were added to an equal volume of 20% trichloroacetic acid and their incorporation into total nucleic acid and DNA was measured. Incorporation into RNA (top panel) was determined by subtracting the incorporation into DNA (bottom panel) from that into total nucleic acid. The initial cell concentration was 6.1 $\times 10^5$ cells/ml.

The rRNA is composed of 90% cytoplasmic rRNA and 10% organelle (largely chloroplast) rRNA (27). Therefore CH prevents the accumulation of tRNA and of cytoplasmic and chloroplast rRNAs. DNA synthesis is completely inhibited by concentrations of either 7.1×10^{-6} or 3.6×10^{-5} M. There is no detectable lag before CH affects DNA synthesis (Fig. 3) and a lag of 0.5 hr, at most, before it affects RNA accumulation.

Since the inhibition of ^{3}H-adenine into nucleic acids could result from a change in the specific radioactivity of nucleotide precursors, I examined the incorporation of $^{32}PO_4$ into nucleic acids. The effects of CH on the accumulation of ^{32}P into RNA were identical (data not presented). Cycloheximide was not inhibiting the apparent rate of macromolecular synthesis by preventing the uptake of precursors into the cell. It stimulated the uptake of arginine and very slightly inhibited adenine uptake (Table I). Treatment with CH did not significantly affect the specific radioactivities of the nucleoside triphosphate precursors of RNA and DNA (Table II). Therefore the effects of CH on the accumulation of label in RNA

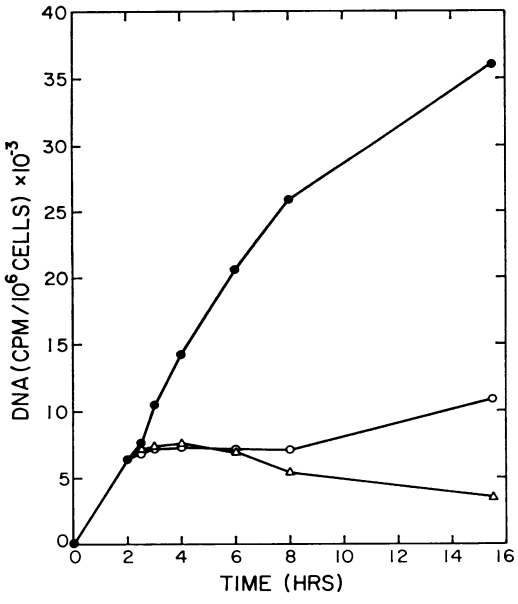

FIG. 3. Cycloheximide rapidly inhibits DNA synthesis. *Arg 2* cells (6.7×10^5/ml) were incubated with ^{3}H-adenine for 2 hr as described in the legend to Figure 2. Then aliquots were made 7.1×10^{-6} M (○) or 2.6×10^{-5} M (△) in cycloheximide, or were left untreated (●). At intervals these were sampled for their incorporation of ^{3}H-adenine into DNA.

Table I. *Uptake of Precursors into Cells in Presence of Cycloheximide*

Cells of *arg 2* were preincubated in the presence or absence of cycloheximide for 30 min and then the uptake of arginine (24) or adenine was measured. To measure uptake of adenine, cells were incubated in medium which contained 2.37×10^{-7} M adenine. The experiment was begun by adding 0.8 μCi/ml of ^{3}H-adenine (26.6 Ci/mmole). The cells were collected at intervals and washed as previously described (24) with medium which contained 5.7×10^{-4} M arginine and 4.7×10^{-6} M adenine. Uptake of precursor is presented as cpm/10^6 cells.

Precursor	Time	Cycloheximide Concn		
		None	7.1×10^{-6} M	3.6×10^{-5} M
	min			
Arginine	10	9168	21288	14510
	20	19662	33173	43854
	30	24868	39402	29408
Adenine	10	9740	8956	10045
	20	19128	17218	16904
	30	30462	28318	26295

did not result from changes in the specific radioactivities of nucleotide precursors.

The decrease in the rate of accumulation of RNA might reflect an inhibition of synthesis or a stimulation of breakdown of the synthesized products. Both, in fact, occur. Figure 4 shows that CH inhibited the rate of RNA synthesis within 1 hr and that the extent of inhibition varied between 25 to 60%. A fraction of RNA synthesis was resistant to inhibition by CH since a 5-fold increase in CH concentration did not inhibit the rate of RNA synthesis. Cycloheximide also causes the breakdown of nucleic acids (Fig. 5). There is a delay of 4 to 6 hr before RNA begins to be degraded and of 6 to 8 hr before DNA breakdown begins.

Since CH stimulates the breakdown of DNA, one might expect that CH would kill cells. This was indeed the case. Figure 6 shows that 7.1×10^{-6} M cycloheximide killed cells exponentially with a rate constant of 0.14 hr^{-1}. The rate increased by more than two times (to 0.32 hr^{-1}) when the concentration of CH was increased by five times. The rate of killing was unaffected by starvation for arginine, a treatment which inhibits net protein synthesis and cell division and which partially inhibits DNA synthesis and accumulation of RNA (26). Cycloheximide did not completely disrupt cellular function, for after 24 hr in 3.6×10^{-5} M cycloheximide, 20 to 50% of the cells remained motile although less than 0.1% formed colonies when plated. Irreversible lesions in macro-

Table II. *Effects of Cycloheximide on Nucleoside Triphosphate Pools*

Cells were grown for 16 hr in low phosphate medium containing 1 μCi/ml ^{32}P and were then centrifuged and resuspended in the same medium. Cycloheximide was added to part of the resuspended cells in addition to 0.4 μCi/ml of ^{3}H-adenine. After 2 hr the cells were harvested and extracted. Then nucleotides were chromatographed and all measurements normalized to the size or specific radioactivities of the ATP pool in the untreated control.

	No Cycloheximide			7.1×10^{-6} M			3.5×10^{-5} M		
	Mean	SD		Mean	SD		Mean	SD	
	Relative Pool Size								
GTP	9.68	3.3	(3)[1]	7.69	1.6	(2)	6.56	3.4	(3)
ATP	100		(3)	58.2	5.5	(2)	62.6	11	(3)
UTP	30.4	21	(3)	26.8	12	(2)	20.5	11	(3)
CTP	8.62	4.8	(3)	6.27	2.4	(2)	5.73	6.1	(3)
dGTP	3.47	4.0	(3)	1.06	0.04	(2)	2.46	3.2	(3)
dATP	2.60	1.9	(3)	0.85	0.21	(2)	1.37	0.88	(3)
	Relative specific radioactivities (mole ^{3}H/mole nucleotide)								
GTP	29.8	5.3	(3)	29.7	8.2	(2)	20.8	10	(3)
ATP	100		(3)	87.3	51	(2)	79.8	52	(3)
UTP	6.68	4.2	(3)	17.4	12	(2)	11.7	14	(3)
CTP	17.4	11	(3)	8.06	6.9	(2)	22.7	34	(3)

[1] No. of determinations are shown in parentheses.

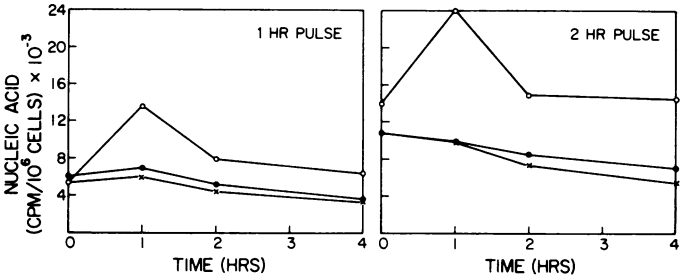

FIG. 4. Cycloheximide partially inhibits the rate of RNA synthesis. *Arg 2* cells (1.1×10^6/ml) were incubated in medium containing arginine with: nothing in addition (○); 7.1×10^{-6} M cycloheximide (●); (×), 3.6×10^{-5} M cycloheximide (×). The cells were incubated in these solutions for the periods of time indicated by the abscissa of the graph and then aliquots were added to enough ^{3}H-adenine to produce a concentration of 0.4 μCi/ml. After 1 hr (left panel) or 2 hr (right panel), the incorporation of ^{3}H-adenine into total nucleic acid was measured. This incorporation is almost totally into RNA because there is a 1 to 2 hr lag before incorporation into DNA begins to be significant.

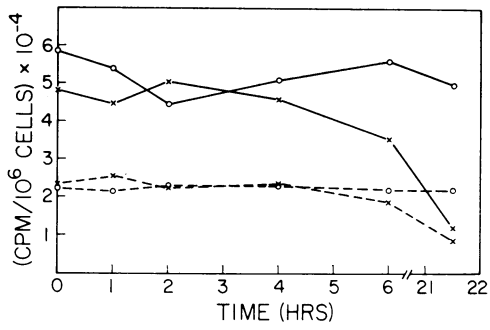

FIG. 5. Cycloheximide stimulates the breakdown of nucleic acids. Cells of *arg 2* were grown in medium containing 0.4 μCi/ml of ^{3}H-adenine (19 Ci/mmole) for 9 hr. They were washed free of ^{3}H-adenine and resuspended in fresh medium containing 3.5×10^{-5} M cycloheximide, (×), or nothing (○), in addition. Samples were assayed for their content of ^{3}H-RNA (——) or -DNA (– –) per 10^6 original cells at intervals.

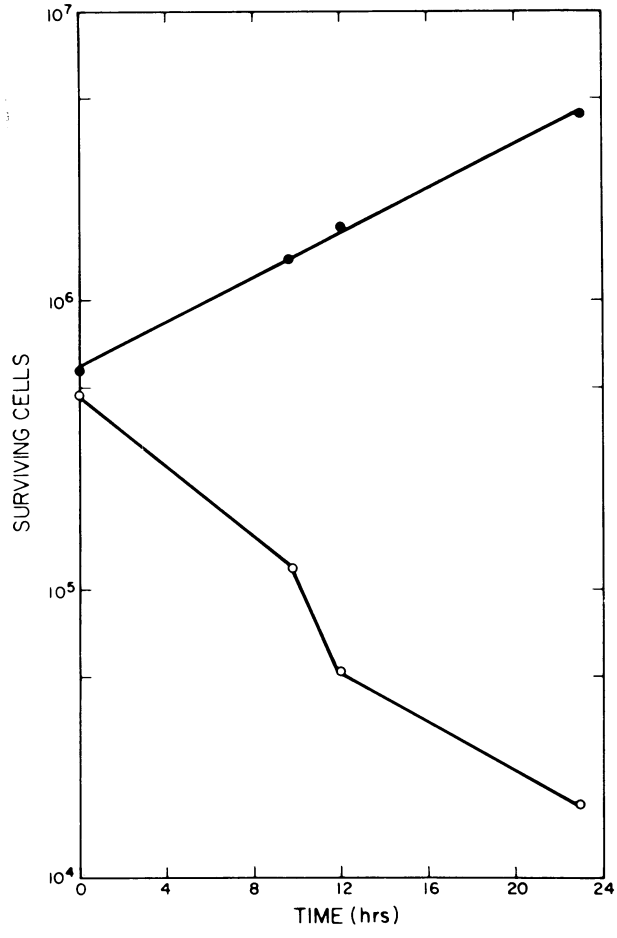

FIG. 6. Cycloheximide kills *C. reinhardi*. Cells of *arg 2* were suspended in medium containing arginine with: nothing in addition (●), or 7.1×10^{-6} M cycloheximide (○). At intervals 5-ml samples of cells were centrifuged from the medium and resuspended in fresh medium containing arginine. Then suitable dilutions were plated on the same medium solidified with agar.

molecular synthesis were produced, however (Fig. 7). After 16 hr in CH, cells made protein at a rate which was 10% of normal, and the rates of RNA and DNA synthesis were 5% and 2.5% of normal, respectively. Much of this residual

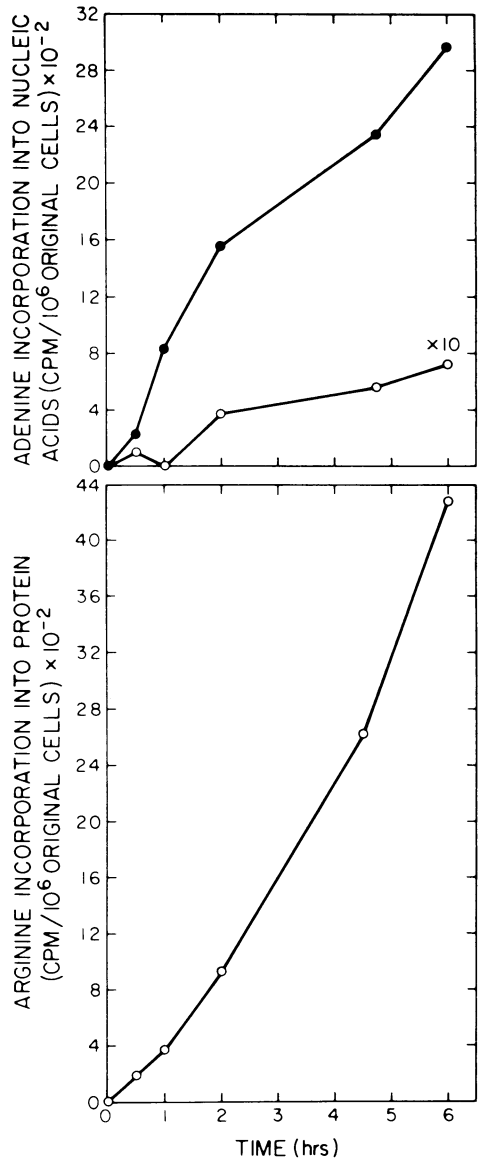

FIG. 7. Cycloheximide inhibits macromolecular synthesis irreversibly. *arg 2* (2.68×10^6/ml) were incubated for 16 hr in medium containing arginine plus 3.6×10^{-5} M cycloheximide. Then the cells were centrifuged, washed, and resuspended in fresh medium containing ^{3}H-arginine (0.5 μCi/ml) to measure protein synthesis, or in arginine plus ^{3}H-adenine (0.2 μCi/ml, 22 Ci/mmole) to measure DNA and RNA synthesis. Top frame: RNA, (●); DNA, (○). Bottom frame: protein synthesis. The activities are plotted in terms of the original number of cells. At the start of labeling 4.5% of the cells were viable.

synthesis could have occurred in the fraction (4.5%) of the cells which remained viable.

Cycloheximide affected the pool sizes of nucleotides (Table II). In these experiments, the concentration of nucleotides was normalized by setting the concentration of ATP in the untreated cells (average from five experiments = 76 pmoles/10^6 cells; range = 30–81 pmoles/10^6 cells) to 100%. All of the pool sizes were apparently decreased by 15 to 60%, but only the decrease in the ATP pool was statistically significant. Ninety-five per cent confidence limits for the extent of this decrease are 42 ± 7.6% and 37 ± 15% after treatment with 7.1×10^{-6} M or 3.6×10^{-5} M cycloheximide, respectively.

If simple cessation of protein synthesis is the reason that

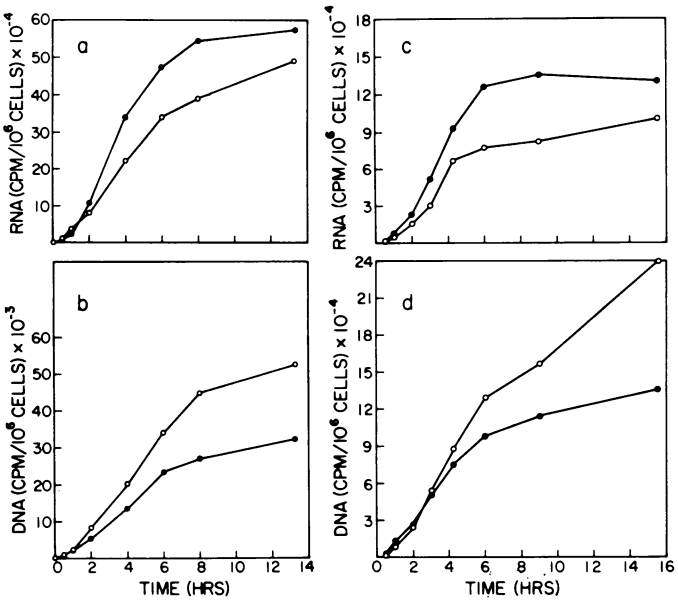

FIG. 8. The effect of inhibition of protein synthesis, in temperature-sensitive mutants, on nucleic acid synthesis. The temperature-sensitive mutants were incubated with 0.4 μCi/ml of ^{3}H-adenine at a temperature (22 C) which allows protein synthesis, (○); or one which inhibits more than 90% of protein synthesis (33 C), (●). In frames a and b the incorporation into nucleic acids in *ts 1* is presented. Incorporation into nucleic acids in *ts 13* is presented in frames c and d. The initial cell concentrations were 2.5×10^5/ml (*ts 1*) and 6×10^5/ml (*ts 13*).

CH inhibits nucleic acid synthesis, then inhibiting protein synthesis in a temperature-sensitive mutant should also inhibit nucleic acid synthesis. Instead, the cellular physiology of mutants which are conditionally defective in protein synthesis was affected to a more limited extent when protein synthesis was exhibited by a nonpermissive temperature. Figure 8 shows that DNA synthesis is inhibited by 30 to 40% under conditions which inhibit at least 90% of protein synthesis in the mutants (24), while RNA accumulation was not inhibited at all. RNA synthesis was stimulated by inhibition of protein synthesis in the mutants (Fig. 9) by 50 to 150% within 1 hr of exposure to high temperature.

The stimulation of RNA synthesis in the mutant did not result simply from exposure to a higher temperature. The rate of RNA synthesis of the parent strain was unaffected by an identical increase in temperature (Fig. 10). This result is not surprising since this strain grows at approximately the same rate at both 22° C and 33° C. The stimulation of RNA synthesis by inhibition of protein synthesis in these mutants contrasts considerably with the effects of CH, but resembles the effects of starvation of *C. reinhardi* for an essential amino acid. This treatment produced similar stimulation of RNA synthesis (26).

Similarly, if the other deleterious effects of CH simply result from inhibition of protein synthesis, they should appear under nonpermissive conditions in the mutants. High temperature completely inhibits cell multiplication in both mutants but their viability does not decrease during 24 hr under nonpermissive conditions. Finally, Table III shows that nonpermissive conditions do not decrease the size of the ATP pool in *ts 1*, although the size of the UTP pool does decrease transiently under nonpermissive conditions. In one determination of pool sizes and specific radioactivity with *ts 13*, a similar result was obtained.

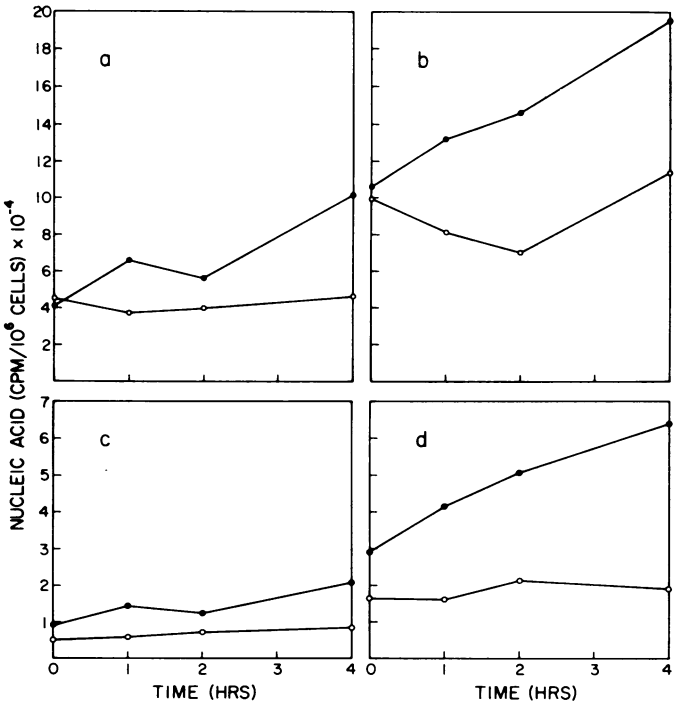

FIG. 9. Stimulation of the rate of RNA synthesis by inhibiting protein synthesis in temperature-sensitive mutants. The mutants *ts 1* (3.7×10^5 cells/ml) and *ts 13* (2×10^5 cells/ml) were incubated at a permissive (○) or nonpermissive (●) temperature. After the intervals indicated on the abscissa, aliquots of cells were added to sufficient ^{3}H-adenine to produce a concentration of 0.4 μCi/ml and the synthesis of nucleic acid in 1 hr (frames a and c) or 2 hr (frames b and d) was measured. The results obtained with *ts 1* are presented in frames a and b, whereas frames c and d contain the results obtained with *ts 13*.

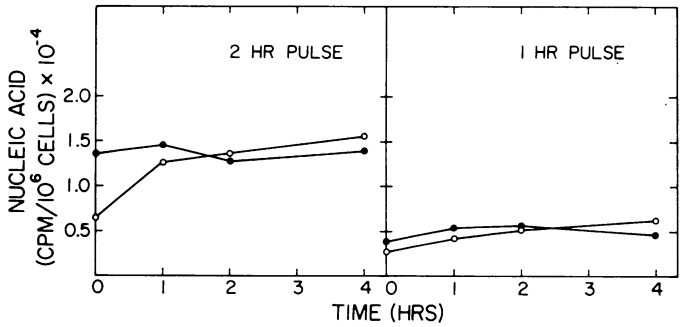

FIG. 10. The effects of temperatures of 22 C or 33 C on the rate of RNA synthesis. *Arg 2* (3.9×10^5/ml) was incubated at 22 C (○) or 33 C (●) for varying lengths of time and then pulse-labeled with ^{3}H-adenine as described in the legend to Figure 9.

DISCUSSION

Cycloheximide has wide ranging effects on cellular functions even when administered at low concentrations. It is unlikely that the "side effects" of CH can be operationally distinguished from its effects on protein synthesis. The sensitivity of protein synthesis and of other processes, such as accumulation of RNA and DNA synthesis, to inhibition by CH is very similar. Similarly, the differences in time of onset of the various effects of CH are so small relative to the periods over which CH is normally used that any attempt to discriminate between "primary" and "secondary" effects of CH on a temporal basis would be extremely difficult or impossible.

Table III. *Effects of Inhibition of Protein Synthesis by Nonpermissive Temperature (33 C) on Nucleoside Triphosphate Pools in ts 1*

	4 Hr at 22 C		2 Hr at 33 C		4 Hr at 22 C		4 Hr at 33 C	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Relative pool sizes								
GTP	11.2	0.99 (2)[1]	9.75	2.8 (2)	11.9	0.57 (2)	10.3	3.1 (2)
ATP	100	... (2)	88.9	5.2 (2)	113	7.1 (2)	87.3	17 (2)
UTP	34.8	3.5 (2)	23.7	0.57 (2)	39.4	0.99 (2)	26.8	4.1 (2)
CTP	7.25	0.45 (2)	10.4	3.5 (2)	9.38	... (1)	14.3	12.0 (2)
dGTP	0.59	0.25 (2)	0.62	0.42 (2)	0.82	0.13 (2)	0.61	0.36 (2)
dATP	0.91	0.41 (2)	0.60	0.19 (2)	1.06	... (1)	1.73	0.38 (2)
Relative specific radioactivities (mole ^3H/mole nucleotide)								
GTP	13.0	2.97 (2)	11.7	5.53 (2)	17.2	5.1 (2)	12.5	4.2 (2)
ATP	100	... (2)	132	109 (2)	67.1	8.8 (2)	76.2	59 (2)
UTP	3.82	0.17 (2)	3.72	2.8 (2)	7.28	1.6 (2)	5.22	4.1 (2)
CTP	9.44	3.19 (2)	5.40	0.47 (2)	5.50	... (1)	5.75	0.55 (2)

[1] No of determinations are shown in parentheses.

Instead, it seems more reasonable to assume that, in the absence of proof to the contrary, inhibition of some process by CH (under conditions where CH inhibits protein synthesis) means very little and that only an absence of any inhibition is likely to be significant.

One kind of experimental evidence argues against my conclusion. At least one mutant of yeast, resistant to CH, has ribosomes which are resistant to this drug *in vitro* (5). Cursory consideration of this result seems to suggest that CH effects on the cell could be mediated only through inhibition of protein synthesis. However, no evidence was presented in this paper that resistance to cycloheximide was the result of a single mutation. This objection is not "trivial," since there are at least four independent genes which produce CH resistance in yeast (10). This reduces the significance of these observations. In addition, this mutant does not begin to grow until three days after being placed in CH (35) and thus some additional change in cell function is necessary for resistance to CH.

Table IV. *Effects of Inhibition of Protein Synthesis by Various Methods upon Cellular Function*

These results are summarized from this paper and from McMahon (24); McMahon and Blaschko (25); and McMahon and Langstroth (26).

Effect	Cycloheximide (7.1×10^{-6} M)	*ts* Mutants	Arginine Starvation	Chloral Hydrate (10 mM)
Inhibits rate of DNA synthesis	Complete	Partial	Partial	Partial
Inhibits rate of RNA synthesis	Yes	No	No	Not done
Inhibits accumulation of stable RNA	Yes	No	Yes	Partial
imulates break-Stdown of nucleic acids	Yes	No	No[1]	No[1]
Decreases ATP	Yes	No	No	Not done
Kills cells	Yes	No	No	No
Extent of inhibition of protein synthesis	95%	90%	Net Protein Synthesis	100%
Reference	This paper	24	26	25

[1] Unpublished results.

These physiological studies indicate that there could be as many as three effects of CH on *C. reinhardi*. The first effect is its ability to inhibit protein synthesis. The evidence presented above has shown that its effects on most other processes measured are separate. These other effects can be provisionally separated into two groups on the basis of their sensitivity to an increase in CH concentration from 7.1×10^{-6} M to 3.6×10^{-5} M. In the group of cellular parameters which show no additional effect of the increased CH concentration are the rate of RNA synthesis and the size of the ATP pool. The rate of killing appears to be at least partially independent from these other effects since it is increased by the increase in CH concentration. The results of Horgen and Griffin suggest that cycloheximide can directly inhibit RNA synthesis *in vitro* (13).

Fortunately, the possibility that the similarity in the effects of CH on protein synthesis and on viability and RNA synthesis was a result of tight coupling between these processes and protein synthesis is not true. If this were true, studies of the importance of protein synthesis for any biological process would be crippled. The experiments performed with the temperature-sensitive mutants show that protein synthesis can be inhibited specifically. These results are supported by studies with other treatments which interfere with protein synthesis (summarized in Table IV). They show that the specificity of the mutants is unlikely to be some unique property of the mutants themselves. Taken together they show that CH treatment is an unsatisfactory way to inhibit protein synthesis in the living cell.

Finally, it is important to call attention to the fact that protein synthesis is not required for RNA synthesis in *C. reinhardi*. This conclusion is in contrast to that of a number of other investigations which were described in the introduction. Instead of inhibiting RNA synthesis, interference with protein synthesis in *ts* mutants or by amino acid starvation (26) stimulates the rate of RNA synthesis.

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LITERATURE CITED

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