

Protein Synthesis in Chlamydomonas reinhardi

1. Characterization of Temperature-Sensitive
Mutants in Protein Synthesis
2. Action of the Drug, Chloral Hydrate

Thesis by

John William Cross, Jr.

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This is dedicated to the one I love. Thanks to your patience,
Susan, here it is!

Acknowledgments

As I have discovered the hard way, a thesis does not spring forth in full glory in a few afternoons of sunbeams and inspiration. During the course of this effort I have had the benefit of advice, comradeship and sympathy from numerous friends and associates here in Pasadena. I pause here to mention a few of them.

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Abstract

I. Mutants of Chlamydomonas which are temperature-sensitive in protein synthesis were analyzed. These mutants were previously shown to be unaffected in RNA synthesis or in nucleotide pool levels at the growth-restrictive temperature (33°) although amino acid incorporation is blocked at least 40%. Here it is demonstrated that exposure of these cells to non-permissive conditions causes their polysomes to break down, resulting in the accumulation of 80S ribosomes which are not bound to mRNA. There was no defect detected in either mutant when their rates of charging of any of the 20 amino acids to tRNA were measured at the restrictive temperature in vitro, or in one of them whose degree of charging of tRNA was estimated in vivo at 33°. It is concluded that the defects in these mutants result from defective initiation of protein synthesis in vivo at 33°.

II. The mechanism by which the anesthetic, chloral hydrate, inhibits protein synthesis was investigated in Chlamydomonas. This drug was previously shown to inhibit protein synthesis and cell division in Chlamydomonas at concentrations similar to those which produce anesthesia in vertebrates. The incorporation of amino acids into protein is rapidly inhibited after the drug is added to cells. At the same time, polysomes in the cells partially break down into monosomes, which are not bound to mRNA, but do contain nascent peptides capable of reacting with puromycin. Maximum polysome

breakdown occurs in 15-30 min and is followed by a gradual reformation of polysomes, which is complete in 4-8 hours. Amino acid incorporation remains maximally inhibited during this period (85-90% inhibition at 10 mM chloral hydrate).

The inhibition of protein synthesis by two compounds which are metabolites of chloral hydrate in other organisms was examined. Protein synthesis is inhibited by trichloroethanol, but not by trichloroacetic acid at concentrations of 10 mM. Trichloroethanol produces the same effects on polysomes as does chloral hydrate. However, in Chlamydomonas no significant amount of chloral hydrate is metabolized to either of these two substances, and so it appears that the effects which are observed are due to the action of chloral hydrate itself. A preliminary investigation of the binding of chloral hydrate to cells shows that Chlamydomonas accumulates the drug to a higher concentration than that found in the medium.

III. An effort was made to enable the selection of mutants of Chlamydomonas conditionally defective in assembly of chloroplast membranes.

Chlamydomonas cells which had formed active chloroplasts were found to be killed by photooxidation in an intense light beam, and inhibition of photosynthetic electron transport afforded significant protection from this killing. Therefore it was reasoned that Chlamydomonas mutants unable to form photosynthetically active

chloroplasts would be protected. It was, however, found that photooxidation does not select for mutants with reduced contents of photosynthetic pigments.

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General Introduction

I. Temperature Sensitive Mutants in Protein Synthesis

Often in cellular physiology it is desirable to know the requirements of the system for protein synthesis. The most direct approach to this problem is to block protein synthesis at some point in time and determine if some part of the process continues to occur. Usually protein synthesis is blocked by the addition of an antibiotic, but the validity of the conclusions obtained depends on the specificity of the drug. Inhibitors, even those which block only specific reactions of protein synthesis in vitro, may have other disconnected effects on cells which may confuse any conclusions (1,2).

An attractive alternative is to employ the properties of temperature sensitive mutants in protein synthesis. If such mutants can be shown to harbor only a defect in a defined reaction of protein synthesis, then it should be possible to employ them to more rigorously define the requirements of cellular phenomena for protein synthesis. Mutants of bacteria (3,4,5), yeast (6,7,8), and mammalian cell lines (9) showing temperature sensitivity in protein synthesis have been isolated. In this Thesis I report the analysis of two such mutants isolated from the green alga, Chlamydomonas reinhardtii, Dangeard (10).

These mutants were isolated using a suicide selection employing an arginine auxotroph, arg 2 (11) and the arginine analogue,

canavanine, which kills *C. reinhardi* only when it is incorporated into protein in place of arginine by cytoplasmic ribosomes (10).

II. Effects of Chloral Hydrate on Protein Synthesis

Chloral hydrate, colloquially known as knockout drops or the Mickey Finn, is an anesthetic drug commonly used in medical practice as a sedative on account of its higher than average factor of safety between sedative and lethal doses (12). In common with other general depressants, it also blocks mitosis (13), and McMahon and Göpel have shown that this inhibition correlates strongly with inhibition of protein synthesis (14). Similar concentrations of the drug inhibit mitosis in a variety of organisms (13, 15, 16), growth of bacteria (17), protein synthesis in Chlamydomonas and mouse neuroblastomas cell cultures (18), and induce anesthesia in rats (19). This universality suggests that the drug may act on some common targets in producing its effects.

The experiments reported here form an effort to understand how chloral hydrate blocks protein synthesis through an analysis of the polysomes of cells exposed to the drug. Some preliminary work towards understanding how chloral hydrate binds to the cell is also included.

III. Attempts at Isolation of Mutants Defective in Chloroplast Assembly

How light stimulates photosynthetic organisms to synthesize new chloroplast material is an as yet incompletely understood problem of great significance. This section reviews the process of chloroplast development as it is now understood and presents the results of an unsuccessful attempt to isolate mutants conditionally defective in their chloroplast development by photo-oxidative killing of those cells which were able to synthesize a functional chloroplast.

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Part I.

Two Mutants of Chlamydomonas Defective in Initiation
of Protein Synthesis in vivo

SUMMARY

Two temperature-sensitive mutants of Chlamydomonas reinhardi Dangeard which are defective in protein synthesis were examined. Both show breakdown of their polysomes at the restrictive temperature into monosomes which do not contain fragments of mRNA. The polysome breakdown involves only cytoplasmic (80s) ribosomes and is prevented or reversed when ribosome translocation is inhibited with cycloheximide. From these results we believe that these mutants are defective in initiation of protein synthesis at the restrictive temperature.

INTRODUCTION

In understanding processes in cellular physiology it is helpful to know how dependent a given process is upon protein synthesis, and it may be desirable to know the site of such protein synthesis in an organism possessing semi-autonomous organelles. Inhibitors of protein synthesis have often been employed in order to help answer such questions, but the conclusions are weak unless the

specificity of the drug for protein synthesis is high (21). As an alternative to the use of inhibitors of protein synthesis we are studying mutants of Chlamydomonas reinhardi which are temperature-sensitive in protein synthesis.

These mutants were isolated from the arginine auxotroph, arg 2, by exposure to the arginine analogue, canavanine, at 33° (20). Since canavanine kills those cells which incorporate it into their cytoplasmic proteins instead of arginine, the survivors of this treatment are enriched in mutants which fail to synthesize protein at 33° (20,23). Preliminary screening showed that 75% of the temperature-sensitive survivors of this treatment were defective in their amino acid incorporation at 33° compared to 22° (20). Two of these mutants, ts1 and ts13, showed at least a 90% inhibition of amino acid incorporation at 33° and were chosen for more intensive analysis.

Since these mutants were selected at 33° and subsequently grown at 22°, it would be expected for their lesions to be reversible, and in fact this was observed (20). Their incorporation of [³H]adenine into RNA was not reduced at 33°, but rather was stimulated 50 to 150% (20, 21). This is unusual, since mutants showing specific defects in protein synthesis (6,9,19,28), including a mutant of yeast defective in initiation (10), have often been reported to show a secondary inhibition of stable RNA synthesis under growth-restrictive conditions. Incorporation of [³H]adenine into DNA

was partially inhibited (30 to 40%), but no more than by other treatments which inhibit protein synthesis, such as arginine starvation in arg2 or treatment with 10 mM chloral hydrate (21). There was no measurable disturbance in arginine uptake in ts1 and ts13 (20) nor in nucleotide pool levels, except for a transient decline in the UTP pool (21). These results indicate that a general disturbance of metabolism is unlikely.

The nature of the polysome patterns from cells shifted to the restrictive temperature can serve as a sensitive indicator of the nature of the lesion in a temperature-sensitive mutant (27). Thus we have employed this technique here to categorize these mutants.

MATERIALS AND METHODS

The isolation of the temperature-sensitive mutants and their growth have been described previously (20,22). Cells were labeled with $^{35}\text{SO}_4$ as described (4).

Analysis of Polysomes. Exponential cells ($0.5-2 \times 10^6$ cells/ml, 200 ml per stirred flask) were incubated at 22° or were put into a constant temperature chamber (Scherer) at 33°. The culture reached 33° within 1/2 hr. In some experiments cells were more rapidly warmed by addition of an equal volume of medium (HSMA) at 43° or by swirling the flask in a 40-50° water bath. Constant illumination was always maintained. At the indicated time cells were chilled rapidly with ice made from distilled water and processed for

polysomes or active ribosomes as described previously (4). Briefly, cells were resuspended in 0.25 M sucrose 25 mM KCl, 25 mM MgCl_2 , 25 mM Tris·HCl pH 7.6 at 0–4° and broken in the French pressure cell at 7×10^7 dynes cm^{-2} . After addition of potassium deoxycholate (12 mM) to lyse the cells and centrifugation at $10^4 \times g$, 10 min, the supernatant (S10) was layered on 0.31–1.1 M exponential sucrose gradients and centrifuged at $1.74 \times 10^5 \times g_{\text{av}}$ for 1.75 hr at 2° in an SW41 rotor. The gradients were then pumped from the bottom through a flow cell in a Beckman spectrophotometer to measure absorbance at 260 nm, or by upward displacement through an ISCO flow cell to measure A_{254} .

Analysis of Ribosomes Bound to mRNA. The association of ribosomes with mRNA was tested using the method of high-salt dissociation in 25 mM Mg^{++} (4,17). S10 lysates were treated with RNase and centrifuged over 0.31–1.2 M exponential sucrose gradients containing polysome buffer but with 0.5 M KCl (4). Sedimentation was 5 hr at $1.74 \times 10^5 \times g_{\text{av}}$ in the SW 41 rotor. Gradients were analyzed by upward displacement through an ISCO flow cell to measure A_{254} .

Isolation of tRNA. tRNA was isolated from Chlamydomonas cells by a modification of previously reported methods (2,11,13). First, discharged soluble RNA was prepared by the methods of Lee-Ping (13) from late-log or early stationary phase cells, and this was further purified by DEAE-cellulose chromatography by the method of

Barnett et al. (2). After a final precipitation of the product from potassium acetate buffer, 2% (w/v) pH 5, with 2 volumes of ethanol at -25° , the precipitate was pelleted and thoroughly dried. After dissolving it in distilled water, the solution had an A_{280}/A_{260} ratio of 0.51, and an RNA concentration of 12 mg/ml, assuming 24 A_{260} units per mg. Purity of the product was assayed by electrophoresis in 0.1% sodium dodecylsulfate on 10% polyacrylamide gels prepared as by Miller and McMahon (25), but with 10% acrylamide instead of 2.4% and without agarose. The major impurity was 5S ribosomal RNA, which composed less than 10% of the RNA.

Preparation of Aminoacyl-tRNA Synthetase Extracts. These were prepared by modification of the method of Barnett et al. (2). Cells in the exponential phase of growth were harvested, washed in cold HSMA and resuspended in 0.2 M KPO_4 , pH 7.5; 0.01 M 2-mercaptoethanol; 10% (v/v) glycerol; 4 ml per g of cells, wet weight. Cells were passed through a pre-chilled French press at 7×10^8 dynes cm^{-2} , centrifuged at $1.05 \times 10^5 \times g$ at 4° for 2 hr, and supernatants passed over a 10 x 0.9 cm column of DEAE-cellulose equilibrated with the same buffer. Crystals of $(NH_4)_2SO_4$ (Schwarz/Mann, ultrapure) to 3.0 M were added to the effluent and the precipitate which formed overnight at 4° was collected. This was dissolved in 0.01 M Tris-HCL, pH 7.5, 0.1 M KCl, 0.01 M 2-mercaptoethanol, 20% (v/v) glycerol and was desalted on a Bio-Gel P-2 column (20 x 2.4 cm) equilibrated with the same buffer. The product (7-9 mg/ml protein by the method of

Lowry et al. (15), using bovine serum albumin as a standard) was frozen at -25° until used.

Assay of Aminoacyl tRNA Synthetase. Aminoacyl-tRNA synthetase activity was assayed by the method of Barnett et al (2). The reaction mix contained per ml: 50 μ mole N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES) pH 7.5, 2 μ mole ATP, 10 μ mole Mg (acetate)₂, 5 μ mole 2-mercaptoethanol, 1 μ Ci [¹⁴C]amino acid (29-338 μ Ci/ μ mole) (Schwartz/Mann), 0.01 μ mole of the 10 other amino acids. Each reaction mix also contained 70-90 μ g synthetase preparation, and 0.2 mg tRNA. Assays were initiated by the addition of tRNA, 20 μ l samples were taken and assayed by the method of Mans and Novelli (16), omitting the hot TCA step. Synthetase activity was estimated during the period of linear incorporation into TCA insoluble material. Control experiments showed that the reaction was entirely dependent upon the synthetase preparation and on the tRNA. The rate of incorporation was linearly related to the amount of synthetase protein and tRNA which was added. The amount of ATP used was saturating.

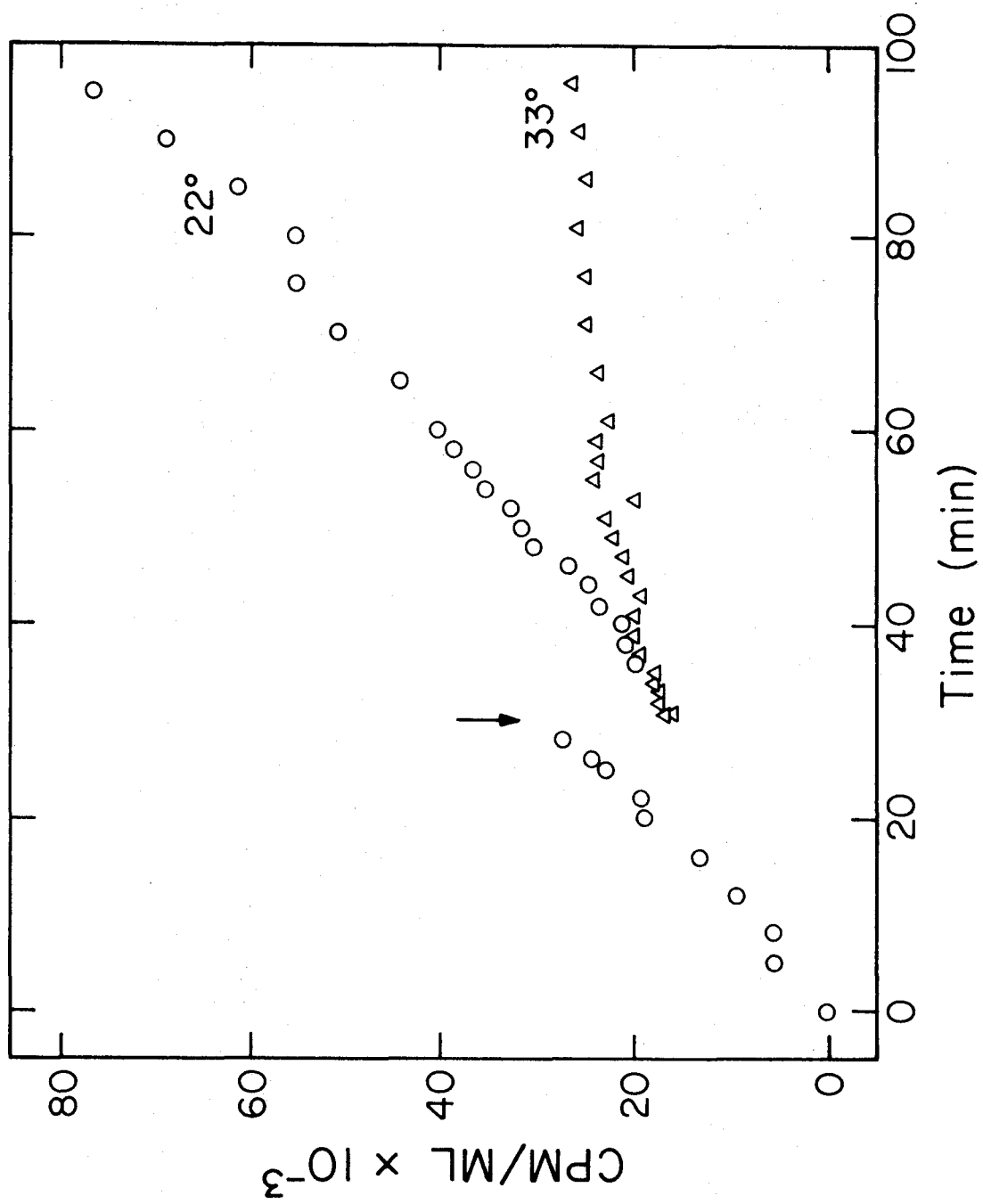
tRNA Charging in vivo. Measurement of the degree of tRNA charging in vivo was done with a modification of the method of Yegian et al. (29). Arg 2 or temperature-sensitive cells in late log phase ($1-5 \times 10^6$ cells ml^{-1} , cultured aerated in portions of 15 l) were brought to 33° , if necessary, by aeration with pre-warmed

humidified air in a 33° incubator for 3 hr. Cells were then poured over crushed ice made from distilled water to cool them rapidly and harvested by continuous flow centrifugation. The rate of flow was about 500 ml per min. Soluble RNA was then prepared from the chilled cells as described (2,11) but without the incubation at pH 8.8 to discharge amino acids. The tRNA was then divided in two. One part was then oxidized in 0.75 mM NaIO₄, 75 mM sodium acetate, pH5.0 in the dark as described (29). The other portion was incubated similarly, but without periodate. After the incubation, remaining periodate was removed by addition of ethylene glycol (29). If the A₂₃₂ did not change drastically after the addition of a test quantity of ethylene glycol, the oxidation was repeated with fresh periodate. The RNA was then precipitated in ethanol, and both portions were discharged at pH8.8 as usual (13). tRNA prepared in this manner had an A₂₈₀/A₂₆₀ ratio of 0.46-0.47.

RESULTS

The inhibition of protein synthesis in these mutants occurs rapidly. When the temperature of their growth medium is raised to 33°, greater than 90% of the incorporation is inhibited within 10 to 15 min (Figure 1). The course of incorporation of ³⁵S₄²⁻ into protein shows a gradual decline in rate over this time period, rather than the abrupt inhibition which is observed upon the addition to the medium of a chemical inhibitor

Figure 1. Incorporation of $^{35}\text{SO}_4^{2-}$ into protein by ts13: 22° → 33° shift. Exponential ts13 cells were resuspended at 22° in medium containing 1/10 the normal SO_4^{2-} concentration at 1.4×10^6 cells/ml. At zero time cells were supplied 1 $\mu\text{Ci/ml}$ $\text{H}_2^{35}\text{SO}_4$ (New England Nuclear, carrier-free) and 1 ml samples were taken into 1 ml 10% trichloroacetic and prepared for counting as described (4). After 30 min of incorporation (arrow) the culture was divided in two portions. One part (open circles) received an equal volume (20 ml) of medium at 22°. The other part (triangles) received an equal volume (15 ml) of medium preheated to 43° to bring the culture to 33°, and the culture was incubated at 33° in a water bath. Incorporation at 33° was 8.8% of that at 22° during the interval from 25 to 65 minutes after the shift in temperatures.



of protein synthesis such as cycloheximide or chloral hydrate (4, 20, 21).

The polysome contents of arg 2, ts1 and ts13 after incubation at 22° or 33° are shown in Figures 2 to 4. No significant change is seen in the pattern for arg 2 polysomes after 2 hr at 33° (Fig. 2 and Table I), but changes can be seen in the temperature-sensitive mutants. The polysomes of ts1 (Fig. 3) and ts13 (Fig. 4) break down into monosomes when the cells are warmed to 33°. When the cells are warmed more rapidly, the breakdown of polysomes also occurs more rapidly (4). Fig. 3 shows that upon return to 22° this breakdown is rapidly reversible in ts1 and in ts13, and Table I shows that this breakdown is blocked by cycloheximide, but not by chloramphenicol.

These findings are consistent with the hypothesis that these mutants are defective in the initiation of protein synthesis. If initiation is limiting for protein synthesis at 33°, then we expect that by inhibiting peptide elongation with cycloheximide we might make elongation the limiting factor and cause polysomes to reform. This is plausible, since the mutants still show 10% of the normal rate of protein synthesis at 33° (20). Lodish (14) has used a similar method to show that initiation is limiting to protein synthesis in rabbit reticulocytes.

We incubated ts13 cells at 33° for 1 hr and then added 7×10^{-6} M cycloheximide, which inhibits 92 to 99% of the protein

Figure 2. Polysomes of arg 2 at 22° (a) and 33° 2 hr (b). Polysomes were analyzed as described in Part I. The computed percentages of polysomes for this figure and for Figures 2 and 3 are shown in Table V.

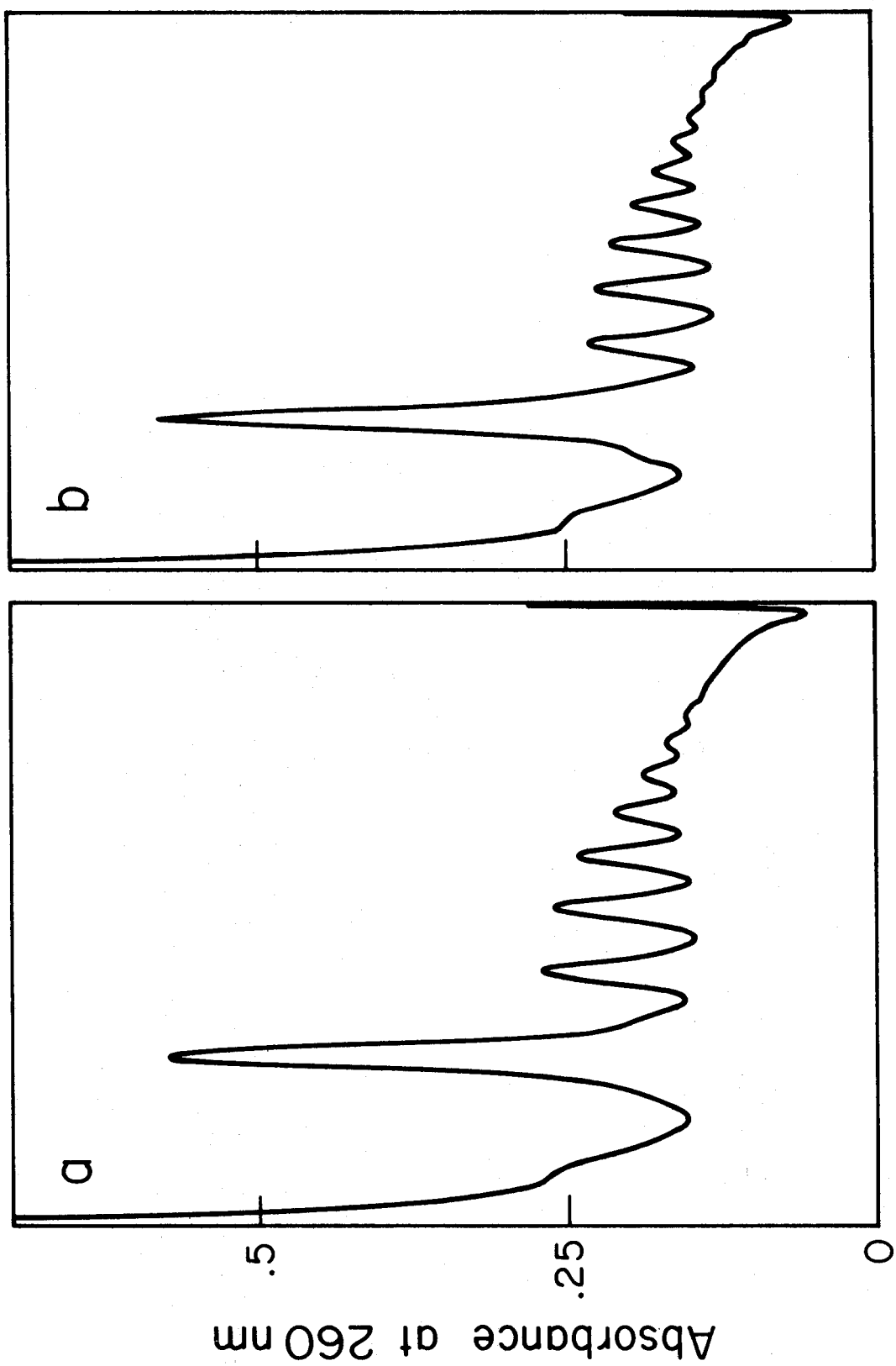


Figure 3. Polysomes of ts1 at 22° and 33°. Cultures (200 ml) were incubated at 22° (a) or placed in a chamber at 33° for one hour (b) before harvest. One culture (c) was incubated at 33° one hour and then quickly returned to 22° by swirling in a water bath. The culture reached 22° in 10 min and then was kept at 22° an additional 5 min before harvesting. Cultures were harvested and polysomes analyzed as described in the text.

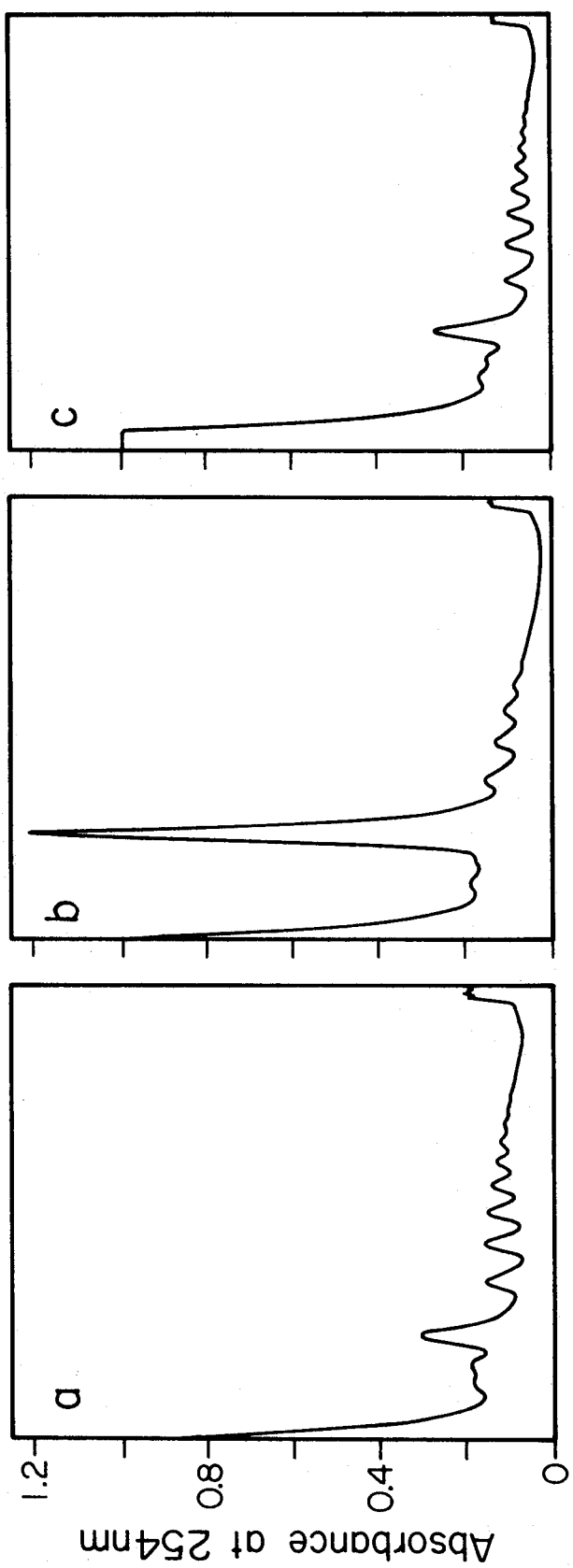


Figure 4. Polysomes of ts13 cells at 22° (a) and at 33° for 0.5 hr (b), 1 hr (c), and 2 hr (d). Cultures (200 ml) were incubated in a 22° room or placed in a 33° chamber for the indicated time, and then harvested and polysomes analyzed as described in the text.

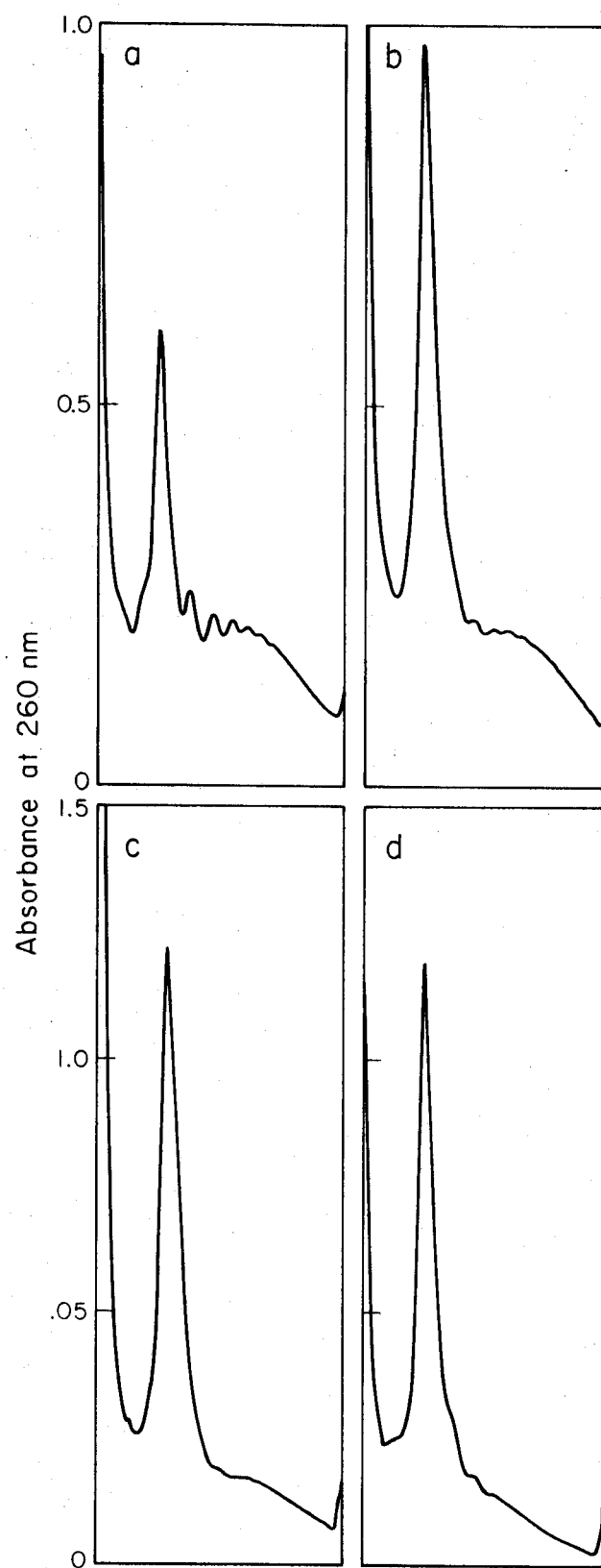


Table I. Effects of Temperature and Antibiotics in vivo on Polysome Distributions

Lysates of cells incubated at 22° or at 33° for the indicated time were prepared as described in the text and analyzed on isokinetic sucrose density gradients in the SW41 rotor. Absorbance at 260 nm (arg 2, ts 13), or at 254 nm (ts 1) was scanned for each gradient using a flow cell and the percentage of the absorbance falling in the polysome region of each curve was obtained as described before (4). In the right column, the RNase resistant absorbance of arg 2 and ts 13 from three experiments (16% ± 1.3%) has been subtracted. In other experiments using arg 2, similar percentages of material resistant to RNase action were found (4). ts 1 (line 7) were returned to 22° rapidly in a water bath. ts 13 cells (line 12) cooled gradually in a 22° chamber.

Table I.

Cells	Treatment	Percent Ribosomal Absorbance in Polysomes	Minus Average RNase-Resistant Background
arg2	22°	67	51
	33° 2 hr.	64	48
ts1	22°	65	49
	33° 1 hr	36	20
	33° 1 hr. then at 22° 15 min	61	45
	33° 1 hr + cycloheximide 1/2 hr	72	56
	33° 1 hr + chloramphenicol 1/2 hr	36	20
	ts13 experiment A 22°	61	45
	33° 1/2 hr	40	24
	33° 1 hr	33	17
	33° 2 hr	28	12
	33° 1 hr, then 22° 1 hr	48	32
	experiment B 22°	66	50
	22° + cycloheximide 1 hr	73	57
	33° 2 hr	23	7
	33° 2 hr + cycloheximide 1 hr	51	35
	33° 2 hr + cycloheximide 2 hr	52	36
	experiment C 22°	55	39
	22° + chloramphenicol 2 hr	61	45
	33° 2 hr	15	0
	33° + chloramphenicol 2 hr	19	3

synthesis in Chlamydomonas (21), and about the same in reticulocyte lysates, in which it limits elongation (14). The result was reformation of an almost normal polysome profile for *tsl* or *tsl3* within an hour (Table I). We have previously shown that cycloheximide treatment of arg 2 cells causes an increase in polysomes above the normal level at the expense of monosomes (4). This reinforces the argument that cycloheximide can cause elongation to be limiting. Cycloheximide is also known to exert nonspecific effects on Chlamydomonas cells (21), but this effect on polysomes is more rapid.

This reformation of polysomes in the presence of cycloheximide also eliminates the possibility that the defect of *tsl3* is in mRNA synthesis or processing or that the polysome breakdown is caused by release or activation of some ribonuclease.

Active Ribosomes. If mutant polysomes break down due to normal termination and lack of reinitiation, then the monosomes formed should be free ribosomes not bound to fragments of mRNA. As in our previous work (4), the resistance of active ribosomes to dissociation by 0.5 M KCl was used as a criterion for their binding of mRNA (17).

Figures 5 to 8 show that indeed the 80S ribosomes of mutant cells become more sensitive to 0.5 M KCl dissociation after shift of cells to 33°. In *tsl3* this increased sensitivity is prevented by cycloheximide (Fig. 7) but not by chloramphenicol (Fig. 8). It can also be seen (Fig. 5, Fig. 6) that the relative absorbance due to the 70S organelle ribosomes and to the 50S subunits of both

Figure 5. Dissociation by high salt of ts13 ribosomes from cells at 22° (a) or 33° for 0.5 hr (b) or 1.0 hr (c). S10 lysates of ts13 cells (300 ml/flask, 2×10^6 cells/ml) grown at 22° or placed in a 33° chamber for 0.5 or 1.0 hr were prepared, treated with RNase A, and centrifuged in isokinetic sucrose gradients as described in (4). Assignment of sedimentation coefficients were made by comparison with mouse liver ribosomes and subunits run on identical gradients and by use of fraction I protein [18S (8)] as an internal standard.

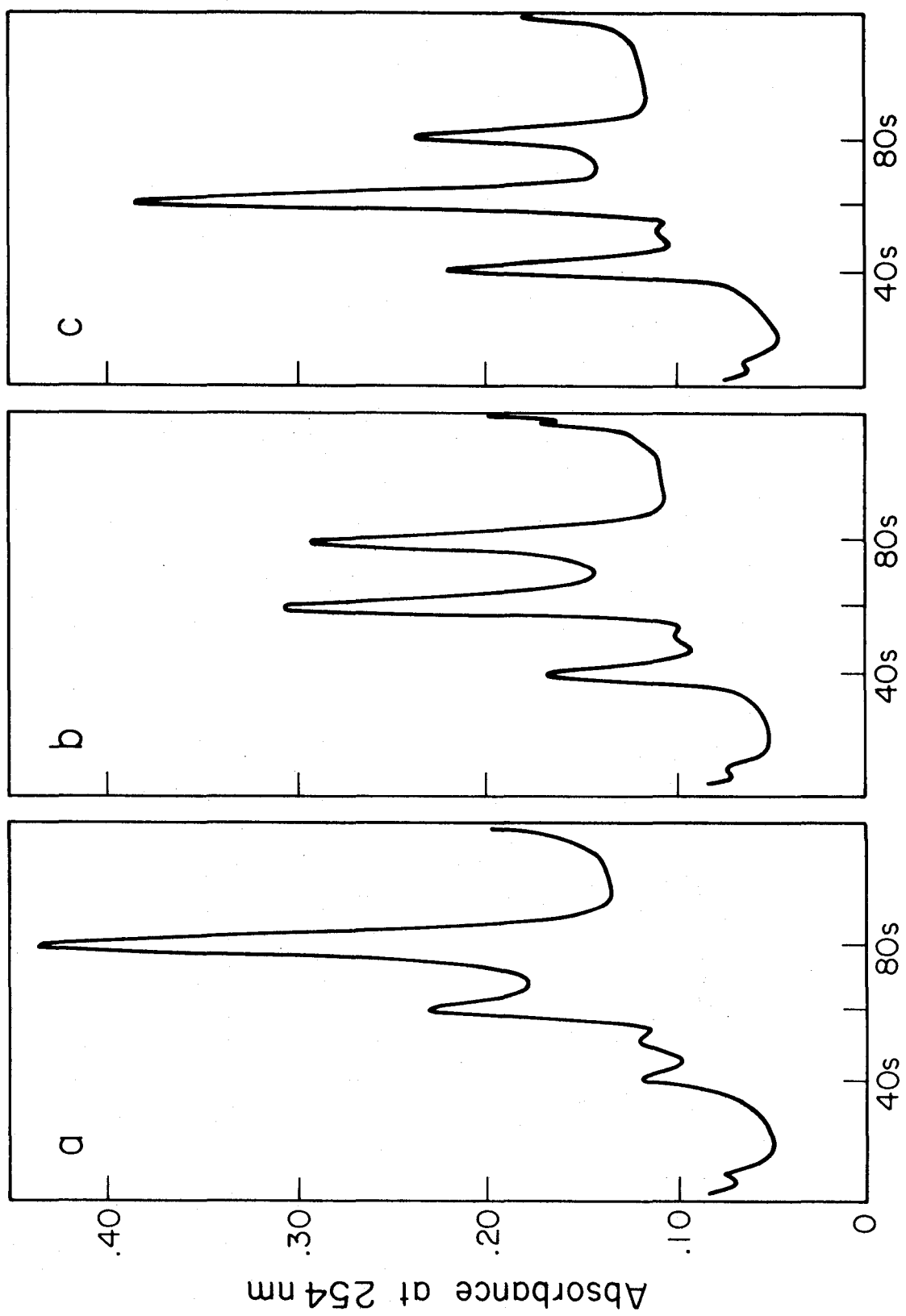


Figure 6. Dissociation by high salt of ts1 ribosomes from cells at 22° (left) or at 33° for 1 hr (right). ts1 cells (200 ml/flask, 2×10^6 cells/ml) were treated and ribosomes analyzed as in Figure 5.

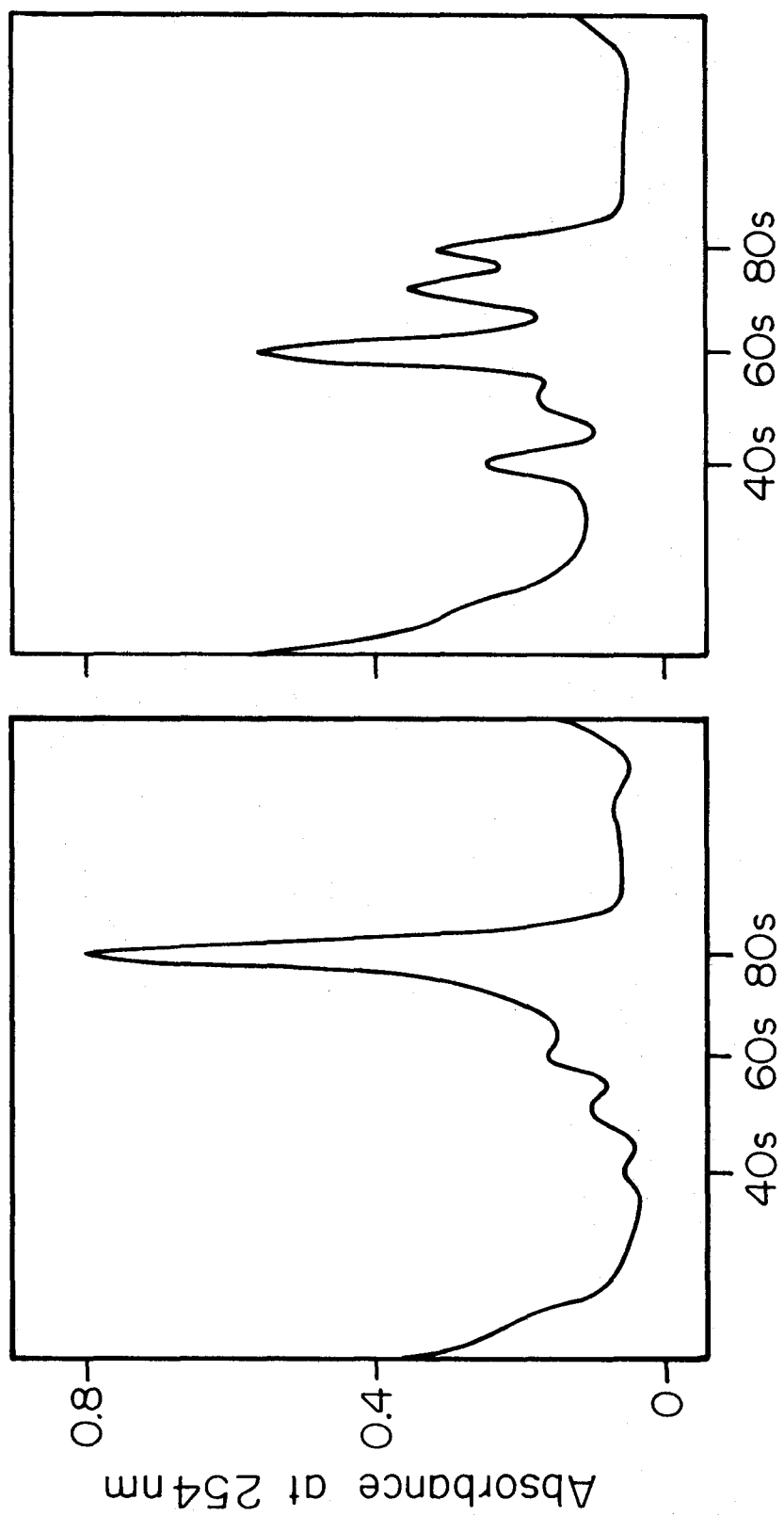


Figure 7. Cycloheximide prevents high-salt dissociation of ts13 ribosomes from cells incubated at 33° 1.0 hr. The experiment is the same as in Figure 5 except that cycloheximide (10 µg/ml) was added 1.0 hr before harvest. Left: cells at 22°, right: cells in 33° chamber 1.0 hr.

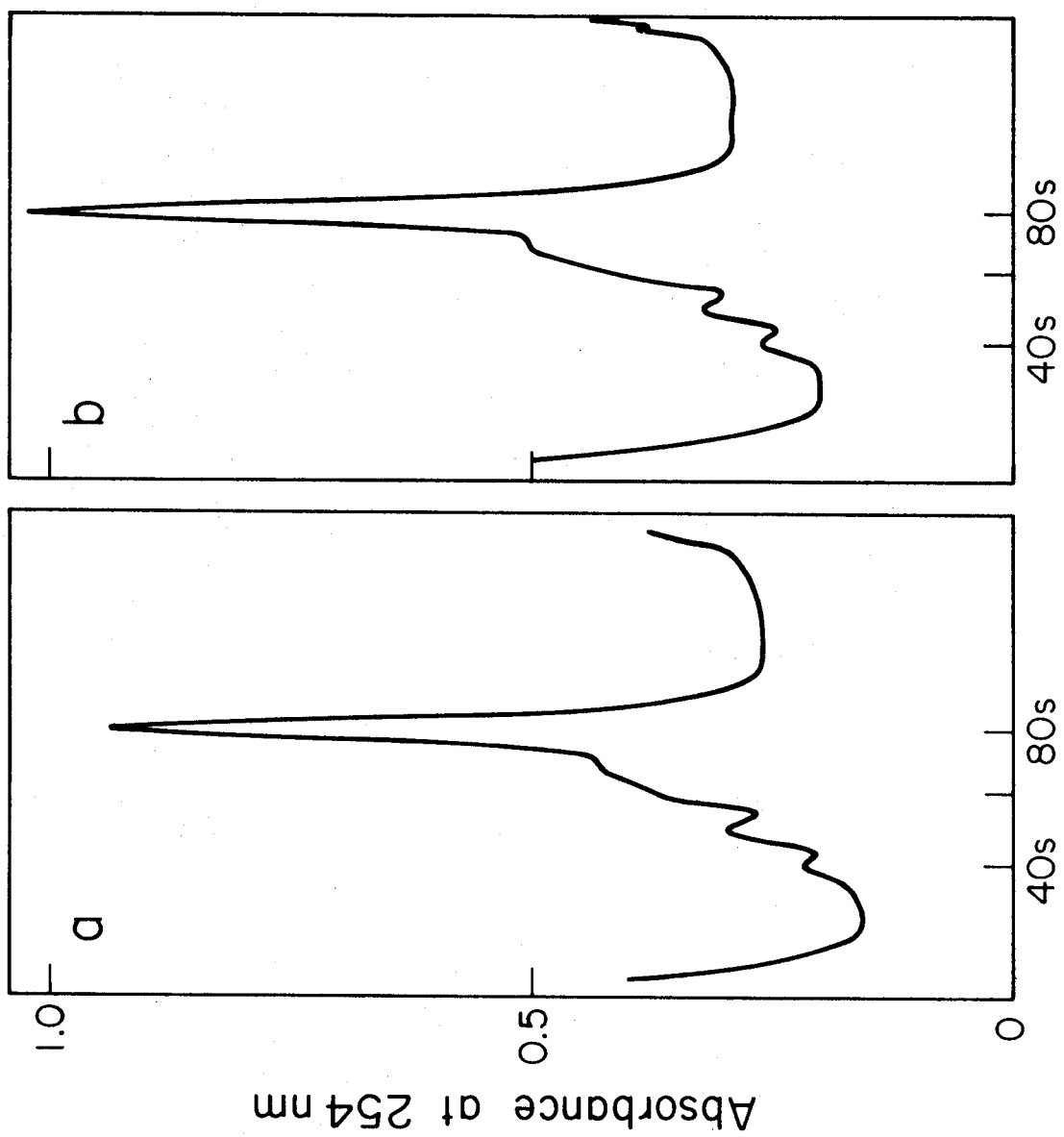
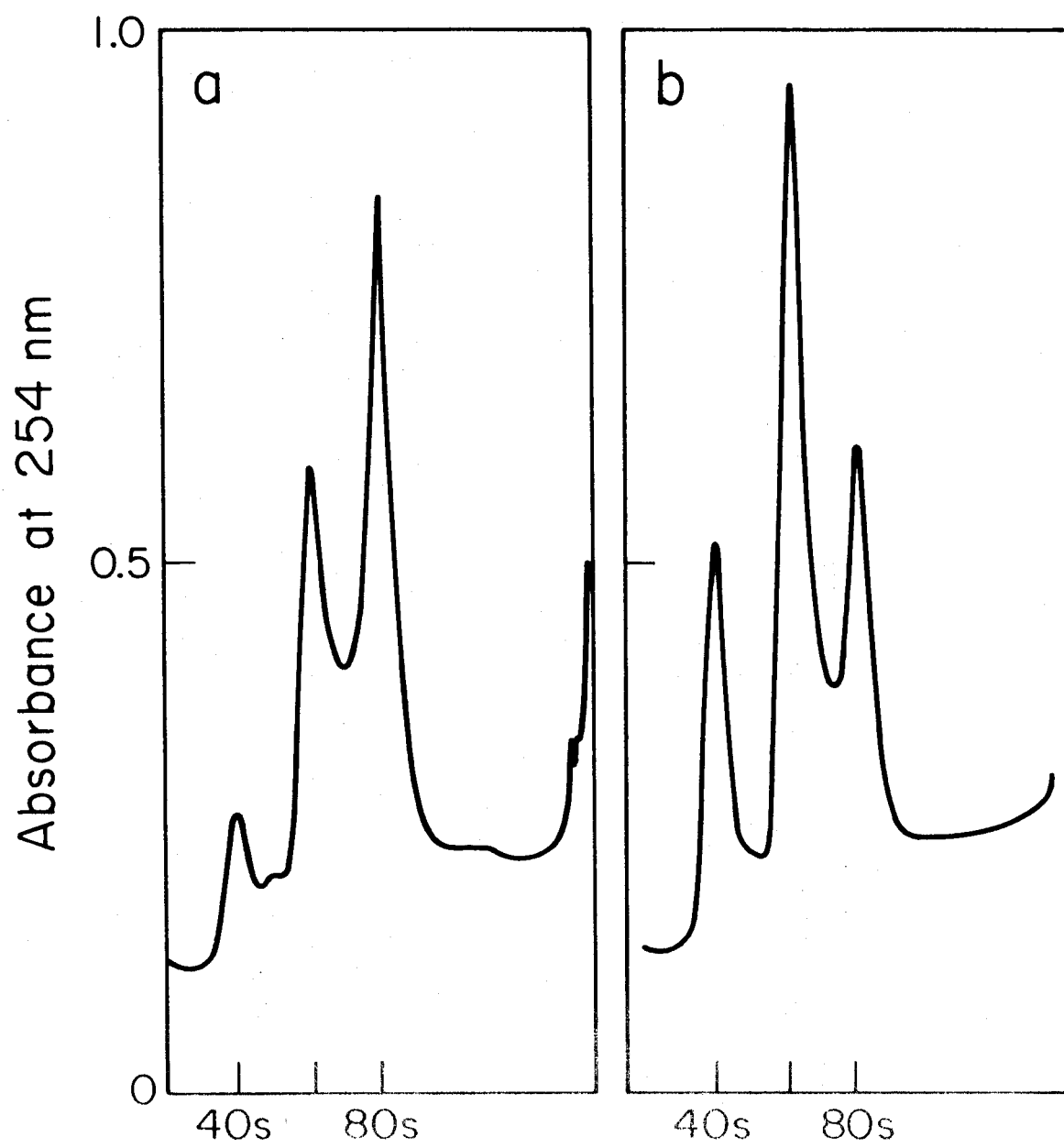


Figure 8. High salt dissociation of ribosomes from ts13 cells treated with chloramphenicol. The experiment is the same as Figure 6, except that chloramphenicol (150 $\mu\text{g/ml}$) was added 1 hr before harvesting cells. Left: cells at 22°, right: cells in 33° chamber 1.0 hr.



mutants is not altered by temperature shift (70S ribosomes do not form a distinct peak in most gradients due to the relative abundance of 80S and 60S material, but 50S subunits are always observed. 30S subunits are only rarely seen above the background).

Amino Acyl tRNA Synthetases. A defect in charging initiator tRNA with methionine or some other tRNA whose codon is commonly near the initiation site might produce the effects observed on polysomes if it results in decreased binding of messenger RNA to ribosomes in high salt. A defective initiator methionyl-tRNA synthetase with a temperature-sensitive defect has in fact been identified in yeast (18,27). Such a defect could lie in the tRNA synthetase or in the tRNA molecule itself (26).

Tables II-IV show the results of a survey of aminoacyl-tRNA synthetase activities. No attempt was made to find the optimum conditions for each reaction, rather the conditions were optimized for the arginyl tRNA synthetase and then used for all of the reactions. Each reaction received 0.2 mg of arg 2 tRNA (discharged) and thus each sample (20 μ l) contained 160 pmoles of tRNA. The sum of the pmoles of amino acid bound to tRNA by all arg 2 synthetases at 33° is 110. Thus the fraction of the tRNA reacting is $110/160 = 68\%$. The rate of the reactions was linear for at least 10 min in each case (not shown).

Tables II-IV. Purified tRNA from Chlamydomonas arg2 (II, IV), or E. coli-B (Sigma) (III) was incubated with extracts of arg2 and ts1 (II, III) or ts13 (V), and a [¹⁴C]amino acid for the indicated time and plated onto Whatman 3 MM filters, plunged into cold 10% trichloroacetic acid, washed and counted as described in the text. The values given in Table II for 22° are the average of three experiments ± standard error.

Table II. arg 2 tRNA, pmoles amino acid incorporated/10 min

Amino acid	22°		33°		40°*		45°	
	arg 2	ts1	arg 2	ts1	arg 2	ts1	arg 2	ts1
1 ALA	1.19 ± .35	2.4 ± .4	2.4	3.9	3.7	5.2	2.2	1.8
2 ARG	9.8 ±2.1	7.7 ± .7	13.	10.	4.8	7.9	0	0.1
3 ASN	3.5 ± .6	2.8 ± .3	3.9	3.4	-----		5.3	5.8
4 ASP	1.52 ± .22	1.82± .14	2.8	2.8	3.8	4.3	2.0	3.9
5 CYS	16. ± .4	16 ± .6	18.	15.	-----		15.	22.
6 GLN	0.68 ± .29	1.48± .36	1.13	1.8	0.5	1.9	0	0
7 GLU	5.1 ±1.4	9.5 ±1.4	5.8	9.6	1.7	4.3	0.2	0
8 GLY	7.6 ± .4	9.8 ±4.5	8.9	11.8	-----		10.9	9.7
9 HIS	3.4 ± .4	4.8 ± .9	3.3	7.1	5.4	9.4	0.8	3.8
10 ILE	0.82 ± .10	.98± .17	1.3	2.0	-----		1.10	1.6
11 LEU	0.81 ± .18	1.04± .30	1.3	1.8	-----		2.5	4.2
12 LYS	10.5 ±1.7	7.3 ±2.4	9.3	7.9	-----		6.3	7.9
13 MET	1.20 ± .35	2.1 ± .5	1.1	2.9	2.1	4.4	1.2	2.2
14 PHE	0.87 ± .23	0.91± .21	0.7	1.4	1.3	3.0	0.2	1.2
15 PRO	1.52 ± .49	2.6 ±1.0	3.4	6.3	2.9	4.2	1.2	1.4
16 SER	0.61 ± .17	0.54± .15	2.0	1.5	1.1	1.2	0.3	0.6
17 THR	2.6 ± .3	5.0 ±2.0	7.2	3.0	-----		7.8	6.5
18 TRP	6.0 ±1.0	10.2 ±1.7	13.	21.	-----		8.8	15.2
19 TYR	0.76 ± .01	1.00± .04	1.8	3.1	-----		0.94	1.50
20 VAL	4.7 ±1.5	7.0 ±2.1	9.0	13.	-----		4.7	7.5

* for comparison, values for 5 min have been doubled in this column.

TABLE III. tRNA from E. coli B, pmoles incorporated/10 min

Amino acid	33°		22°	
	arg 2	tsl	arg 2	tsl
1 ALA	2.4	6.0	1.1	4.6
2 ARG	7.8	9.8	5.8	3.9
3 ASN	2.6	1.8	1.4	0.9
4 ASP	5.3	5.8	4.4	4.6
5 CYS	28.	36.	24.	27.
6 GLN	0.29	0.44	0.33	0.28
7 GLU	2.6	4.2	1.6	2.6
8 GLY	11.2	14.2	5.8	8.0
9 HIS	2.1	1.5	3.8	2.1
10 ILE	1.29	1.97	0.71	0.99
11 LEU	1.66	2.96	0.78	1.22
12 LYS	3.06	5.45	1.98	2.93
13 MET	4.4	7.5	2.6	0.9
14 PHE	0.70	1.4	0.38	0.58
15 PRO	0.9	3.9	0.9	1.5
16 SER	0.4	1.1	0.4	1.1
17 THR	3.2	1.7	1.9	3.3
18 TRP	3.9	3.5	4.9	3.6
19 TYP	0.75	1.17	0.43	0.65
20 VAL	4.81	14	2.47	3.8

TABLE IV. 33° and 22° charging ts13 extracts (pmoles)/10 min

Amino acid	33°	22°
1 ALA	2.8	1.9
2 ARG	12.8	7.4
3 ASN	3.8	1.7
4 ASP	6.5	4.7
5 CYS	50.	40.
6 GLN	1.9	0.6
7 GLU	22.	13.
8 GLY	21.	14.
9 HIS	7.7	4.6
10 ILE	4.2	2.0
11 LEU	6.5	2.7
12 LYS	46	42
13 MET	2.5	2.6
14 PHE	3.2	1.5
15 PRO	5.0	3.4
16 SER	1.4	0.4
17 THR	16.7	8.6
18 TRP	16.3	10.1
19 TYR	3.9	1.4
20 VAL	18.6	10.4

As can be seen, there is no amino acid for which the corresponding synthetase activity is abolished or greatly reduced in either *ts1* (Tables II-III) or in *ts13* (Table IV) compared to arg 2. I employed tRNA from E. coli B in one set of reactions since it is known that there exist in Chlamydomonas some synthetase activities, presumably of chloroplast origin, which prefer prokaryotic tRNA to Chlamydomonas whole-cell tRNA (13). This method also did not detect any defective activities. Using higher temperatures beyond the growth range of Chlamydomonas (40°, 45°, our unpublished observations) did not serve to detect any defective enzymes. In one case (9) it was necessary to employ such conditions in order to identify the lesion in a mutant with a defective synthetase (isoleucyl-tRNA synthetase in Saccharomyces).

In cases where a defective synthetase has been identified in a temperature-sensitive strain (6,9,12,18,19,28), such in vitro assays have sufficed to identify the lesion. However, in one case where a synthetase was implicated in a glycine auxotroph (7), it was detected only by its in vivo charging of tRNA or by the use of a suppressor tRNA molecule as the substrate. Thus, such assays may not always be relied upon to identify these mutants.

Therefore, some estimate of in vivo charging seemed in order. The method chosen depended on the fact that uncharged tRNA contains a vicinal pair of hydroxyls at the -CCA terminal end, and hence is

sensitive to periodate oxidation(29). Once oxidized, it cannot be recharged in vitro, hence the ratio of amino acids charged in vitro by the oxidized molecules to those charged by the untreated tRNA is the fraction of tRNA which was charged in the cell, assuming complete charging in vitro. In practice this is difficult to achieve for several reasons. First, it is difficult to completely discharge the tRNA for recharging (26). Second, extracts containing synthetase are contaminated with RNase activity to a small but measurable degree (my unpublished observations and those of W. E. Barnett). Nevertheless, an estimate of in vivo charge was attempted.

[^{14}C]amino acids, ts1 synthetase-containing cell extract, and ts1 tRNA (either oxidized with NaIO_4 or untreated) were incubated as described in the Methods section at 33° for 15 min, 30 min or 60 min. For most amino acids the reaction was near completion at 30 minutes, and none of the amino acids showed any decrease between 30 and 60 min, as might be expected if product RNA were being degraded by RNase. In general, rates of amino acid incorporation in the first interval using unoxidized ts1 tRNA (Table V) were similar to the values obtained in previous experiments with arg 2 tRNA and ts1 enzyme extracts. Several amino acids (such as aspartic acid) did show reduced rates of incorporation, but this does not correlate with their relative level of protection from oxidation (Table V). It is therefore unlikely that the tRNA molecules themselves are the site of the lesion (26).

Table V. Cells were incubated at 22° or 33° and collected by continuous flow centrifugation. They were extracted with phenol and half of each preparation was then oxidized with NaIO_4 to render any uncharged tRNA molecules unable to be recharged. Then both halves were discharged at pH 8.8 and recharged at 33° with [^{14}C]amino acids as described in the text.

TABLE V. ts1 tRNA

Amino acid	pmoles amino acid bound to untreated (22°) tRNA per min at 33°		cpm (oxidized) cpm (untreated) (ts1)		column A/column B 33°/22°
	ts1 extract and tRNA	arg 2 extract and tRNA	A 33°	B 22°	
1 ALA	0.22	0.24	.07	.09	0.78
2 ARG	0.16	0.78	.06	.11	0.82
3 ASN	0.097	0.26	.17	.23	0.73
4 ASP	0.082	0.53	.19	.30	0.62
5 CYS	0.83	2.8	1.85	3.27	0.56
6 GLN	0.079	0.029	.20	.08	2.3
7 GLU	0.22	0.26	.12	.17	0.71
8 GLY	0.26	1.12	.20	.17	1.17
9 HIS	0.092	0.21	.18	.18	1.0
10 ILE	0.108	0.13	.58	.78	0.74
11 LEU	0.106	0.17	.14	.13	1.07
12 LYS	0.078	0.31	.06	.06	0.93
13 MET	0.18	0.44	.18	.23	0.78
14 PHE	0.034	0.08	.18	.19	0.94
15 PRO	0.22	0.09	.05	.05	1.0
16 SER	0.02	0.04	.9	.4	2.
17 THR	0.30	0.32	.03	.07	.36
18 TRP	0.27	0.39	.22	.33	.66
19 TYR	0.06	0.08	.12	.18	.66
20 VAL	0.37	0.48	.73	.81	.90

An unexplained and unusual observation is the 1.8-3.2-fold stimulation of cysteine binding caused by oxidation of the tRNA. It is possible that the oxidation produces a new site on the tRNA, probably an aldehyde, which reacts with cysteine to form a thioacetal (1).

The tRNA molecules, in general, are not protected to the degree found for 16 amino acids in E. coli by Yegian et al. (70-120%) (29). However, my average for 19 amino acids (excluding cysteine, see above) is 23% protection, and this figure was not reduced at 33°.

Since no amino acid exhibits a precipitous decline in protection on incubation at 33°, tRNA molecules or aminoacyl-tRNA synthetases are unlikely to be responsible for the lesions in these mutants. It must be noted, however, that the rate of charging of all amino acids using ts1 tRNA and synthetase at 33° was only 36% of that obtained using arg 2 tRNA and synthetase under similar conditions. This deficit is unlikely to be the cause of the temperature sensitive lesion since it could be due to a variety of trivial reasons, and since protein synthesis is inhibited by 90% or more in vivo.

DISCUSSION

In order to unambiguously define a temperature-sensitive mutant it is necessary to determine biochemically what specific molecule is altered by the lesion. For a mutant in initiation of protein synthesis, this would require development of a system for in vitro protein synthesis entirely dependent on natural initiation. However, we have as yet had little success in finding conditions suitable

for protein synthesis from endogenous messenger or added natural messengers which are efficient either in an S30 extract or in a more fractionated system similar to that of Chua et al. (3). Thus at this point we are unable to assay for defects in specific initiation factors in Chlamydomonas. Further work will allow the identification of factors required for synthesis from endogenous messengers and make if possible then to identify the lesions in these mutants. An alternative might be to assay for initiation factors from Chlamydomonas using another fractionated protein synthesizing system, such as that from wheat germ, in which much progress has been made towards understanding initiation in eukaryotic protein synthesis.

Otherwise, our data strongly points to these mutants being defective in initiation. When raised to 33°, their ribosomes breakdown into monosomes not bound to mRNA. The breakdown of polysomes and release of ribosomes as salt-dissociable ribosomes are inhibited by cycloheximide and involve only 80S ribosomes and their subunits. This latter fact is a curious one. It is surprising since greater than 90% of amino acid incorporation is concurrently inhibited, despite the fact that about 30% of cellular protein synthesis is on organelle ribosomes as estimated either from their percentage of the total number of ribosomes (4), or the maximal percentage of amino acid incorporation inhibitable by chloramphenicol or spectinomycin (unpublished). This fact may reflect some not yet understood coupling between cytoplasmic and organelle protein

synthesis. It might be that any inhibition of cytoplasmic protein synthesis signals a halt to organelle protein synthesis. We know, for instance, that cycloheximide can inhibit 90 to 100% of cellular protein synthesis at modest levels ($< 10 \mu\text{g/ml}$), which have no effect on amino acid incorporation by isolated 70 S ribosomes (3,21).

These mutants should be useful in studying a variety of cellular processes which may require protein synthesis to occur, since these mutants appear affected only in protein synthesis and not in synthesis of RNA (20,21). DNA synthesis is also partially blocked, but since all treatments which interfere with protein synthesis in Chlamydomonas also partially block DNA synthesis (21), this may be the result of a secondary effect.

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Part II.

A. Chloral Hydrate Causes Breakdown of Polysomes
in Chlamydomonas reinhardi in vivo.

SUMMARY

Chloral hydrate produces a biphasic change in the proportion of polysomes in the cell. Within 1-2 min after addition to cells it inhibits protein synthesis and causes polysomes to break down. The ribosomes dissociate from mRNA by a process which requires protein synthesis but which is apparently abnormal. Released ribosomes do not appear to be bound to fragments of mRNA, but do carry a nascent polypeptide chain. Protein synthesis remains inhibited by more than 85% for over 24 hr, but the apparently normal polysomes reform after 90-120 min. These effects result from interaction of the cells with chloral hydrate itself and not from its conversion to one of its usual metabolic products, trichlorethanol or trichloroacetic acid.

INTRODUCTION

Chloral hydrate, after a history of notoriety as knockout drops and the Mickey Finn, has become an important sedative and anesthetic for children and the elderly. It is also an antimitotic agent, as are most anesthetics (1). While it interferes with the formation of mitotic poles (2) and the elongation of the spindle (3), it does not prevent shortening of previously formed spindle fibers (2, 3). Mitosis is prevented by concentrations (5-13 mM) which produce anesthesia in a broad range of animals including fish and mammals (1).

Chloral hydrate also prevents cell division in Chlamydomonas reinhardi at these concentrations, and we have shown that these concentrations inhibit protein synthesis (4). The correlation between the extents of inhibition of these two processes by different concentrations of chloral hydrate is very good, $r = 0.99$ (5). Therefore we have investigated the effects of chloral hydrate in greater detail.

Other anesthetics have been shown to partially inhibit protein synthesis, but have not been subjected to systematic study. Ether inhibited the incorporation of [^{35}S]cysteine into rat brain protein by 35%, but rat liver protein synthesis was unaffected (6). The concentrations of ether in the two tissues were not measured and so it is impossible to say whether the difference in the degree of inhibition resulted from differential tissue sensitivity to ether or some other reason, such as differential accumulation of ether. Chloroform partially inhibited the incorporation of [^3H]leucine into rat liver (7). Inhibition of amino acid incorporation by these anesthetics, however, could have been caused by relatively trivial reasons, includ-

ing some gross physiological change or an alteration in the specific radioactivity of precursor pools.

Many of these technical difficulties can be avoided by using chloral hydrate on C. reinhardi. It blocks protein synthesis at 10 mM, an effect which is rapidly reversible. There are no deleterious effects on the cell at this concentration (4). Chloral hydrate inhibits cell division and produces anesthesia in a variety of organisms at concentrations which overlap those which inhibit protein synthesis (1, 2, 4). It also completely inhibits protein synthesis in cultures of mouse neuroblastoma at a concentration of 10 mM (G. Magnus and D. McMahon, unpublished results).

MATERIALS AND METHODS

Cultures. Most experiments used arg 2, a mutant of Chlamydomonas lacking arginosuccinate synthetase (8). It was obtained from W. Ebersold, University of California at Los Angeles. ts13 is a mutant derived from arg 2 by selection with canavanine at 33° (9). It grows normally at 22° but protein synthesis is inhibited at 33°, apparently because of a defect in initiation (our unpublished observations). CW-15, a mutant lacking a normal cell wall, was obtained from D. Roy Davies, John Innes Institute, Norwich, England. Cells were grown as described previously in HSMA medium (4).

Reagents. $\text{H}_2^{35}\text{SO}_4$ (carrier free) and [methoxy- ^3H]puromycin were from New England Nuclear Corp., while [2- ^3H]arginine was from Schwarz/Mann. Chloral hydrate (USP) from Merck was used directly or after distillation under 10 cm mercury and recrystallization from hot water. The purification did not result in any change in the effects of chloral hydrate on [^3H]arginine incorporation into protein or $^{32}\text{P}_1$ incorporation into total nucleic acids (4). Trichloroethanol (98%) from Aldrich Chemical Company was dissolved immediately before use to avoid light stimulated decomposition. Amino acids, cycloheximide, deoxycholic acid, and unlabeled puromycin dihydrochloride were from Sigma Chemical Company. Chloramphenicol was from Parke, Davis and Company and RNase A and T were from Worthington Biochemicals. Sucrose for analytic gradients was analytical reagent grade, decolorized with Norit A (Braun Chemical Company), while lysis buffers and preparative

gradients used Schwarz/Mann Ultrapure RNase-free sucrose. Other chemicals were analytic reagents.

Analysis of Polysomes. Cells ($0.5 - 2 \times 10^6$ cells/ml, 200 ml/flask) were incubated as described in the figures and then poured through a funnel containing ice made from distilled water. Subsequent operations were at $0-4^\circ$. They were resuspended in 2.0 ml of lysis buffer containing 25 mM Tris-HCl pH 7.6, 25 mM KCl, 25 mM $MgCl_2$ and 0.25 M sucrose (10). The cells were passed through a French press at a pressure of no more than 7×10^7 dynes cm^{-2} , 0.1 vol of 0.12 M potassium deoxycholate, pH 8, added to lyse > 90% of the cells, and the tube mixed gently. CW-15 cells could be lysed with the deoxycholate without French pressing. The key to isolation of intact polysomes in high yield is the use of minimal force with the French press and reliance on the deoxycholate to lyse the cells. The suspension was centrifuged $10^4 \times g$ for 10 min and the supernatant carefully removed. About 0.1 ml of 25% (w/v) Triton X-100 was added per 2 ml of lysate and the contents gently mixed. Next the lysate was layered on 0.31-1.2 M convex exponential sucrose gradients (9) and centrifuged at $1.74 \times 10^5 \times g_{av}$ for 2 hr at 2° . The absorbance of each gradient at 260 nm was recorded continuously as the gradient was pumped from the tube. The percentage of polysomes was determined by weighing Xerox copies of the mono- and polyribosomal regions. Eighty-five per cent of the UV-absorbing material in the polysome region is sensitive to degradation by RNase (Table 1). The background resistant to RNase has not been subtracted from these determinations. The baseline of absorbance of the sucrose gradient itself was zero,

since decolorized sucrose solutions were used.

Labeling of Nascent Peptides with [^3H]Puromycin. Arg 2 cells (1 liter, 2×10^6 cells/ml) either untreated or given chloral hydrate for 15 min were chilled and lysed in polysome buffer + 40 mM 2-mercaptoethanol and 50 $\mu\text{g/ml}$ dextran sulfate (Sigma) as described above. The $10^4 \times g$ supernatant was then layered over 2.0 ml 1.8 M sucrose in polysome buffer and centrifuged at $1.05 \times 10^5 \times g_{\text{av}}$ for 18 hr at 2° . The pellet was rinsed and resuspended in polysome buffer containing 5 mM MgCl_2 and 10 mM 2-mercaptoethanol. RNA was determined by the Schmidt-Thannhauser (19) method, modified by Fleck and Munro (20). The number of nascent peptides present were assayed by incubating the resuspended ribosomes with [^3H]puromycin in a 0.5 ml reaction mixture containing: 40-160 μg ribosomal pellet RNA, 0.18 mM [^3H]puromycin (5.2 $\mu\text{c}/\mu\text{mole}$), 0.2 mM GTP, 25 mM Tris-HCl pH 7.6, 25 mM KCl, 5 mM MgCl_2 , 10 mM 2-mercaptoethanol. The reaction was not further stimulated by the addition of pH 5 enzymes prepared according to (10). Incubations were started by addition of puromycin and incubated at 33° . Normal incubation was for 30 min. Reactions were terminated by addition of 1.5 ml ice-cold 10% trichloroacetic acid, were collected on Whatman GF/A filters and washed with 3 x 5 ml 10% trichloroacetic acid and with absolute ethanol and counted (4). Efficiency of counting was determined by internal standardization.

Gel-filtration Chromatography. Nascent peptides were labeled with puromycin by incubation at 33° for 60 min as above. The mixes were

precipitated in four volumes of 15% trichloroacetic acid, and collected on a Whatman GF/C filter. These were washed with 4 x 10 ml of 10% trichloroacetic acid, and dried at 50°. The filter was then extracted overnight with 0.5 ml 8 M urea + 0.05 M Tris-HCl pH 7.6, 0.3 M 2-mercaptoethanol at room temperature. The next day 0.1 ml 1 mg/ml blue dextran was added and the entire contents were layered over a 51 x 1 cm column of Bio-Gel P2 equilibrated with 8 M urea + 0.05 M Tris-HCl pH 7.6, and eluted with the same buffer at 20 ml/hr. Fractions of 0.5 ml were taken and of these 0.40 ml aliquots were taken and counted in 6 ml scintillation fluor (3.75 g/l diphenyloxazole in xylene (technical)-Triton XL00 3:1 (v/v) at an efficiency of 40%. The recovery of radioactivity from the column was about 100%.

Chromatography of [1-¹⁴C]Chloral Hydrate. Exponential phase Arg 2 cells were resuspended in fresh medium at 8×10^6 cells/ml and to 0.90 ml cells was added 0.10 ml [1-¹⁴C]chloral hydrate (2 µc/ml, 100 mM, made up in HSMA). At 0, 15, 60 or 120 min, 50 µl aliquots were put into chilled tubes containing 50 µl ethyl acetate. The sealed tubes were incubated for two hours at 0° with periodic mixing. Then the samples were placed in a bath of dry ice-acetone until chromatographed the same day. At this time the samples were melted in an ice bath and then refrozen in dry ice-acetone briefly so as to freeze only the aqueous layer. Ten microliter aliquots of the organic layer were spotted on 5 x 20 cm x 0.25 mm sheets of silica gel (E. M. Laboratories) along with standards of chloral hydrate, trichloroethanol and trichloroacetic acid (which were also extracted from aqueous solution into ethyl acetate).

The plates were developed in ethyl acetate-hexane 1:1 (v/v). All chromatographic steps were performed in a cold room at 4°. The lanes of the plates containing label were scraped by 0.5 cm lanes into scintillation vials and counted in xylene-Triton X100 liquid scintillation fluor at 44% efficiency. The remainder of each plate was then sprayed with ethanolic diphenylamine solution (0.5% w/v) and irradiated at close range with an unfiltered 4 W ultraviolet lamp until the spots of the chlorinated compounds appeared (31).

RESULTS

Breakdown of Polysomes. Chloral hydrate (10 mM) inhibits the incorporation of [^3H]arginine into protein by an arginine auxotroph of *C. reinhardi*, arg 2, by 85-90% (4) for more than 24 hr. Fig. 1 shows that the rate of protein synthesis labeled with $^{35}\text{SO}_4^{=}$ is inhibited by 78% within 1-2 min. Polysomes extracted from chloral hydrate-treated cells (Fig. 2) begin dissociating into monosomes after 1 min and are reduced in amount by 50% in 6-15 min. Several hours later, larger than normal amounts of polysomes are present in spite of continuing inhibition of protein synthesis. This biphasic result was totally unexpected and led us to further investigate the nature of the events which cause it.

Although it may appear from Fig. 2 that total recovery of ribosomes is altered by the addition of chloral hydrate, analysis of several experiments shows that this appearance is due entirely to variations in recoveries. When the total areas under mono- and polyribosomal peaks

Fig. 1. Effect of 10 mM chloral hydrate on $^{35}\text{SO}_4$ incorporation into protein. 0 - no addition; Δ - 10 mM chloral hydrate added at the arrow. Arg 2 cells were washed and resuspended to 7×10^5 cells/ml in medium (12) with 0.1 the normal sulfate concentration (normal = $81 \mu\text{M}$), containing $1 \mu\text{Ci ml}^{-1}$ of $\text{H}_2^{35}\text{SO}_4$. At intervals 1 ml samples were added to 1 ml of 10% (w/v) trichloroacetic acid + 10 mM Na_2SO_4 + 1 mM l-cysteine + 1 mM reduced glutathione, mixed and placed at 0° for >30 min. An additional 1.0 ml of 10% trichloroacetic acid was added, the tubes heated to 90° for 30 min, then chilled to 4° . Samples were filtered onto GF/A filters and washed with 10% trichloroacetic acid and then washed 3 times with 4 ml methanol:chloroform:water (12:5:3 v/v) (13). Their radioactivity was determined by scintillation counting (4).

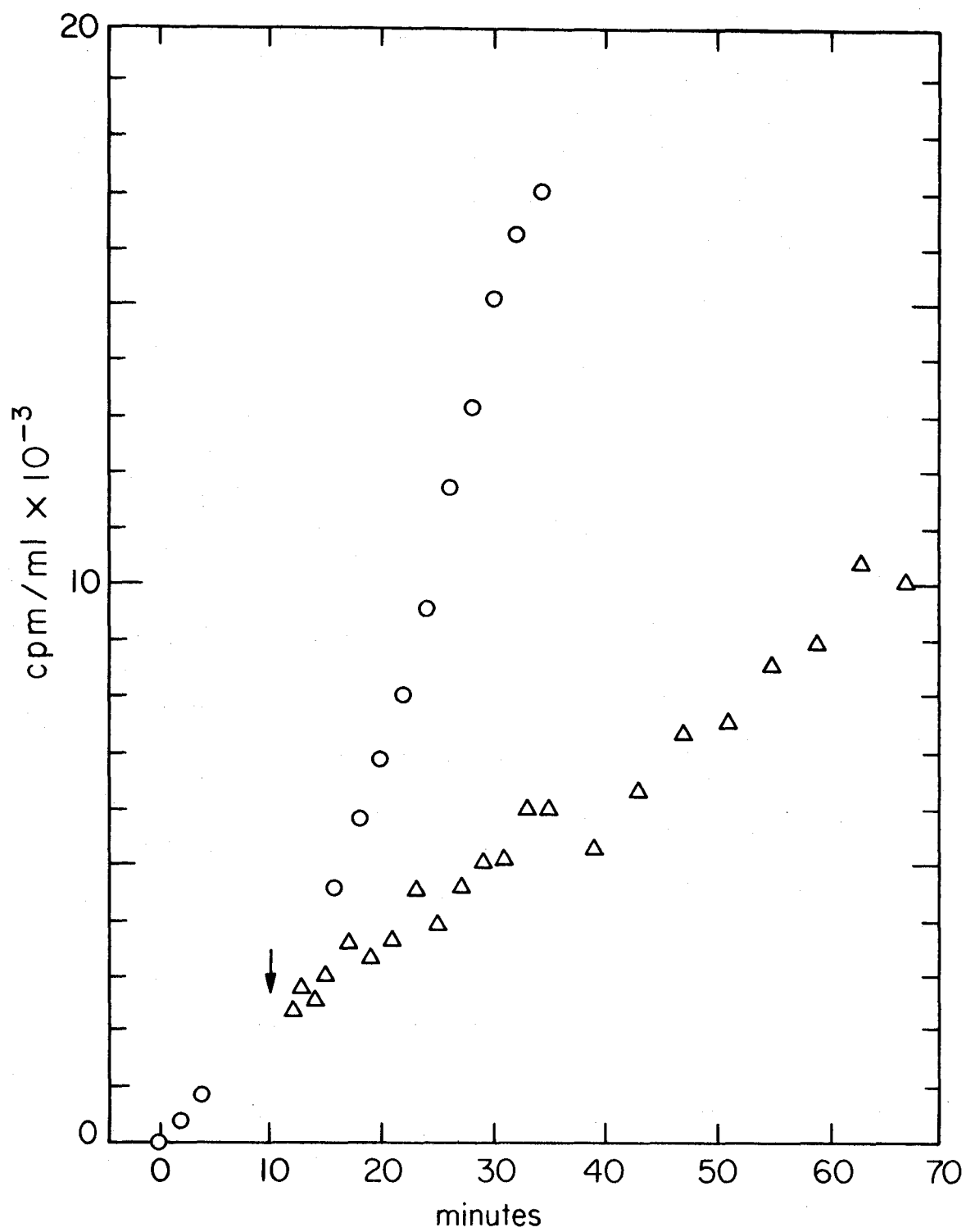


Fig. 2. Size distribution of polysomes in cells treated with chloral hydrate. Cells (Arg 2, 5×10^5 cells/ml, 200 ml/flask) were incubated with or without 10 mM chloral hydrate, and after the indicated incubation they were chilled and polysomes analyzed on 12 ml sucrose gradients in the SW41 rotor as described in the text. (a) A_{260} pattern of polysomes, centrifugation left to right; (b) per cent polysomes relative to control (0-120 min), error bars represent $1 \times$ S.E.; (c) per cent polysomes relative to control (0-8 hr).

Figure 2a

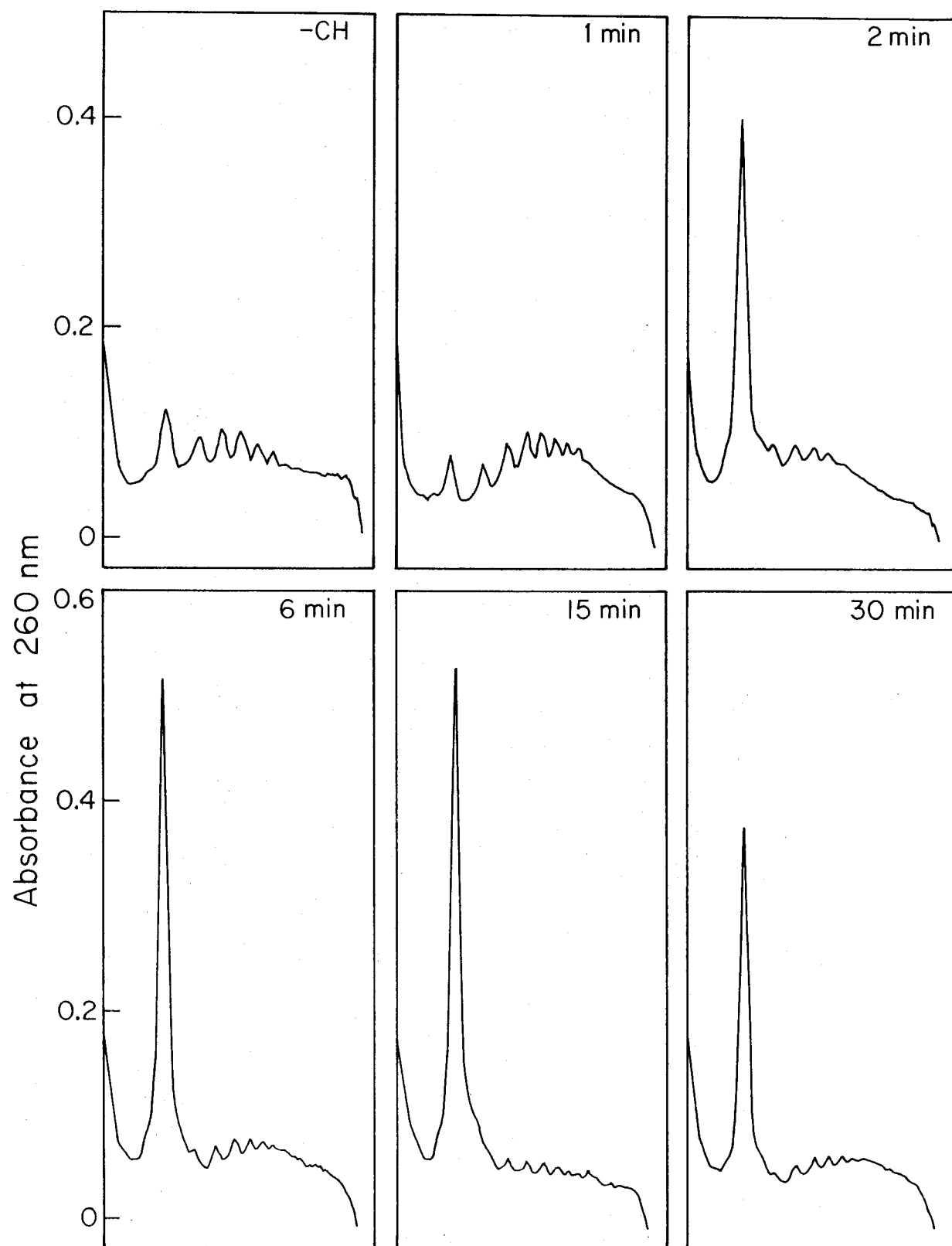
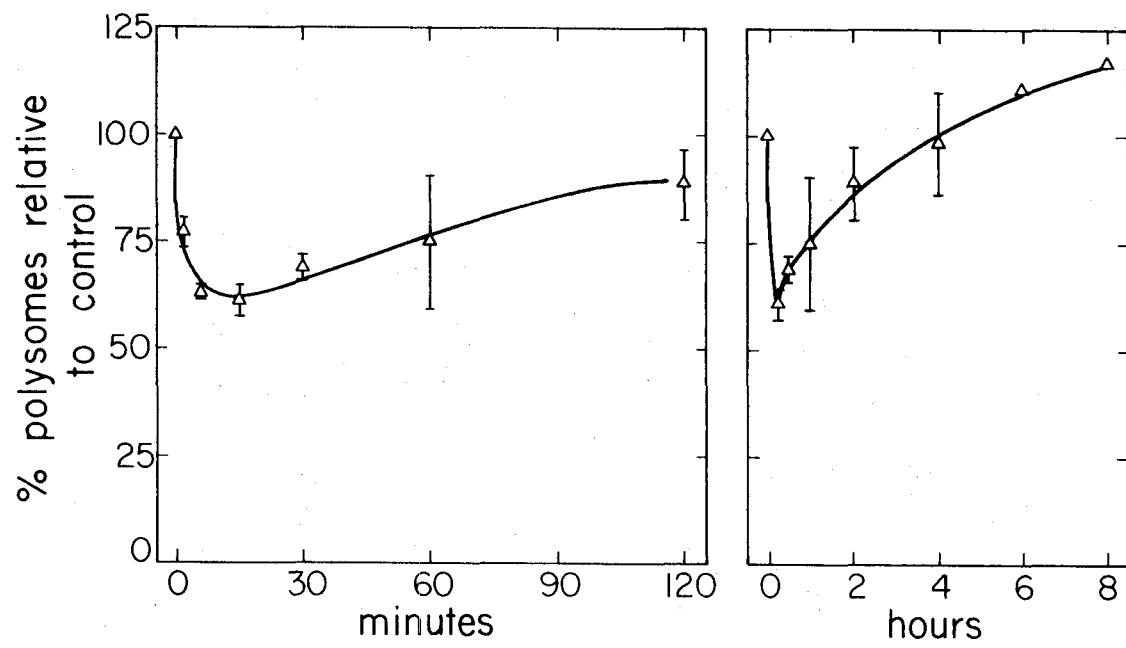


Figure 2b

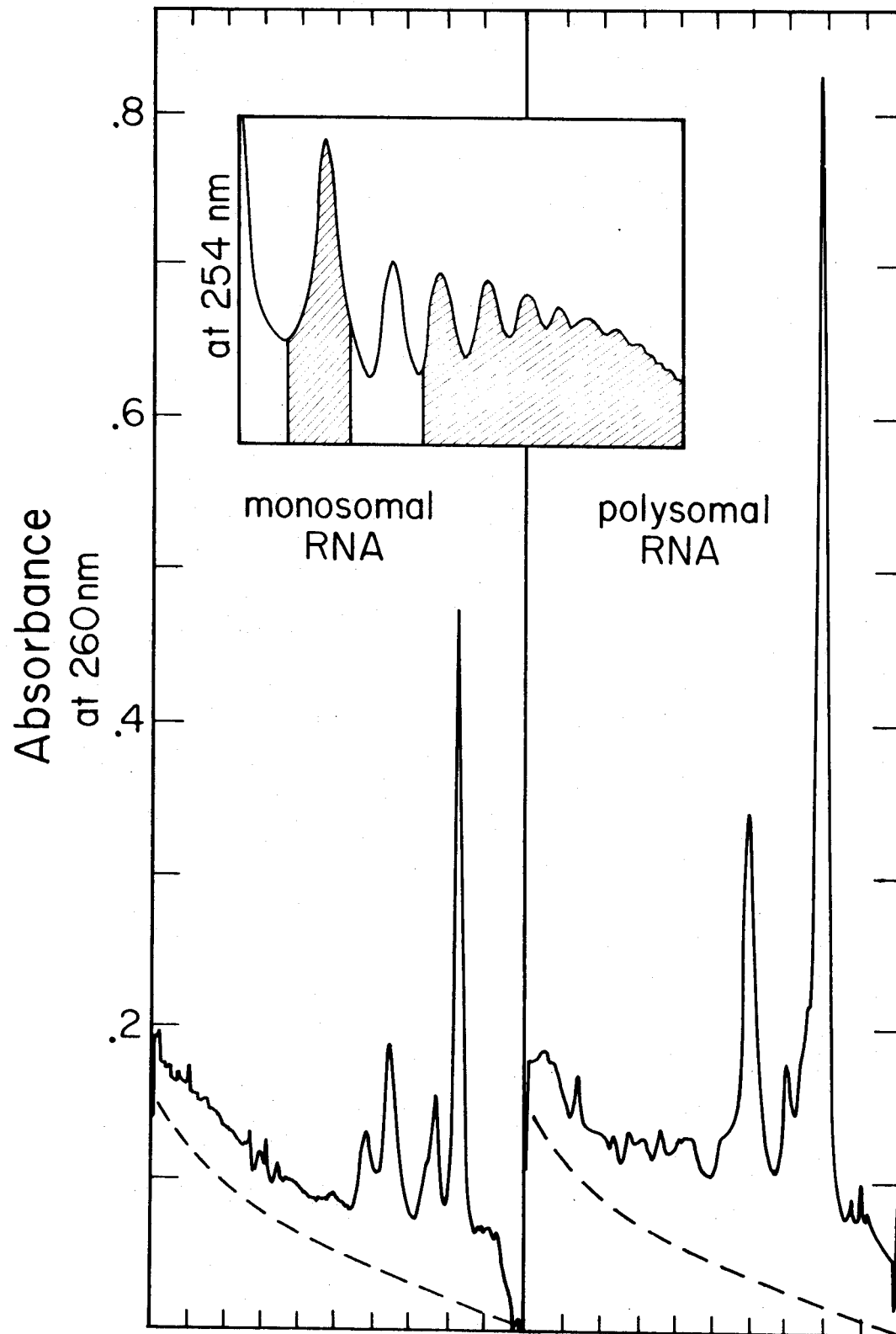


were integrated by weighing Xerox copies and compared to the control, the following relative recoveries were obtained, corrected in each case for variation in the rate at which the gradient was pumped from the tube (weight of absorbance from treated cells/weight of absorbance from untreated cells): 10 mM chloral hydrate 1 min, 0.91 (1)*; 2 min, 1.06 \pm 0.10 (2); 6 min, 1.07 \pm 0.15 (5); 15 min, 0.99 \pm 0.05 (5); 30 min, 0.96 \pm 0.07 (4); 60 min, 0.89 \pm 0.08 (4); 120 min, 0.91 \pm 0.12 (5); 240 min, 0.77 \pm 0.01 (2). The decline in recovery for the 60 min and greater time points is entirely due to growth in the control culture during the period after the drug was added to the experimental cultures and the time that all the cultures were harvested. This interpretation is supported by the measurement of protein (30) and RNA (19) contents of the lysates from several experiments, which showed greater protein and RNA in the untreated samples, as expected.

The polysomes consist largely of cytoplasmic ribosomes. The RNA extracted from monosomal and polysomal regions of sucrose gradients was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3) (14). The 25S and 18S RNA species are characteristic of Chlamydomonas cytoplasmic 80S ribosomes, while the 23S and 16S species derive from the chloroplast 70S ribosomes (10). Judging from the ultraviolet absorption of the peaks of rRNA, the polysomal region contains 76% cytoplasmic ribosomes and the monosomal region contains 63% cytoplasmic ribosomes. These values compare to the 65-80% cytoplasmic ribosomes determined for whole cells by ourselves and other workers (10,14). Polysomes containing

* \pm standard error, number of experiments in parentheses.

Fig. 3. RNA from Chlamydomonas monosomes and polysomes. Arg 2 cells were chilled and lysed as in Fig. 2 and the $10^4 \times g$ supernatant was layered on 0.31-1.2 M exponential sucrose gradients and centrifuged 4 hr at $9.5 \times 10^4 \times g_{av}$ at 2° in the SW27 rotor. The tubes were scanned at 254 nm in an ISCO gradient fractionator and 1 ml fractions taken. The fractions from the monosomal and polysomal regions were pooled (shaded areas of inset), RNA extracted (12) with phenol-m-cresol-8-hydroxyquinoline (90:10:0.1, v/v/w), and the RNA in the aqueous phase precipitated with 2 volumes of -25° absolute ethanol. The pellet was washed with 70% ethanol and was dissolved in distilled water. Electrophoresis was on 2.4% acrylamide, 0.4% agarose gels (12). Left panel: Monosome region RNA. Right panel: Polysome region RNA. Sedimentation on the sucrose gradients is shown left to right, and electrophoresis is shown right to left. The major RNA peaks are (l. to r.) 16S, 18S, 23S and 25S RNA. The baseline is indicated as a dashed line.



both types of ribosomes appear to break down after treatment with chloral hydrate (unpublished results).

This decrease in polysomes might have been caused by inhibition of ribosome attachment to mRNA caused by a ribosome which was "frozen" by chloral hydrate. Such a decline in polysomes has been observed with low concentrations of chlortetracycline, where an average of one ribosome is frozen per message (15). This mechanism, however, should not decrease polysomes by more than 50% since the average position of a frozen ribosome (at which point it would block the translocation of other ribosomes) should be in the middle of the mRNA. Higher concentrations of chloral hydrate would then restore normal amounts of polysomes. Table 1 shows that 20 mM chloral hydrate diminishes polysomes by 62%, so a mechanism involving an average of one frozen ribosome per mRNA molecule is unlikely. Moreover, we shall show that the accumulating ribosomes have not naturally run off the mRNA, but have terminated abnormally.

The dissociation produced by chloral hydrate requires protein synthesis. It does not occur at 0°, nor does it occur in the presence of a low concentration of cycloheximide (Table 1). The inhibition by cycloheximide argues against a trivial explanation for the polysome breakdown: that chloral hydrate activated or released an endogenous RNase which then degraded the polysomes. This also appears unlikely since the polysomes reform rapidly when the chloral hydrate is removed, and for one other reason. Degradation of polysomes by RNase should leave a fragment of mRNA on the ribosome. The ribosomes of a variety

TABLE 1. The effects of various treatments on the fraction of ribosomes contained in polysomes

Treatment	Per cent polysomes relative to control
Control, no chloral hydrate	100
10 mM chloral hydrate, 6 min 22°	63
10 mM chloral hydrate + 2 µg/ml cycloheximide, 6 min 22°	110
10 mM chloral hydrate, 6 min 0°	98
10 mM chloral hydrate, 30 min 0°	104
10 mM chloral hydrate, 60 min 0°	108
20 mM chloral hydrate, 6 min 22°	32
20 mM chloral hydrate + 2 µg/ml cycloheximide, 6 min 22°	115
control + RNase <u>in vitro</u>	15

Arg 2 cells were incubated as indicated after the addition of chloral hydrate. Cycloheximide, if included, was added immediately before the chloral hydrate. Cells incubated at 0° were funneled through ice immediately after addition of chloral hydrate. Polysomes were analyzed as described under Fig. 2. RNase (last line) was added to a control S10 extract at 94 U ml⁻¹ RNase A + 20 U ml⁻¹ RNase T₁, and it was incubated 15 min at 37° before layering on a gradient. It should be noted that cycloheximide is a relatively nonspecific inhibitor of protein synthesis. However, its effects on this system are produced more rapidly than its "nonspecific" effects (11).

of organisms, including Chlamydomonas, carrying such fragments of mRNA cannot be dissociated into subunits by 500 mM KCl (10,16,17), but the ribosomes released by chloral hydrate are dissociated by this treatment (Fig. 4). Ribosomes which are dissociated by high salt have increased after 15 min of treatment and have begun to decrease by 2 hr as we would expect from the kinetics of polysome breakdown and reaggregation (Fig. 2). This result also excludes the possibility that the polysome size distribution changes because the average size of mRNA molecules has decreased.

If free ribosomes accumulate simply because initiation of protein synthesis is inhibited more than peptide elongation and chain termination, then the released ribosomes should not carry nascent peptides. We determined whether nascent peptides decreased on ribosomes isolated from cells treated with chloral hydrate by measuring the ribosome-catalyzed incorporation of [^3H]puromycin into a trichloroacetic acid insoluble form (18) (Table 2). If there is no reinitiation of protein synthesis in vitro, then the molecules of puromycin precipitated in vitro should correspond to the number of active ribosomes which carry nascent peptides in vivo. Since we have added no tRNA, amino acids or soluble factors for protein synthesis, reinitiation is rather unlikely. Table 2 shows that the reaction is saturated with puromycin and linear with increasing amounts of added ribosomes. The kinetics and extent of the reaction are quite comparable to those observed in other systems (18,21).

Equivalent amounts of [^3H]puromycin-labeled peptides were recovered

Fig. 4. Dissociation of ribosomes in 500 mM KCl. CW-15 cells were exposed to 10 mM chloral hydrate for 0, 15 or 120 min. S10 extracts were prepared as described in the text, cycloheximide (10 μ g) and chloramphenicol (125 μ g) added per 0.8 ml of lysate and treated with RNase A (59 U ml⁻¹ Worthington, 10 min at 21°). They were rechilled and centrifuged on 0.31-1.1 M exponential sucrose gradients in 500 mM KCl, 25 mM MgCl₂, 25 mM Tris-HCl pH 7.6 at $1.91 \times 10^5 \times g_{av}$ for 270 min at 4°. Gradients were scanned at 254 nm using an ISCO gradient fractionator. Centrifugation is left to right. The total UV absorption due to ribosomes and their subunits is slightly greater in the 15 min than in the 0 min gradients, possibly indicating a release of ribosomes which are tightly membrane bound, while the 120 min gradient contains only 74% of the absorbance in the control. This difference is consistently seen, but is not observed when UV absorbing material on the gradient is normalized to S10 protein or to S10 RNA, so we believe it to be due to growth of the cells in the 105 min period after chloral hydrate is added to the 120 min culture and before the drug is added to the 15 min culture. S-values were obtained by comparison with the A₂₅₄ patterns of mouse liver ribosomes sedimented under identical conditions. Material sedimenting between 60S and 80S is believed to represent partially unfolded 70S organelle ribosomes.

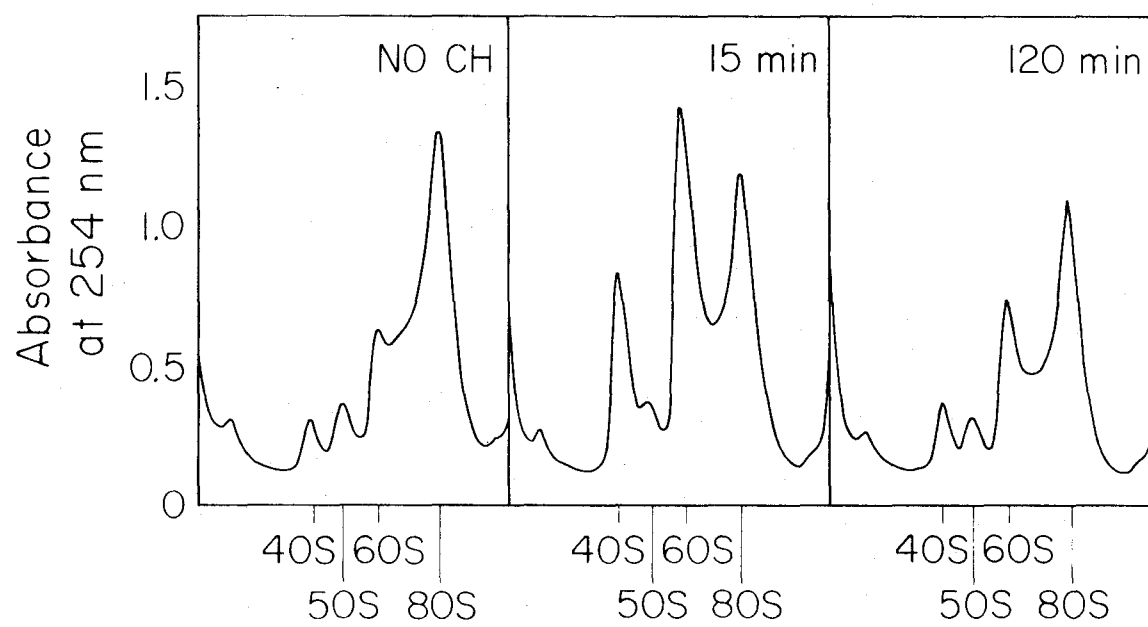


TABLE 2. [^3H]puromycin labeling of nascent peptides

Tube	μg RNA in added ribosomes	Reaction conditions	pmoles [^3H]puromycin precipitated
Experiment A: ribosomes from control cells			
1	40	normal	12
2	80	normal	28
3	120	normal	44
4	160	normal	68
5	80	no GTP	23
6	80	0.09 mM [^3H]puromycin	26
7	80	0.18 mM [^3H]puromycin	28
8	80	0.36 mM [^3H]puromycin	30
9	80	2.5 mM Mg^{2+}	20
10	80	5.0 mM Mg^{2+}	21
11	80	7.5 mM Mg^{2+}	21
12	80	10.0 mM Mg^{2+}	15
Experiment B: ribosomes from control, or chloral hydrate treated cells			
1	80 from control cells	normal, 0 min incubation	1
2	80 from control cells	normal, 5 min incubation	6
3	80 from control cells	normal, 60 min incubation	22
4	80 from control cells	normal	21
5	80 from control cells	5 mM chloral hydrate	18
6	80 from control cells	10 mM chloral hydrate	20
7	80 from control cells	20 mM chloral hydrate	19
8	80 from cells treated with 10 mM chloral hydrate	normal	44
9	80 from cells treated with 20 mM chloral hydrate	normal	32

(continued next page)

Table 2 [^3H]puromycin labeling of nascent peptides (continued)

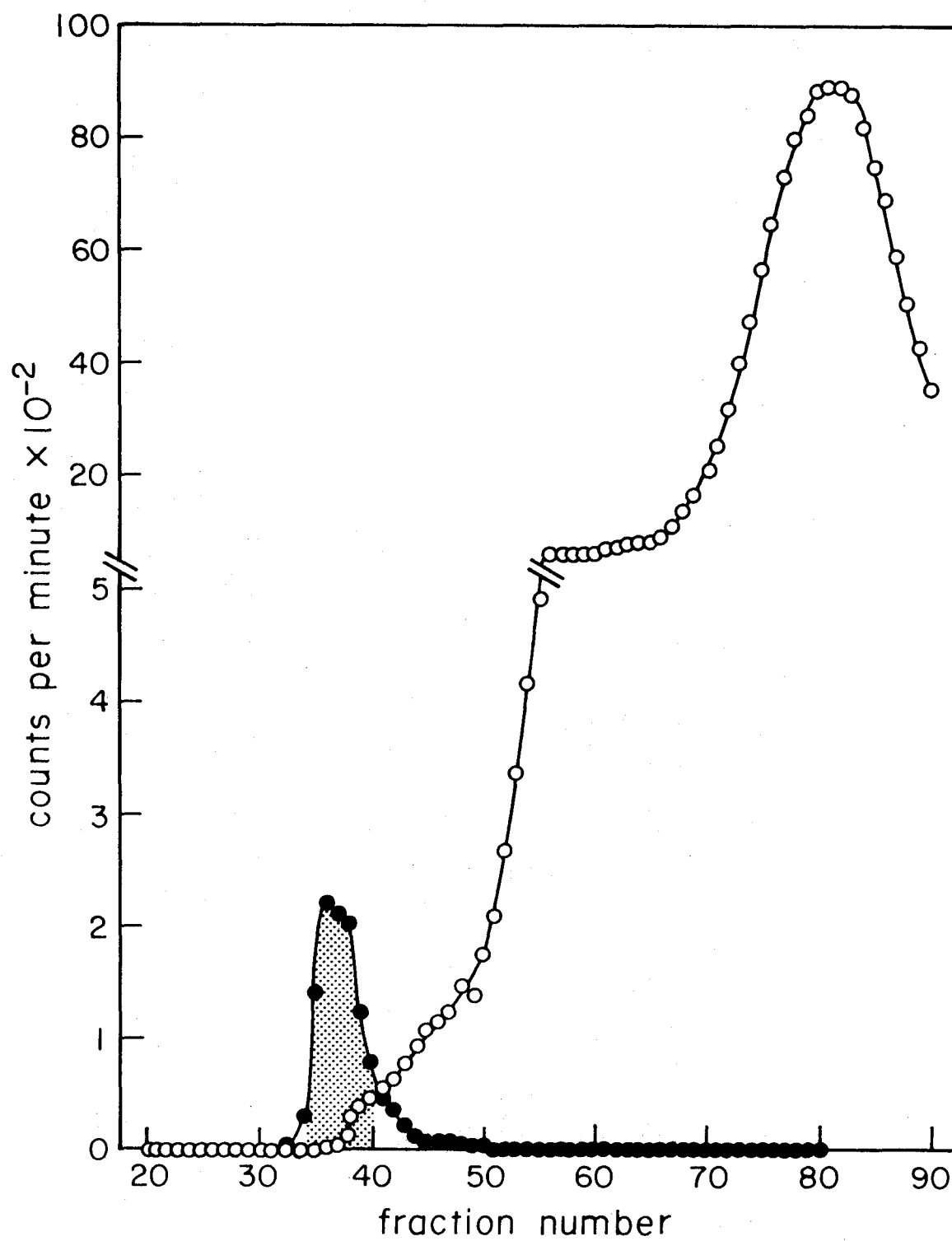
Ribosomes prepared from untreated cells or those given 10 or 20 mM chloral hydrate for 15 min were incubated as described in Methods at 33° for 30 min in 0.18 mM [^3H]puromycin (5.2 $\mu\text{C}/\mu\text{mole}$) unless stated otherwise. Incubations were ended by precipitation with cold trichloroacetic and ^3H was counted as described in the text.

by precipitation with 3 volumes of 100% ethanol at -25° , as with tri-chloroacetic acid, and the acid-insoluble material was completely excluded on a Bio-Gel P2 column when it was redissolved and eluted in buffered 8 M urea (Fig. 5). If the reaction mix was incubated with pronase for 90 min at 33° , acid precipitated less than 10% of the radioactivity of an undigested control (not shown).

In most reactions we have employed an amount of ribosomes containing 80 μ g RNA. This corresponds to 40 pmoles of ribosomes if each ribosome contains 2×10^6 daltons of RNA ($1.3 \times 10^6 + 0.69 \times 10^6$, for 80S ribosomes (14)). This number of ribosomes from untreated cells incorporated 28 pmoles of puromycin indicating that 70% of the ribosomes bear nascent peptides. This fraction compares favorably with the fraction of ribosomes engaged in protein synthesis as estimated by the fraction of ribosomes in polysomes (67-75%) or the fraction resistant to dissociation by 500 mM KCl (73%). When ribosomes from chloral hydrate-treated cells were used, the fraction of ribosomes carrying nascent chains was higher than the control, even though there were fewer ribosomes which were "active" by the two criteria above. Every ribosome from cells treated with 10 mM chloral hydrate 15 min bears a nascent peptide, as do 80% of those from cells treated with 20 mM chloral hydrate. This result indicates that the termination of the ribosomes from their messenger RNA is abnormal. Finally, the data in Table 2 show that chloral hydrate does not inhibit the puromycin release reaction.

Reformation of Polysomes. The reformation of polysomes which occurs in chloral hydrate could either require normal initiation or could be an

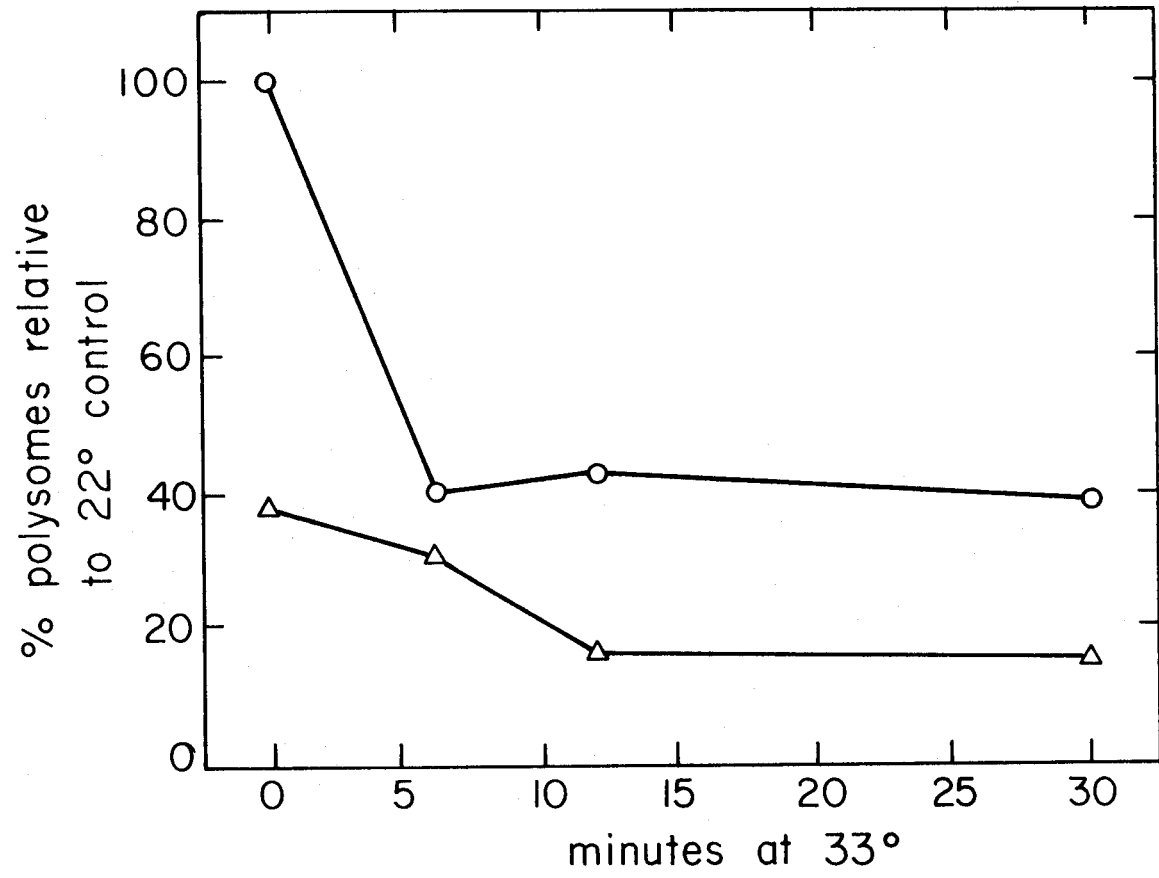
Fig. 5. Chromatography of [^3H]puromycin-labeled peptides in 8 M urea on Bio-Gel P2. Ribosomes (150 μg) from untreated Arg 2 cells were incubated with 50 μM [^3H]puromycin (25 $\mu\text{c}/\mu\text{mole}$) as described in the legend of Table II, and chromatographed as described in the test. The recovery of precipitated radioactivity from the excluded volume of the column was 100%. Filled circles: precipitated material which had been redissolved in urea. Open circles: [^3H]puromycin alone chromatographed on the same column. Shaded area: fractions containing blue dextran.



abnormal aggregation of ribosomes. Using a temperature-sensitive mutant defective in initiation of protein synthesis, tsl3 (22), we attempted to resolve this question. When raised to the restrictive temperature, 33°, tsl3 shows a rapid decay of polysomes to monosomes (Fig. 6), and cannot initiate protein synthesis on natural messengers in vitro (J. Cross, unpublished observations). The extent of breakdown of polysomes from tsl3 incubated in 10 mM chloral hydrate is greater than in its parent strain, arg 2, and recovery of polysomes is less in tsl3. We incubated tsl3 with 10 mM chloral hydrate for 9-1/2 hr, a time which allows complete recovery of polysomes in arg 2. In tsl3, only partial recovery occurs (Fig. 6), suggesting that the effects of chloral hydrate and the defect in this cell may be acting synergistically. When the temperature of the cells was raised to 33°, the polysomes which had reformed broke down, although more slowly than in the culture which did not receive chloral hydrate. Thus, the polysomes in tsl3 cells treated with chloral hydrate decrease when initiation is inhibited, as we would expect if they were normal polysomes as opposed to being nonspecific aggregates of ribosomes.

Chloral Hydrate is the Active Agent. In mammalian tissues chloral hydrate is reduced to trichloroethanol, which has been suggested to be the active agent for some of chloral hydrate's effects (23). It or trichloroacetic acid, an oxidation product of chloral hydrate (23), might conceivably be the real inhibitor of protein synthesis in vivo. An alternative explanation for the transient nature of the polysome breakdown is that it results from metabolic conversion of chloral hydrate to

Fig. 6. Polysome breakdown in tsl3 in the presence or absence of chloral hydrate. Cells of tsl3 were incubated at 22° for 9.5 hr in the presence (Δ) or absence (O) of 10 mM chloral hydrate. At time = 0 an equal volume of medium at 43° (containing 10 mM chloral hydrate, if appropriate) was added, and the cells incubated at 33°. At intervals, the cells were chilled and their polysomes were analyzed as described in the text.



trichloroethanol or trichloroacetic acid. This second possibility would be true only if the metabolic product did not produce polysome breakdown and only if the compound is produced in the cells from chloral hydrate. Trichloroethanol does cause polysome breakdown (Fig. 7), and it inhibits protein synthesis at a concentration of 10 mM (Fig. 8). When we assayed for trichloroethanol (24,25) in the medium of cells incubated over a 24 hr period in 10 mM chloral hydrate, a maximum of 0.4 mM trichloroethanol was detected. This concentration did not measurably inhibit protein synthesis (Fig. 7). No trichloroacetic acid (26) was found in the cultures to the limit of detection (0.2 mM) and neither this concentration nor 10 mM trichloroacetic acid inhibited protein synthesis in vivo (unpublished results). No significant decrease in the concentration of chloral hydrate was observed by the Fujiwara reaction (26) in the medium of cells incubated in 10 mM chloral hydrate over a 24 hr period.

To confirm this result we incubated cells with [1-¹⁴C]chloral hydrate for intervals from 0-120 min and chromatographed ethyl acetate extracts of the medium (Fig. 9) or the unextracted medium itself (not shown) on thin layers of silica gel. In both cases the only labeled peak seen corresponded to the position of the unlabeled chloral hydrate standard in an adjacent lane.

DISCUSSION

These experiments show that chloral hydrate itself produces an effect on the cell which results in the inhibition of protein synthesis

Fig. 7. Polysome breakdown caused by 10 mM trichloroethanol.
Arg 2 cells were incubated for various times with freshly dissolved
10 mM trichloroethanol and were chilled, and the polysomes analyzed.

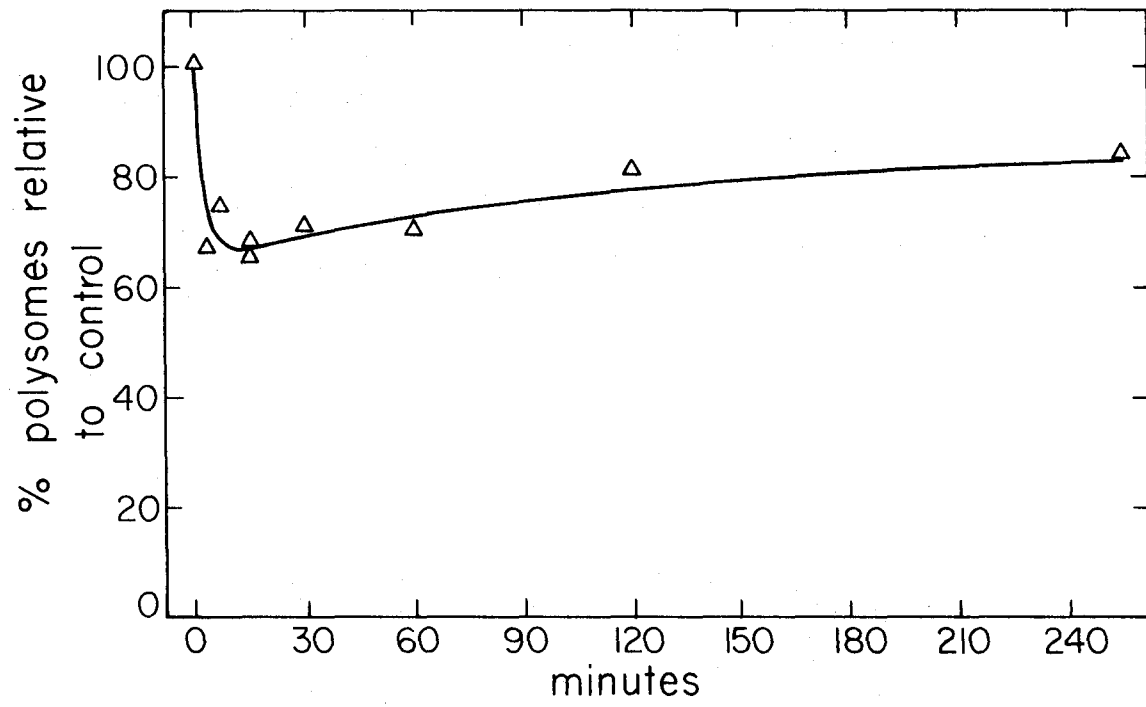


Fig. 8. Effects of 0.4 and 10 mM trichloroethanol on [^3H]arginine incorporation into protein. Arg 2 cells were washed and resuspended in labeling medium at 3.5×10^5 cells ml^{-1} . At initial time, trichloroethanol and [^3H]arginine ($0.5 \mu\text{g ml}^{-1}$, $0.02 \mu\text{C ml}^{-1}$) were added, and samples were taken at intervals and prepared as described in the legend to Fig. 1. Cells contained 0 mM (○), 0.4 mM (●), or 10 mM trichloroethanol (▲).

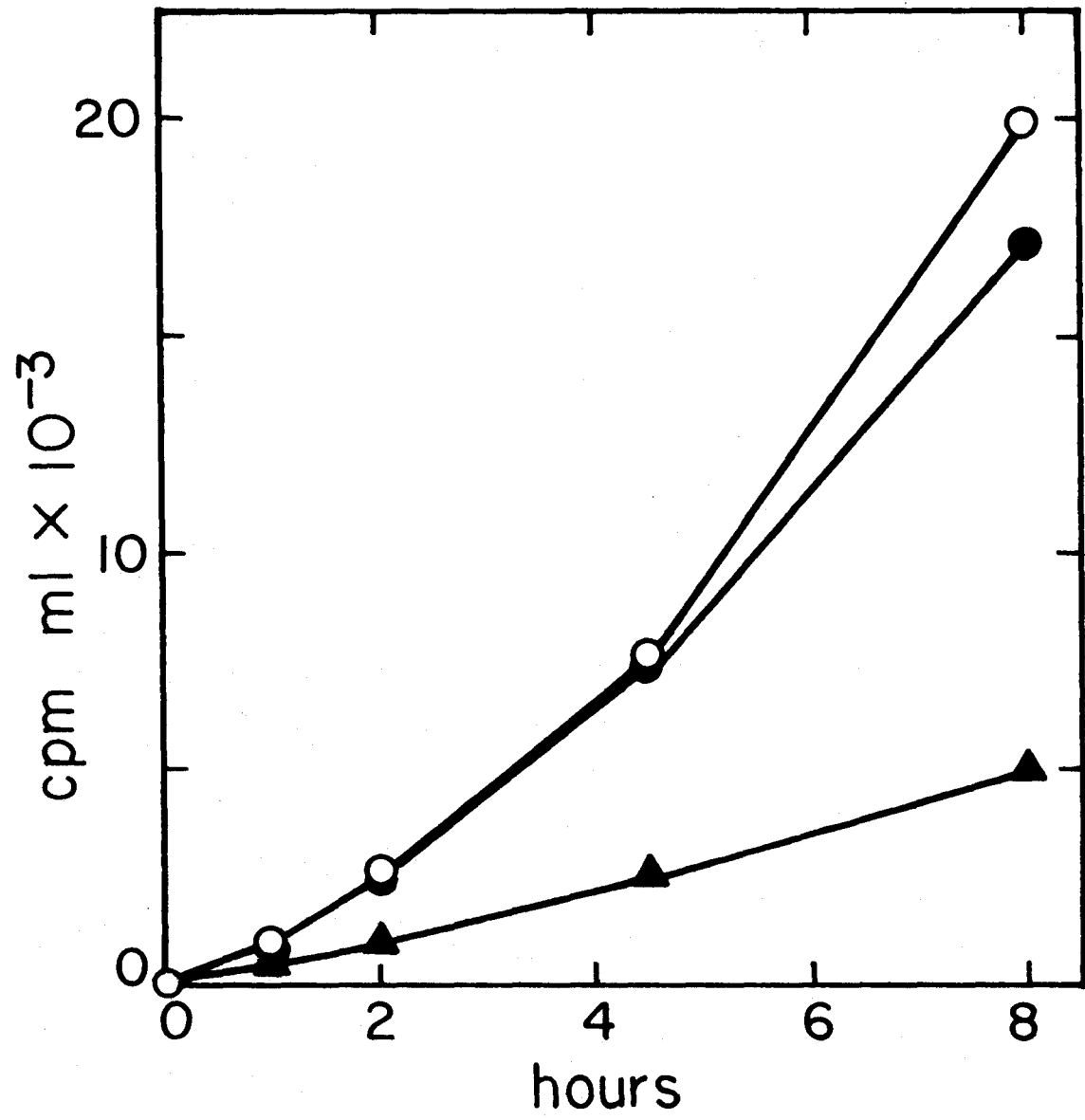
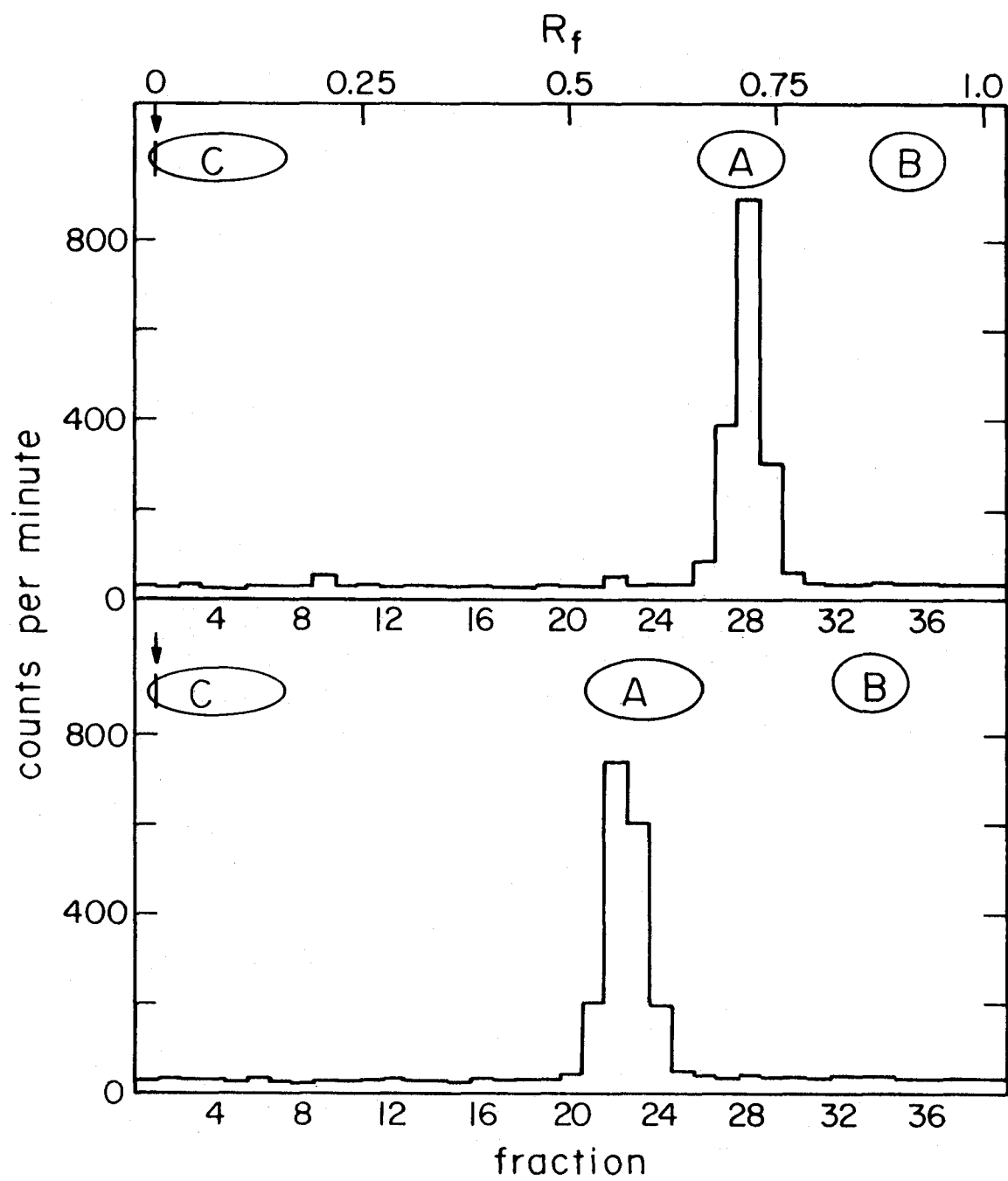


Fig. 9. Chromatography of extracts of Arg 2 cells exposed to [^{14}C] chloral hydrate. Arg 2 cells were exposed to [^{14}C] chloral hydrate (0.2 $\mu\text{C}/\text{ml}$, 10 mM) for 0 min (above) or 120 min (below) before aliquots were extracted with cold ethyl acetate for chromatography on thin layers of silica gel as described in the text. Standards run in adjacent lanes are shown above each histogram. For convenience, standards are shown along a single lane. A = chloral hydrate, B = trichloroethanol, C = trichloroacetic acid.



and polysome breakdown. No trichloroacetic acid and insignificant trichloroethanol appear in the culture, and chloral hydrate does not disappear when incubated with cells.

The biphasic effect which chloral hydrate produces on polysomes is quite unusual. The initial breakdown in polysomes seems to result from abnormal termination of protein synthesis as is indicated by the presence of a nascent peptide on every ribosome. The decrease in polysome size could result either from premature release of ribosomes from the mRNA or from an inability of a released ribosome carrying a nascent peptide to reinitiate on the mRNA. Peptidyl tRNA does appear to inhibit protein synthesis in E. coli in vivo (27,28). Gradual loss of peptidyl tRNA from these ribosomes may allow reformation of polysomes.

Since protein continues to be inhibited even after polysomes have reformed and since the extent of inhibition of protein synthesis is greater than the extent of breakdown, treatment of the cell with chloral hydrate may affect an additional aspect of protein synthesis. The rate of peptide bond formation or of termination could be involved. Both possibilities are consistent with the data presented here but can be distinguished by measuring the effect of chloral hydrate on the rate of polypeptide chain growth.

We can detect no lag between the initial addition of chloral hydrate and its inhibition of protein synthesis. This indicates that there are likely to be very few processes which intervene from the time chloral hydrate is added to the cells until protein synthesis is inhibited. Although chloral hydrate inhibits protein synthesis more

specifically than cycloheximide (11), only additional work can resolve whether the locus of its action is on the ribosome or upon some other cellular component.

Chloral hydrate seems to inhibit cell division as a result of its ability to inhibit protein synthesis. Only additional understanding of the biochemical mechanism by which it inhibits protein synthesis will reveal whether there is some coupling between this effect of chloral hydrate and its anesthetic effect. Since chloral hydrate is equally effective in inhibiting protein synthesis in a mouse neuroblastoma (D. McMahon and G. Magnus, unpublished experiments), this possibility is worthy of consideration.

Our observations, however, already seem to be directly relevant to a current clinical observation with chloral hydrate. When it is used to treat cerebral irritation of infants, it prevents weight gain (29) by the baby. It could be valuable to see whether protein synthesis is being inhibited or reduced in the infant, and if so, whether there is danger of producing defects in the infant which may have serious consequences in the adult.

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Part II.

B. BINDING OF CHLORAL HYDRATE TO CHLAMYDOMONAS CELLS

It is not known whether chloral hydrate produces its effects on Chlamydomonas by action at the surface or within the cell. There are several reasons to suspect that the drug can act without actually entering the cytosol. First, changing the concentration of inorganic ions in the medium can alter the effect of chloral hydrate on the cell (D. McMahon unpublished observations). If Na^+ , K^+ , Ca^{++} or Mg^{++} is added to the medium in higher amounts than normal, the rate of protein synthesis in 5 mM chloral hydrate (the concentration which produces a 50% inhibition of protein synthesis) is altered. Depending on the original ionic strength of the medium, inhibition either is increased (especially with monovalent ions) or is reduced (especially with Ca^{++}). Non-ionic substances, such as urea and cellobiose, in equiosmolar concentrations did not have such effects. In addition, the survival of cells in 20 mM chloral hydrate (which kills cells after a 5 hr lag) was strongly affected. In medium containing 1/10 the usual phosphate, I found that 30 mM Ca^{++} caused a ten-fold increased killing rate over that obtained in 20 mM chloral hydrate alone. 30 mM Ca^{++} alone did not kill cells. Cells were also killed 30 x faster in distilled water than in growth medium. Distilled water by itself did not kill cells. Although these results are, of course, indirect and subject to varying interpretations, they are suggestive of an action at or in the cell surface. As an aid in

evaluating this possibility, I have measured the amount of chloral hydrate bound by Chlamydomonas cells.

METHODS

Chloral Hydrate Binding

Exponential cells (1.1×10^6 cells/ml) were given 10 mM [$1-^{14}\text{C}$] chloral hydrate, 1.0 $\mu\text{C}/\text{ml}$ (prepared by hydration of [$1-^{14}\text{C}$] chloral from California Bionuclear Corp., Sun Valley, California, and dilution with USP chloral hydrate from Merck and Co.). After 0, 15, 60 or 120 min triplicate 1.0 ml samples were added to 9.0 ml of HSMA + 10 mM chloral hydrate (unlabeled) and immediately washed onto GF/A filters and then washed with 3 x 10 ml HSMA + 10 mM chloral hydrate. Filters were immediately placed into scintillation vials containing 0.1 ml 0.1 N HCl and capped. The next day, 15 ml of scintillation fluid was added and the samples were counted. The fluor contained 2.67 g/l diphenyloxazole in toluene: Triton X 100 2:1 (v/v). Triton X 100 was from Sigma. Counting efficiency (36%) was determined using [$1-^{14}\text{C}$] chloral hydrate as the internal standard.

Average Cell Volume

Cell volume was determined by electronic particle counting. Exponentially growing arg 2 cells were washed twice and resuspended in medium which had been millipore filtered after autoclaving. Size distributions were obtained with the Coulter Model Z Particle Counter and Sizer*, using Coulter latex beads of 9.69 μm diameter as standards.

*I thank John Wells for obtaining the size distributions on his instrument.

The size distributions were analyzed as described by Brecher et al (1) to obtain the average cell volume of $202 \mu\text{m}^3$. From this a diameter of $7.29 \mu\text{m}$ was calculated, assuming a spherical shape. The cells show a broad distribution of sizes, which is not surprising, since under our conditions the cells grow to four times their initial size before dividing into four cells by two consecutive mitoses. The modal cell volume is $120 \mu\text{m}^3$, which corresponds to a diameter of $6.1 \mu\text{m}$.

As a check on this determination, cell volume was also estimated from packed cell volume. Cells packed in precision bore capillary tubes occupied $340 \mu\text{m}^3/\text{cell}$, corresponding to a diameter of $8.7 \mu\text{m}$. This value is an overestimate, since it neglects intercellular space, which is probably considerable, since the cells are surrounded by a rigid cell wall.

Cell Surface Area

This computed from the average radius assuming a smooth sphere plus the surface area of the flagella, assuming smooth cylinders. Because of lack of smoothness, these estimates probably err by a factor of 1-2.

Flagellar surface (from published micrographs 2,3) length: $11.4 \mu\text{m}$,

diameter $0.241 \mu\text{m}$. surface = $8.6 \mu\text{m}^2$

Cellular surface: $167 \mu\text{m}^2$

Total surface: $176 \mu\text{m}^2$

Space Occupied by a Chloral Hydrate Molecule

Without any knowledge of how chloral hydrate molecules might pack together, a rough estimate was obtained by measuring the dimensions of a CPK model. The chloral hydrate molecule occurs as an aldehyde in CDCl_3 solution (S. Chan, unpublished) as determined by proton-NMR, so both this form and the dihydroxy-form were measured. Approximate volume occupied: 0.109 nm^3 hydrate, 0.183 nm^3 aldehyde (including a bound H_2O molecule).

RESULTS

Table 1 gives the amounts of chloral hydrate bound per cell at various intervals after addition of the drug. Much of the chloral hydrate binding occurs rapidly after the drug is added to cells. As soon as the initial value can be measured, cells have already bound 2.2 fmoles of chloral hydrate, 54% of the value at 2 hr. After this initial binding, additional chloral hydrate is bound more slowly. If this initial value is divided by the volume per cell a concentration of 10.7 mM is obtained, assuming the drug is uniformly distributed throughout the cell. This concentration is similar to that in the medium (10 mM), so the additional chloral hydrate added later indicates selective binding of the drug to the cells. The fact that binding is resolved into fast and slow components may also indicate two separate compartments of drug binding.

Most of the biochemical effects of chloral hydrate are already observed before 15 min when only 38% additional chloral hydrate

TABLE I. Chloral Hydrate Bound by Cells

time (min)	Amount bound per cell fmoles/cell	Amount bound per cell volume f moles $\mu\text{m}^{-3} \times 10^3 = \text{mM}$	moles $\mu\text{m}^{-2} \times 10^{18}$	Amount bound per surface area $\mu\text{m}^{-2} \times 10^{18}$ molecules nm^{-2}
0	2.2 (± 0.7)	10.7 (± 3.4)	12.4 (± 3.9)	7.4 (± 2.3)
15	2.9 (± 0.7)	14.2 (± 3.4)	16.3 (± 3.9)	9.9 (± 2.3)
60	3.1 (± 0.5)	15.1 (± 2.4)	17.4 (± 2.8)	10.5 (± 1.7)
120	4.1 (± 0)	20. (± 0)	23.1 (± 0)	13.9 (± 0)
avg (15-120 min)	3.5 (± 0.5)	17.1 (± 2.3)	19.7 (± 2.7)	11.9 (± 1.7)

Cell binding of chloral hydrate was determined as [$1\text{-}^{14}\text{C}$] chloral hydrate retained on filters after exposure of cells to chloral hydrate for different periods. The results of two separate experiments are averaged. Values in parentheses are standard errors.

has been added, so the fast binding component may be responsible for these effects.

Although the concentration of chloral hydrate is higher in the cell than in the medium, it is still possible that the drug has merely bound to the surface of the cell. To evaluate this possibility, the concentration of drug molecules produced if all drug bound at the surface was calculated. At zero time this figure is 7.4 ± 2.3 molecules per nm^2 . Since a single molecule has a profile of about $0.3\text{--}0.7 \text{ nm}^2$, this implies that the chloral hydrate molecules would have to stack from 2-5 deep in or on the membrane. The plasma membrane is about 75 \AA thick (4), and this would require an increase in the membrane thickness of $14\text{--}35 \text{ \AA}$, or an increase in the area of the plasma membrane. Such a small increase in thickness might prove difficult to observe. Since each cell binds at least 2.2 fmoles chloral hydrate, a monolayer of chloral hydrate would occupy at least $400 \text{ }\mu\text{m}^2$, or twice the surface of the cell at a minimum. No change in cell volume is seen after addition of 10 mM chloral hydrate to CW15, a cell-wallless mutant, with the light microscope or the Coulter counter. Such a small difference in surface area might, however, easily be missed with these methods, especially if the increased surface area appeared as a roughening of the surface.

SUMMARY

Chloral hydrate binds to cells in higher concentrations than those in the external medium. Much of the binding occurs in the first seconds of exposure to the drug, while additional chloral hydrate is accumulated over a period of hours. There is as yet, however, insufficient evidence to exclude binding of drug at the cell surface.

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Section III

Attempts at Isolation of Chlamydomonas Mutants
by Selection with High Intensity Light

GOALS

The object of this work was to devise a scheme for selecting mutants in a photosynthetic organism which are conditionally unable to assemble a functional chloroplast. Such mutants could then be used to study the assembly of photosynthetic membranes, and might even give some insight into the mechanism of photosynthesis. The selection which was tested depended upon the photo-oxidative killing of green cells with high intensity visible light. However, the scheme was found not to select for the class of mutants expected, those with reduced photosynthetic pigments.

BACKGROUND

Chloroplast development

The process of photosynthesis in eukaryotes occurs entirely within the chloroplasts. These organelles contain the complex systems of chlorophyll-containing membranes which trap light energy and there convert it to chemical energy. Because of this special, extremely important function, the organization and workings of these membranes are particularly worth studying. The material which follows is a brief overview of chloroplast development in green plants.

The chlorophyll-containing membranes of the chloroplast develop originally from invaginations of the inner of the two membranes which surround the organelle (1, 2 p 54, 3 p 137). In the meristematic tissues of higher plants there are present undeveloped organelles called proplastids (2, p 63). As the tissue containing these proplastids differentiates in the light, these bodies synthesize chlorophyll and their inner membrane envelopes invaginate and expand to form complex systems of flattened pouches called thylakoids. These membranes consist both of individual membranes running through the matrix of the chloroplast (stroma lamellae), and of closely appressed stacks of thylakoids called grana. The grana may contain from 2-100 thylakoids (2 p 30, 4) and can often be seen with the light microscope. Thylakoids in the grana often connect with those found unstacked in the chloroplast matrix. Differences in the degree of thylakoid stacking are found in different tissues of higher plants (1, 5),

and in certain mutants (6). Some chloroplasts contain no grana (1).

If kept in the dark, many species of plants are arrested in their chloroplast development. Such plants contain a type of plastid without chlorophyll whose inner membranes have invaginated to form a network of tubular membranes rather than the lamellar membranes characteristic of fully developed chloroplasts (2 pp 66-69). Such plastids are termed etioplasts from the French etiolier, to blanch. Species which form etioplasts include the angiosperms and a mutant of Chlamydomonas reinhardi called y-1 (7, 8). Other plants such as pines, ferns, mosses, and most algae, including C. reinhardi wild type, form thylakoids and chlorophyll in the dark (2 p 403). Eglina species form only proplastids in the dark, thus resembling the meristematic tissues of higher plants.

Apparently there are control mechanisms here which determine what degree of plastid development will occur in the dark. Chlorophyll is involved directly, since the final enzymatic reduction of protochlorophyll to chlorophyll generally requires light and the action spectrum for the accumulation of chlorophyll and thylakoid membranes is that of protochlorophyll (2 p 403-412). Etioplasts contain a small amount of protochlorophyll which is promptly reduced to chlorophyll on exposure to light. Protochlorophyll synthesis may be regulated by control over the synthesis of the prophyryin precursor, δ -amino levulinic acid, or its transport into chloroplast compartment (9), since feeding plants with δ -amino levulinic acid greatly elevates protochlorophyll levels in the dark (10, 11).

Following this initial burst of protochlorophyll reduction, protochlorophyll levels and the rate of chlorophyll synthesis usually remain low for several hours. This period, termed the lag phase of chloroplast development, is followed by a period of chlorophyll synthesis which may last many hours. The length of the lag phase is not constant but appears to depend on several factors. For instance, it may be reduced or eliminated when cultured cells or tissues are given δ -amino levulinic acid to increase protochlorophyll in the dark (11). During the lag period, endogenous carbohydrate reserves break down in etiolated corn seedlings (12), Chlamydomonas y-1 cells (7) and in Euglena cells (13). Since in some algae a form of glucose repression can prevent chloroplast development even in the light (14, 15), carbohydrate metabolism may play more than an energy-supplying role in the regulation of chlorophyll synthesis. Since sucrose does not have such an influence on etiolated bean leaves, however, the phenomenon may not be of general importance (16). In many plants the lag is eliminated by a short pre-illumination with white or red light followed by a dark period (17, 2 pp 457-476, 18). The phytochrome system governs this response, as the lag period can be restored by a far-red light after the initial red (17, 19, 20). Pre-illumination apparently functions rapidly to elevate δ -amino levulinic acid synthetic activity (17, 21), but synthesis of other chloroplast lipids and proteins, including enzymes of photosynthesis, are also stimulated after a period of incubation in the dark (20, 21, 22).

After the end of the lag phase, the rate of chlorophyll synthesis increases over a period of hours. Careful analysis of the kinetics of chlorophyll synthesis in Chlamydomonas mutant y-1 shows that this period follows exponential kinetics (23). In other words, the rate of chlorophyll synthesis during this period is proportional to the amount already synthesized. This can be interpreted as reflecting a requirement either for a certain amount of nucleating chlorophyll or for some other component before the synthesis of a new lamella can begin. Matsuda believes that this plastid component is chloroplast ribosomes, since their synthesis occurs mainly during this period (23). However, many components are also increasing rapidly during this period. In Euglena the plastid ribosome synthesis occurs mostly before the main period of chlorophyll increase (24).

This sketch of the phases of chlorophyll synthesis during chloroplast development and of some of the mechanisms by which it may be regulated is intended to help orient the reader to the general outlines of this process. A great deal more is known about its specifics than I have attempted to present. The most complete coverage of the field is to be found in reference 2. A more recent review is (25).

Most of this knowledge has been gained from kinetic studies of the appearance of various chloroplast structures and functions, such as membrane stacking, Hill reaction, CO_2 fixation, and the activities of specific enzymes. Little is known, however, about the mechanisms of their synthesis or their activation into functional units.

Since, however, it is now known to a great extent what units are synthesized when, we can now better proceed to ask how they are synthesized and regulated.

Synthesis of Plastid Components Since plastids contain their own DNA and peculiar system for protein synthesis, it is widely assumed that many important plastid components must be coded for by plastid DNA and synthesized in the plastid. Maternally inherited mutations which cause altered plastic phenotypes have also been known for many years (2 pp 222-228). However, it has proven difficult to determine which of the plastid components are coded by the plastid genome. The existence of this organelle does require the presence of chloroplast DNA, for mutants of Euglena which have lost all of their plastic DNA are permanently bleached and unable to form a chloroplast or even a proplastid (26). However, transcription of chloroplast DNA to form chloroplast rRNA is not required to perpetuate the plastid in Chlamydomonas over many cell generations (27). In the experiments of Surzycki rifampicin depleted the chloroplast of chloroplast ribosomes and yet no decrease was observed in the rate of chloroplast DNA replication. Since DNA synthesis in Chlamydomonas is largely dependent on protein synthesis (28), it may be inferred that the chloroplast DNA polymerase is translated on cytoplasmic ribosomes from a nuclear-coded messenger.

Results, such as this, which show that an inhibitor has no effect on a particular process are more likely to yield valid inferences than those which show that a process is indeed inhibited.

This is because inhibitors such as chloramphenicol (29, 30) or cycloheximide (28) may have non-specific effects on cells which can produce confusing results. Thus, the finding that chloramphenicol and spectinomycin, which inhibit plastid protein synthesis, do not inhibit synthesis of ferridoxin, ferridoxin-NADP reductase or phosphoribulokinase (31) is more likely to imply that the synthesis of these does not involve plastid ribosomes than the finding that cycloheximide does inhibit accumulation of these enzymes (31). This argument is reinforced by the finding of Armstrong et al (31) that synthesis of no chloroplast component is blocked by chloramphenicol and spectinomycin but not by cycloheximide and by the findings of McMahon that cycloheximide is a non-specific inhibitor of protein synthesis in Chlamydomonas in vivo (28).

Thus, the site of translation of many plastid components remains in doubt. For instance, the synthesis of ribulose diphosphate carboxylase is inhibited by cycloheximide, chloramphenicol or spectinomycin (31). Clearly, methods with greater resolution must be employed in this area than can be secured by the employment of inhibitors.

In fact, recently the site of coding of several proteins has been deduced through a combination of genetic and chemical analysis. S. G. Wildman and friends identified tobacco species which differ in their peptide finger-prints for ribulosediphosphate carboxylase in either the large or the small peptide subunit and demonstrated by

reciprocal crosses that the finger-print pattern belonging to the large subunit is maternally inherited while the pattern belonging to the small subunit is inherited in Mendelian fashion (32, 33, 34). Since plastid DNA is known to be inherited in a maternal fashion in higher plants, this result implies that the subunits of this enzyme are coded in separate compartments of the cells. Isolation of polysomes translating these subunits will eventually resolve the site of synthesis of this enzyme through identification of the type of ribosome involved.

Kung, Thornber and Wildman (35) have produced similar evidence to demonstrate that the Photosystem II chlorophyll-protein complex of tobacco chloroplasts is coded at least in part by nuclear DNA. Three proteins of the 50S subunit of the tobacco chloroplast are also coded by nuclear DNA (36). Work in Bogorad's laboratory has also demonstrated that different proteins of the Chlamydomonas chloroplast ribosome may either be inherited maternally or uniparentally (34). Such data serve to indicate that the concept of the chloroplast as an autonomous organelle is not correct. Rather it is a subordinate center of genetic information which specialized in supplying some key information for its own perpetuation.

The Genetics of Chloroplast Biosynthesis.

A vast array of mutants are known in algae and higher plants which affect chloroplast development. Many of these phenotypes, however, have been found to be caused by disturbances in cellular metabolism such as auxotrophy for vitamins or amino acids (37, 38), or in uptake of nutrients from the soil (39). Many mutants are also known which produce such gross pleiotropic alterations in chloroplast structure and function that any attempts to define the lesion seem hopeless (2 p 353).

The most productive work has aimed at isolation of mutants with a more defined phenotype, particularly those defective in chlorophyll synthesis or in photosynthesis. This was accomplished by isolating mutants which accumulate certain defined precursors of pigments such as porphyrins and carotenoids (2 pp 338-348, 40) or those which require only acetate for growth or exhibit increased chlorophyll fluorescence (41, 42, 43). Increased chlorophyll fluorescence is particularly specific, since it indicates a decrease in ability of the organism to capture energy from excited chlorophyll molecules by electron transport.

The work on precursor-accumulating mutants has served to clarify the pathways of pigment synthesis (2 pp 338-348, 40) and has demonstrated that most genes involved in pigment synthesis are inherited in Mendelian fashion (2 p 341). Although mutants affecting control of pigment synthesis have been isolated from a variety of organisms,

little seems to have been learned from them about the mechanisms of control, perhaps because such mutants are difficult to define biochemically.

The results from studies of acetate-requiring and elevated chlorophyll fluorescence mutants of Chlamydomonas are more positive. Similar mutants have been isolated from Scenedesmus, but no genetic system is known in this organism (44). Levine and co-workers have isolated a large number of mutants from Chlamydomonas and characterized some of their phenotypes biochemically. Among the functional sites affected are: P700 (the reaction center of photosystem I); plastocyanine (involved in photosynthetic electron transport and of cyclic and noncyclic photophosphorylation); cytochrome 553 and 559 (involved in photosynthetic electron transport and of noncyclic photophosphorylation); Q (the quencher of photosystem II chlorophyll fluorescence); phosphoribulokinase; chloroplast 70S ribosomes and their 50S subunits (43).

None of these mutants are known, however, to reside in structural genes, and unlinked mutants of identical phenotypes are known in several cases (43). Also, some of these mutants revert frequently by accumulation of unlinked suppressor mutations (45). Since such a high frequency of suppressor accumulation is not a constant feature of Chlamydomonas genetics, these reversions may be an important clue to the control of synthesis of chloroplast components. The study of the mutants has helped to order the

photosynthetic electron transport chain and has predicted the existence of functional components not yet identified by physical techniques (43), but has not yet helped clarify the process of assembly of components into chloroplast lamellar membranes or how that is controlled.

Sensitivity of Chloroplasts to Intense Illumination.

At intensities of light 10^3 times of that required to saturate the rate of photosynthesis, the rate again falls (46). This occurs at intensities above 100 Klux, (the intensity of sunlight on a clear day with the sun at its zenith). This process, called photo-inhibition, is dependent upon absorption of ultra-violet or visible quanta. The visible light absorbed in this process is that absorbed by chlorophyll (47, 48) and both photosystems I and II are affected (49, 50). The process is at least in part irreversible, as the photosystem I reaction center is destroyed (49). This process does not, however, require oxygen (47).

Even greater light intensities, 200 Klux or more, produce an extensive permanent destruction of photosynthetic activities due to photooxidation of the chloroplasts (51, 52). This process is sensitized by chlorophyll and requires a partial pressure of oxygen of only 2-10% at standard pressure to produce more than half maximal oxygen consumption (51). This process is believed to be due to the reaction of excited states of chlorophyll with oxygen to produce free radicals, which in turn attack the chloroplast (52).

An extensive line of experiments involving chlorophyll in solution (52), mutants of photosynthetic organisms unable to produce carotenoids (53, 54, 55, 56, 57) and inhibitors of carotenoid synthesis (58, 59, 60) has established that one function of the

chloroplast carotenoids is to prevent the occurrence of photooxidation at lower light intensities. In organisms lacking protective carotenoids the photooxidative process occurs rapidly even at moderate or low light intensities. Apparently, the carotenoids serve as an energy buffer by accepting energy from toxic singlet oxygen and subsequent transfer of energy back to chlorophyll for photosynthesis (52).

When chlorophyll synthesis is induced by light, carotenoid synthesis is also (2 p 429-34), but not necessarily with parallel kinetics (61). Surprisingly, there is no correlation between the amount of light received by a plant in its niche and its ratio of chlorophyll to carotenoids (2 p 487). One explanation for this finding might be that there is an optimal ratio of chlorophyll to carotenoid for all levels of illumination to provide protection from photooxidation.

MATERIALS AND METHODS

Cells and Their Growth.

Chlamydomonas reinhardi wild type was strain 89 (+). The y-1 mutant isolated by Sager (62) was obtained from W. Ebersold as strain 189 (y-1, (-)). It was cloned before use, and produced lemon-yellow colonies when grown in the dark and deep green colonies in the light. In some experiments I used y-1 strain 431 (y-1, ac17, nic13, pf2, P^r, M^r, A^r, S^r (-)) (63) which I also cloned before use. Cultures were grown in HSM medium (64) supplemented with 2 g/l Na acetate (HSMA), or HSMA supplemented with nicotinamide (0.75 mg/ml) or yeast extract (2 g/l) for the growth of strain 431. Growth conditions in the light have been described (65). In order to produce y-1 cells with etioplasts, green, light-grown y cells were inoculated into fresh medium in Al-foil covered flasks or 1.75 x 15 cm culture tubes and incubated at 22° four or five days in a room with a dim green safelight. The cells, at a density close to stationary phase, were harvested by centrifugation at $10^3 \times g$ for 1 min and were suspended in fresh medium at $1-2 \times 10^6$ cells ml⁻¹. Care was taken to insure that the cells received no light until the beginning of the experiment. For instance, the centrifuge tubes were placed into the rotor under dim green safelight and the rotor sealed to the light before the rotor was placed in the centrifuge. Chlorophyll was determined by the method of Arnon (66).

Cells were mutagenized as described by McMahon (67) with N-methy, N-nitro, nitroso guanidine or by an irradiation 23 cm from a 5W Ultra Violet Products UV-lamp sufficient to kill 90% of the cells. Exponential phase cells were resuspended to about 1×10^6 in HSMA, irradiated in a thin layer on a stirred petri dish bottom, and pipetted in 1 ml aliquots into 9 ml of HSMA + nicotinamide or HSMA + yeast extract. After 18-24 hr in a black box, the cultures were placed under 10^4 lux from daylight fluorescent tubes and grown to stationary phase. The tubes were vortexed daily. Next the cells were diluted 10^{-2} in HSMA + yeast extract and grown in the dark 3-4 days prior to high light intensity selection. Viability was determined in all cases as colony-forming units of treated compared to identical control cultures plated on agar containing HSMA, plus yeast extract, if required.

High Light Intensities

High intensities were obtained in the preliminary experiments using a 500 w CZA projector bulb (G. E.) in a reflector mount. The colimated beam passed through a layer of heat absorbing glass and 2 cm of distilled water in a stirred, colorless polystyrene-walled bath which was maintained at 22° by evaporation. The temperature in the bath was measured at frequent intervals and the temperature in the culture tube, occasionally, using a clean, alcohol-sterilized mercury thermometer. The temperature of the exposed cells with the lamp on was 2-3° warmer than the surrounding bath, but well within

the optimal growth range of Chlamydomonas (22°-33°, D. McMahon, unpublished results). In order to insure aeration of the cells and uniform light exposure, cells were vigorously bubbled throughout the experiment with filtered, humidified air or high purity N₂. The entire profile of the culture was in the light beam. In one experiment, the exposed culture tube was partly out of the beam, and survival was greatly increased, indicating that continuous exposure of the cells may be necessary to obtain killing. The intensity of 2×10^5 lux was estimated by extrapolation of the intensity versus $1/(\text{distance})^2$ plot obtained using a General Electric photometer. This plot was linear, as expected. Red light was obtained by filtering the same light beam through a 1 cm layer of 0.02% erythrocin B dye. This concentration produces a sharp cut-off of light below 560 nm, while passing the light above this wavelength, some of which can be absorbed by chlorophyll. For selection of mutants, a more powerful light source was used, allowing a greater cross-section of cells to be exposed at a given time. The light source was a 6.5 kw Osram xenon arc lamp operated at 5 kw in a Dana-Hughes projector mount, model XTL. The beam first passed through 6.6 cm of distilled water cooled by a heat exchanger. Next, the beam entered the plexiglas wall of a circulating water bath at 33° and exposed the cells, which were aerated in 1.75 x 15 cm culture tubes at a density of about 2×10^5 cells/ml.

RESULTS

High intensity light exposure does in fact kill green wild type cells. Figure 1 shows the results of two experiments in which cells were continuously exposed to an intensity of visible light of 200,000 lux or about twice the maximum solar flux density. It can be seen that a rapid killing phase in the first 3-4 hr is followed by a much slower phase which continues for an unknown period. This biphasic killing conceivably could be due to the existence of two genetically or physiologically distinct cell types within the original culture. Therefore, the survivors of 24 hr of exposure in one of these experiments (open circles) were suspended in fresh medium, grown in the light to 2×10^5 cells/ml and re-exposed to high-intensity light for 3 hr. As can be seen in Fig. 1 (triangles), these survivors of the first experiment again died at a rapid rate for 3 hr. This demonstrates that the green survivors of an unmutagenized culture are no more resistant to this killing than were the original cells.

In order to further characterize this effect of high light intensities, its dependence on oxygen was examined. Fig. 2 shows that death is prevented if oxygen is removed from the medium by N_2 bubbling. Thus, this phenomenon may be similar to the photooxidation which has been described in higher plants (51) and in the Chlamydomonas mutant Light Green (NTCC 18302) which has low levels of carotenoids. (55).

If the killing is to be useful in mutant selection as proposed,

Fig. 1

Mt⁺ cells at 5.8×10^5 (○), 2.9×10^5 (□), or 2.1×10^5 (Δ) cells per ml were exposed to high intensity incandescent illumination for periods of time as described in the text and plated on HSMA + 1.5% agar. They were counted after 5 days at 22° in the light. Standard deviations are less than 10% except where error bars are shown. At the end of the experiment, the control cultures were 1×10^6 (18.2 hr) (○), 4.3×10^5 (1.3 hr) (□) and 2.8×10^5 (3 hr) (Δ) cells per ml respectively. Least squares fits of the early and later rates of killing give exponential killing coefficients of 0.67 hr^{-1} and 0.13 hr^{-1} respectively. These lines are plotted.

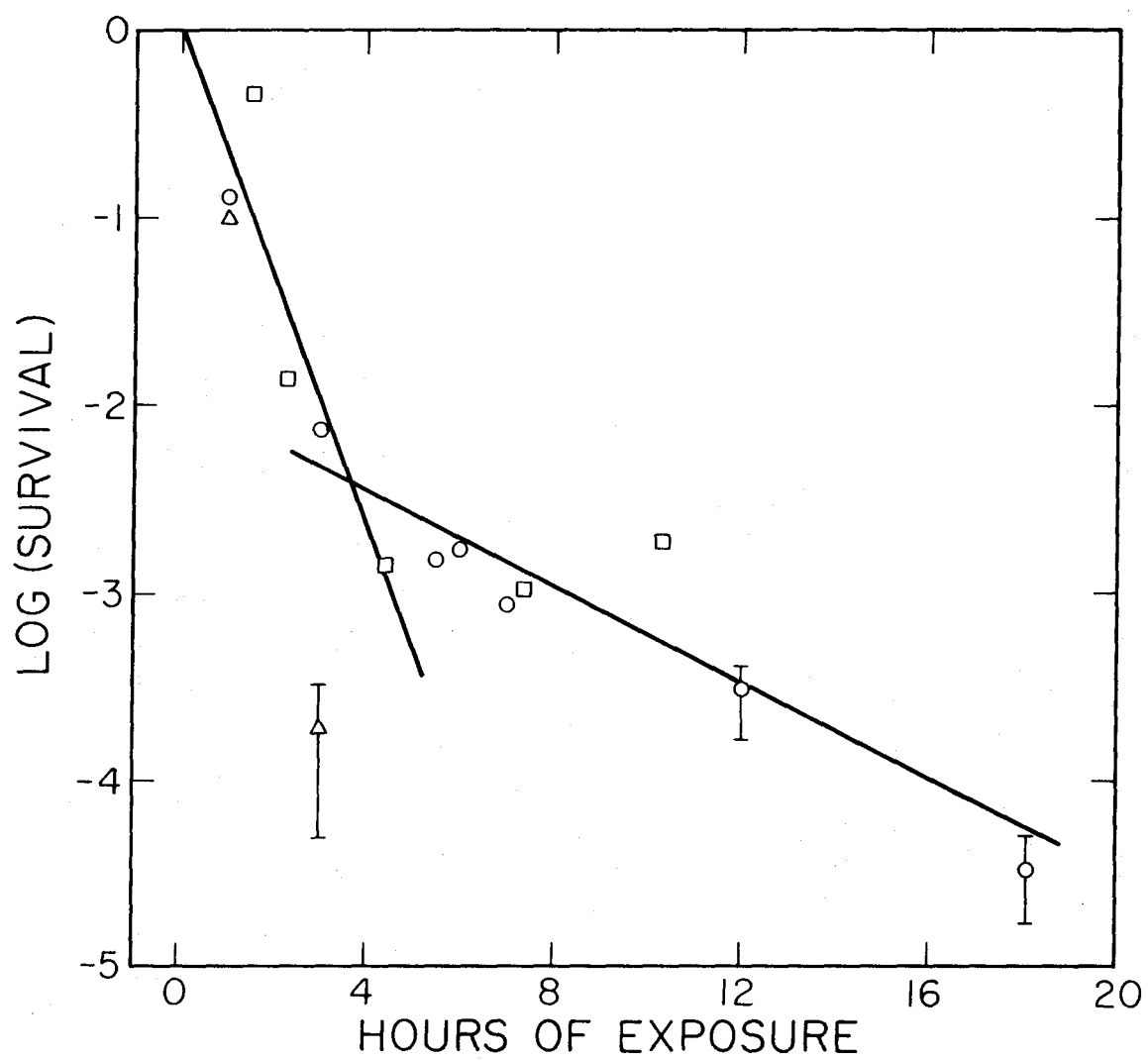
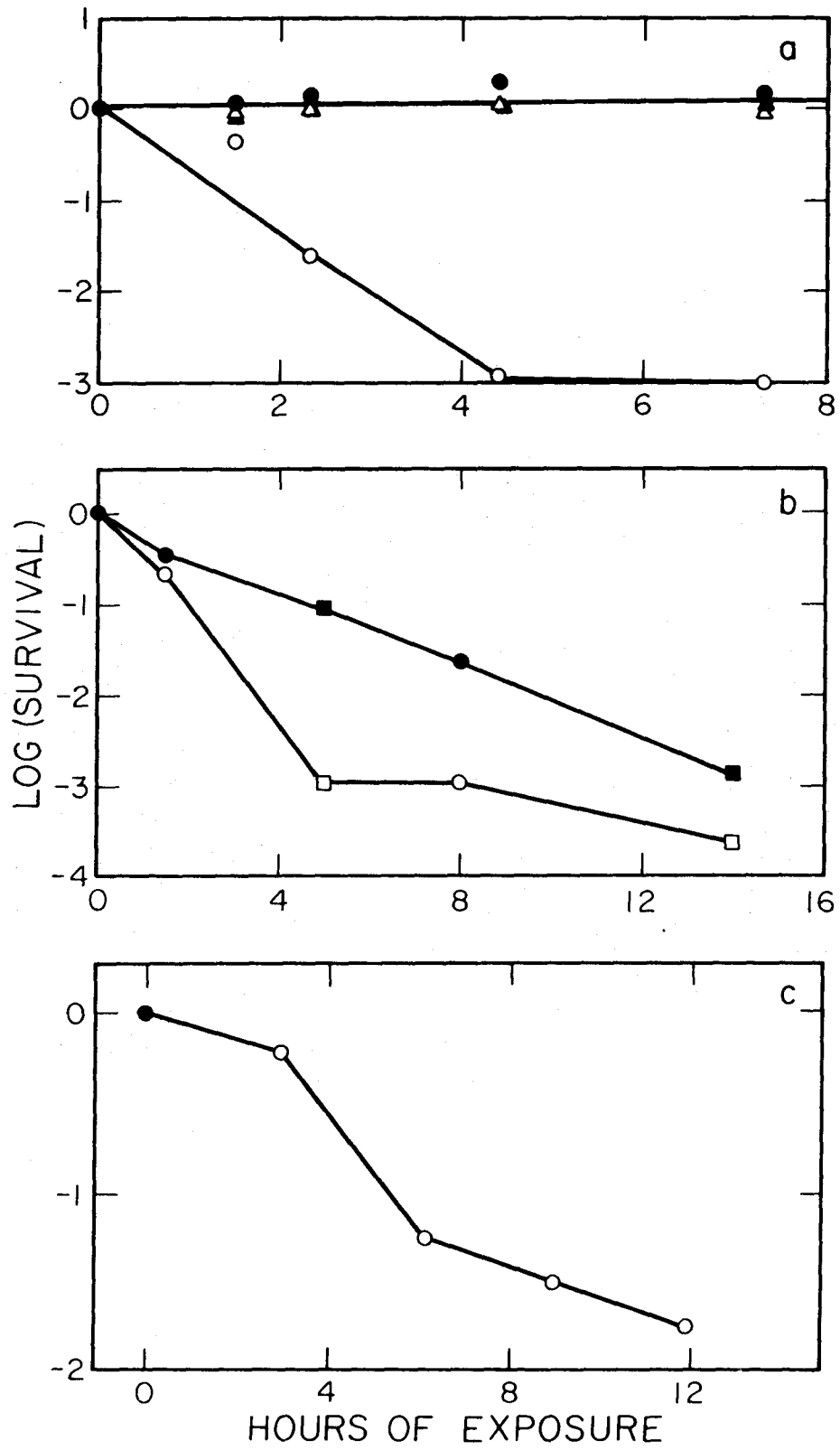


Fig. 2

a. (●) control illumination (10 Klux), aeration, (○) 200 Klux, aeration, (▲) 10 Klux, N₂ (△) 200 Klux, N₂. Cells (mt⁺, 2.9 x 10⁵ cells per ml) were incubated for the times indicated and plated as in fig. 1.

b. (□, ○) 10 μM DCMU, (■, ●) no DCMU. Aerated cells (mt⁺ at 7.6 x 10⁵ (□, ■) or 3.9 x 10⁶ (○, ●) were exposed to 200 Klux and plated. There was no loss in viability for cells receiving 10 μM DCMU at 10 Klux over the time of this experiment.

c. Aerated cells (y-1, grown in the light) at 5.9 x 10⁵ cells per ml were exposed to 50 Klux light of wave length > 560 nm and plated. At the end of the experiment there was no loss in viability in the control culture receiving 450 lux of red light.



it ought to depend on the photosynthetic activity of the cells and on the presence of photosynthetic membranes. Therefore, I exposed cells to high light intensities in the presence of 10^{-5} M dichlorophenyldimethyl urea (DCMU), an inhibitor of photosynthetic electron transport. DCMU is thought to prevent the transfer of excited electrons from the primary electron acceptor of photosystem II, Q (68). This localization is based on the fact that DCMU enhances general PSII chlorophyll fluorescence by preventing electron transfer to acceptor pool A of the electron transport chain, likely plastoquinone. As I expected, DCMU conferred a significant degree of protection on exposed cells (fig. 2b).

Additionally, if the killing is due to a photosynthesis-dependent process, it ought to occur using red light which excites the photosynthetic apparatus but not carotenoids, flavonoids and other blue-absorbing pigments. Fig. 2c shows that this is the case.

As a final check of the suitability of this selection to recover mutants defective in their chloroplasts, y-1 cells grown in the dark were exposed to high intensity light after periods of chloroplast development under ordinary light intensities. y-1 cells grown in the dark for 4 days contained $8.5 \mu\text{g}$ chlorophyll/ 10^7 cells. They were placed under 10^4 lux from fluorescent tubes and allowed to green. A plot of log (survival) versus chlorophyll content of the control culture at the end of the high intensity exposure (fig. 4) shows that

Fig. 3

Cells (y-1, grown in the dark 4 days) were washed and resuspended in fresh HSMA to 1.0×10^6 cells per ml in the dark. The culture (200 ml) was placed under 1000 lux from fluorescent lamps at initial time and sampled for total chlorophyll at intervals (Δ). Aliquots (5 ml) were taken at intervals and placed under the 200 Klux beam of incandescent light for 2 hr periods. Survival at the end of each 2 hr exposure is plotted (0).

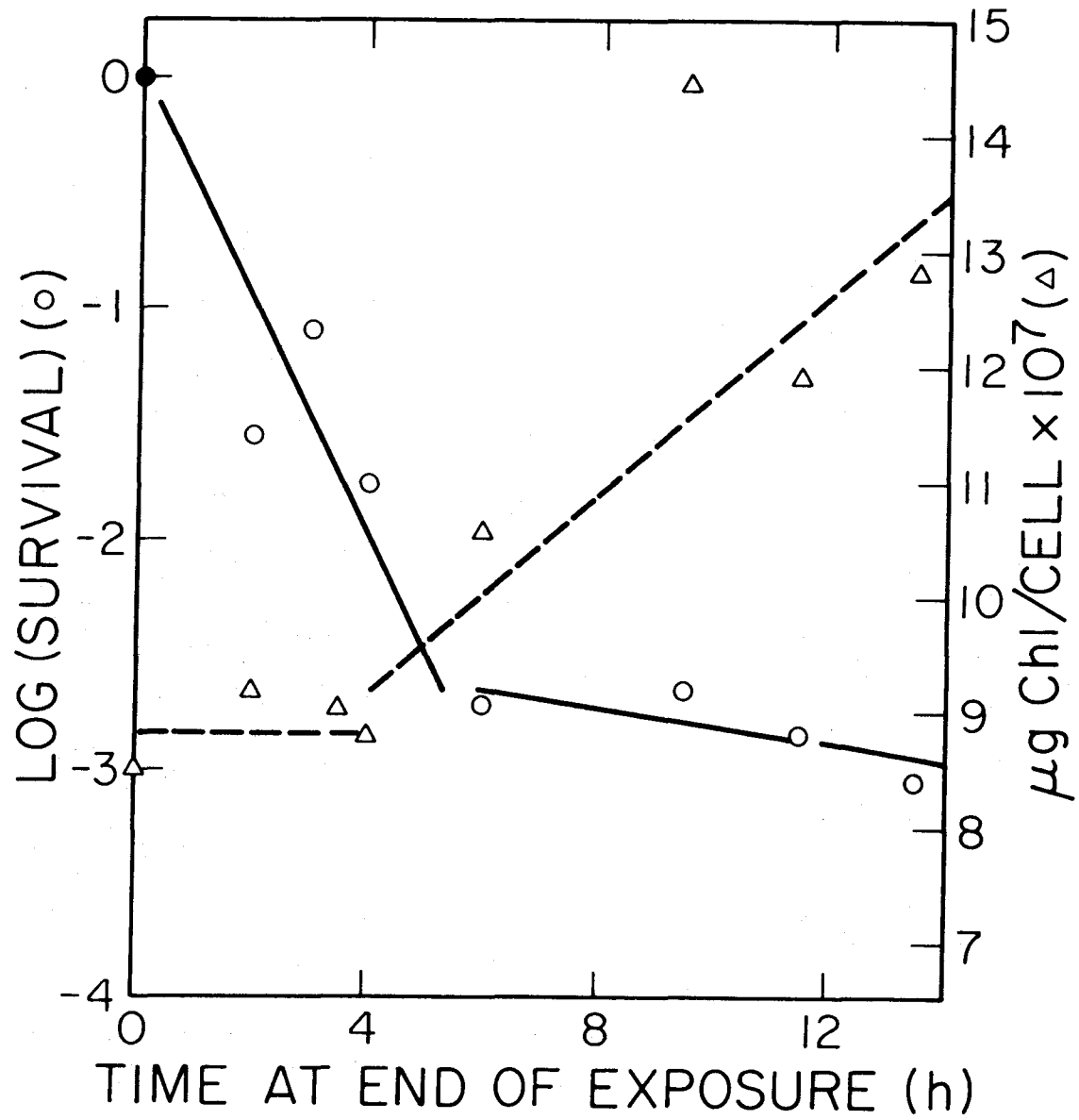
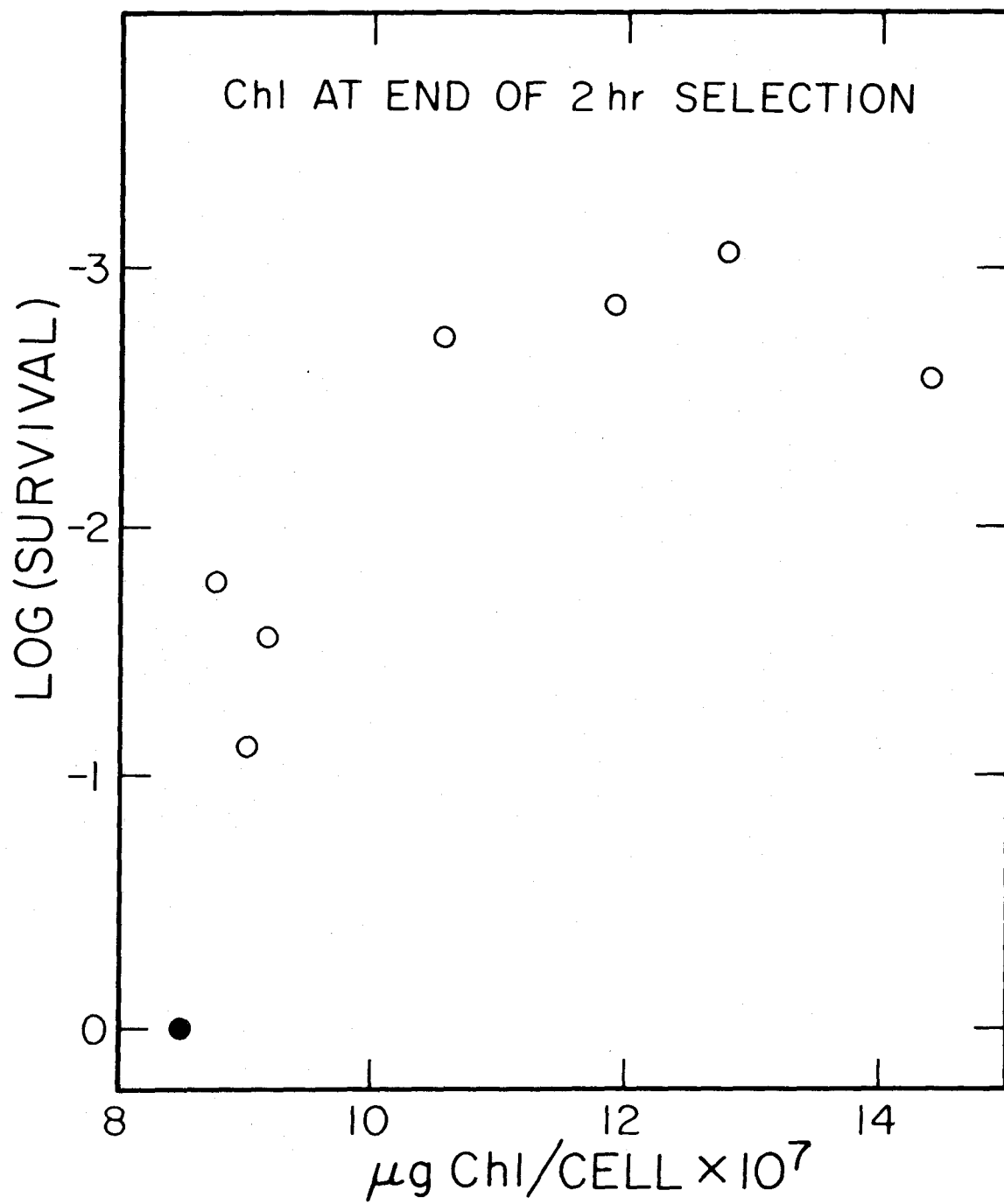


Fig. 4

Data from the experiment in fig. 3 is plotted as log survival vs. total chlorophyll content.



there is a remarkable discontinuity in the sensitivity of greening cells to high intensity light rather than a gradual increase in sensitivity during greening. Since we know that the photosynthetic apparatus in *y-1* is assembled in distinct stages (69), this may point to the requirement for a specific component to be added to the system before the cells become sensitive. Since less than $10.5 \mu\text{g Chl}/10^7$ cells can activate this sensitivity, it may be that early components are necessary for the activation.

Next the selection was tested using mutagenized *y-1* cells grown 3 or 6 days in the dark respectively at 22° and then exposed to the light 15 min or 3 hr at 33° in two separate experiments in hopes of later detecting mutants, among the survivors capable of greening at 22° but not at 33° . Since killing could occur in the presence of only small amounts of chlorophyll ($10 \mu\text{g}/10^7$ cells), it was hoped that the technique would select mutants in a stage of chloroplast assembly early enough that little chlorophyll would be seen in a colony on a petri dish. Thus, only colonies with reduced chlorophyll levels were to be picked for further study.

In order to obtain larger yields of surviving cells after light exposure, an Osram Xenon Arc lamp was used instead of the incandescent bulb to irradiate a larger culture. The rate of killing using this arrangement at a similar light intensity and a stirred water bath at 33° was similar to the rate obtained with incandescent illumination.

As before, with a green culture, killing was rapid for the first 4 hr and then slower. Also, cells were not killed until their chloroplasts began to green.

The result of this selection using y-1 mutagenized with NNG was a recovery of 6 clearly yellow colonies at 33° in the light (3 from each of two experiments). Of these, two were yellow at 33° and green or greenish on 22° plates. About 18,000 colonies were examined, so the frequency was about 3×10^{-4} . There were numerous other colonies with a less well defined phenotype, and others which appeared to form green colonies which matured to yellow or colorless phenotypes. Attempts were made to culture these, but it was found that they were difficult to grow and attempts to study them were gradually abandoned when they died off during regular transfers or were overgrown rapidly by revertants showing normal green colonies.

The two mutants showing apparent temperature sensitive phenotype as colonies on agar were, however, studied further. They, however, may have been subject to instability also, as when they were grown in liquid culture, their chlorophyll content was not reduced at 33° nor was their fixation of [^{14}C] bicarbonate reduced at 33° when corrected for chlorophyll, compared to wild type (data not shown). Levine has isolated similar mutants with reduced chlorophyll but normal CO_2 fixation when normalized to chlorophyll content (6).

These factors led me to question whether these mutants were going to be suitable for further study and to question whether the

selection procedure had in fact selected for stable mutants defective in chloroplast assembly. The second question was answered by an analysis of the effectiveness of the selection. I mutagenized y-1 431 cells, and after growth in the light they were plated for growth in the dark. Lightly pigmented colonies which remained so after transfer to the light at 33° were scored. Six such colonies were detected out of 30,000 examined or 2×10^{-4} . Thus, the frequency of high-intensity survivor-light pigmented colonies does not differ significantly from unselected light-pigmented colonies, and the high intensity light beam must not select for such mutants in a mutagenized culture of Chlamydomonas.

DISCUSSION

How can the inability to select for reduced-pigment mutants be reconciled with the earlier findings which suggested that the scheme would work? There are two obvious explanations. Either there were no mutants within the mutagenized population of Chlamydomonas cells which were favored by the scheme, or there were such mutants, but they were not recognizable as light-pigmented colonies.

The first explanation is easily understood. It might be that resistance to photooxidative killing requires multiple mutations to alter existing enzyme or membrane systems and that these mutations are very unlikely in my mutagenized population. Or perhaps resistance mutations are likely, but they produce physiological conditions which greatly reduce the growth rate or survival under the conditions used in other portions of the selective scheme. In fact, it was observed that in an unmutagenized culture the survivors of one round of killing were again killed at the same rate during the second exposure. This result indicates that resistance mutations are likely to be rare, at least in the absence of a mutagen.

The other possibility is that unrecognized resistance mutations occurred. If resistance unexpectedly produced either no change or an increase in the pigment content of colonies, then they would not have been picked for study. Since we know that depletion of carotenoids causes photosensitization at ordinary light intensities, (55) it is

possible that a resistant phenotype might contain elevated levels of carotenoids relative to chlorophyll. Such cells would be expected to be more darkly pigmented than normal. As mentioned before, however, there is no correlation between chlorophyll/carotenoid ratio and habitat (2 p 487).

It was also shown (fig. 4) that cells are sensitized to the photo-oxidation when they still contain only a small amount of chlorophyll ($10.5 \mu\text{g}/10^7$ cells). This suggests that resistance to photooxidation might be attained without any effect on pigment synthesis through alteration of some other component of the photosynthetic membranes which appears early in y -1 chloroplast development. Such a component might like DCMU affect photosynthetic electron transport, since this inhibitor provides protection from the killing. Since the activation occurs as the cells come out of the lag phase of chlorophyll synthesis at about 4-6 hr, it is unlikely that this component is involved in electron transport or in cyclic or non-cyclic photophosphorylation, activities which are activated during the lag phase (69, 70). Some components, such as cytochromes f (71, 72) and 554 (73) or ferredoxin (71, 74) are already present in the etioplast in an inactive form so the synthesis of these components may also be excluded. Since the synthesis of chlorophyll normally coincides with the synthesis and insertion of membrane proteins and other pigment and non-pigment lipids (7, 74, 75, 73), the activation or photosensitization may,

therefore, coincide with the insertion or activation of a membrane structural component which occurs at this time.

Since photooxidation apparently occurs because of the production of activated oxygen (52), it is possible that increase in peroxisome activity could be involved in resistance.

How might these possibilities be distinguished? The first hypothesis of no mutants being present to select is not directly testable. It can only be proven wrong by the demonstration that a particular class of mutant is present and is being selected. Thus, one approach to this problem would be to test the survival in high intensity light of a variety of preisolated mutants in different aspects of photosynthesis. Chlamydomonas mutants isolated by R. P. Levine (43) are available which show defects in P700, stages of photosynthetic electron transport, the photosystem II reaction center and in phosphoribulokinase. (43). If one such mutant(s) showed significantly more resistance to the high intensity beam than wild type, then it could be reasonably asserted that similar mutants in the mutagenized pool from wild type would be selected by the scheme. However, such mutants might not prove to have increased resistance to high light intensities and the selective value of high light intensity would still be unknown.

An alternative would be to screen the survivors for a variety of parameters other than lack of pigmentation, such as Levine has done: $^{14}\text{CO}_2$ fixation (76); chlorophyll fluorescence (42); acetate

requirement for growth (10); or increased pigments. This method has the disadvantage that there is no advance way of predicting whether which, if any, of these parameters may be involved in increasing survival in high light intensities.

Since the light pigmented mutants which were isolated in this work were not reduced in CO_2 -fixation relative to their chlorophyll contents, it can be argued that this proves that an intense light beam does not select for mutants in photosynthesis. This argument neglects, however, to consider the fact that the reduced-pigment mutants were a minor fraction of the total survivors. Thus, if photosynthetic mutants were being selected independently of a constant proportion of mutants in pigment synthesis, only a very small percentage of the survivors would be double mutants unless the frequency of photosynthetic mutants was particularly high. Levine (43) found a frequency of 10^{-2} for mutants in photosynthesis after UV mutagenesis. Using my data I can employ the binomial distribution to calculate confidence for various possible mutant frequencies. Since none of the three mutants tested were defective in CO_2 fixation when normalized to chlorophyll content, I calculate that with 88% confidence the frequency of photosynthetic mutants is less than 0.5 in the measured population. Since this confidence is rather weak, there is a strong probability that photosynthetic mutants would have been detected among the survivors if more colonies had been tested.

Nevertheless, it has been shown that changes in pigment content in Chlamydomonas do not necessarily result in alterations in photosynthetic ability on a chlorophyll basis. Only further experimentation can conclude whether the selection technique described in this thesis selects for other classes of chloroplast mutants besides those examined.

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