Adaptation to Trichodermin and Anisomycin in *Physarum polycephalum*

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ABSTRACT The effects of the protein synthesis inhibitors trichodermin and anisomycin on the growth of the eucaryotic myxomycete *Physarum polycephalum* have been examined. When either of these drugs is added to log phase monoxenic cultures of myxamoebae, cell division is immediately arrested, but on continued incubation, growth resumes at a rate only slightly lower than that of drug free cultures. The length of the drug induced growth lag is roughly proportional to drug concentration. When adapted cells are transferred to fresh drug containing medium, growth is not inhibited. However, if the drug concentration is increased, transient inhibition is again exhibited. Measurement of the antibiotic concentration in used media demonstrates no significant external inactivation of either drug during adaptation. The resumption of growth cannot be attributed to the selection of stable drug-resistant mutants: single amoebal colonies arising on drug plates are found to be as drug-sensitive as control colonies when retested after subculture. In addition, when adapted cells are transferred to drug free medium, the phenotypic drug-resistance is completely lost after several generations of growth. As recovery occurs in the continuous presence of drug and is not due to the accumulation of drug-resistant mutants, this response appears to be an example of drug adaptation. Cross adaptation between anisomycin and trichodermin is also demonstrated, suggesting a common system is involved in adaptation to these structurally dissimilar, but functionally similar, drugs.

The sesquiterpene antibiotic trichodermin is a fungal toxin which inhibits protein synthesis in a variety of eucaryotic organisms, including yeast (Stafford and McLaughlin, '73) and mammalian cells (Carrasco et al., '73). This drug acts at the ribosomal level, inhibiting the peptidyl transferase reaction (Schindler, '74; Wei et al., '74). In *Saccharomyces cerevisiae,* trichodermin-resistant mutants have been used to confirm the observations that this drug acts at the level of the 60S ribosomal subunit (Jiminez et al., '75; Schindler et al., '74). In addition, these trichodermin-resistant mutants show cross resistance to the structurally unrelated antibiotic anisomycin (Schindler, '74; Jiminez et al., '75). These studies suggest overlapping ribosomal sites for these two antibiotics.

The effects of trichodermin and anisomycin on the growth of *Physarum polycephalum* myxamoebae have been examined. It was observed that the myxamoebae have the capacity to adapt to initially inhibitory concentrations of these drugs. While adaptation to a wide variety of drugs has been observed in ciliates (Frankel, '70), this phenomenon has not been previously reported in the myxomycetes.

This transient inhibition presents an unanticipated problem in association with the use of antibiotics, affecting both the use of drug sensitivity as a phenotype for genetic analysis and usefulness as a tool for biochemical studies. This paper describes the effect of trichodermin and the functionally similar antibiotic anisomycin on the growth of *P. polycephalum.*

MATERIALS AND METHODS

Strain

The amoebal strain of *P. polycephalum* employed, DJ4-37, is a wild-type, mating-type 2 haploid (Jacobson and Dove, '75).

Culture techniques

The techniques for growing amoebae and

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estimating cell number and viability have been previously described (Gorman and Dove, '74). Amoebae were routinely cultured on LIA (0.5% liver infusion, 2% agar) with a feeder layer of *Escherichia coli,* as the genetically defined strains employed do not grow in axenic culture media. For growth curves, cells were cultured on Millipore membranes which were supported over liquid medium with stainless steel grids. Replicate cultures were established by co-inoculating 1×10^5 amoebae and 1×10^{10} formalin killed *E. coli* per 80 mm filter. In mid-log phase ($1\text{-}2 \times 10^6$ cells per filter), the Millipore cultures were transferred to fresh dishes containing experimental medium (time 0 in the experiments shown). For sampling, the Millipores were transferred to empty dishes, flooded with 5 ml of PBS and gently scraped with a glass spreader. The cell suspension was counted in a hemocytometer and plated for viable count. Phosphate buffered salts solution (PBS) at pH 6.8 was employed as the liquid medium for all experiments. Cell counts are normalized to the count at time 0 (N/N_0) to facilitate comparison of separate experiments. Values of N/N_0 during the lag phase are given as the mean $\pm$ standard error of the mean.

Antibiotics

Trichodermin was the generous gift of W. O. Gotfredsen of Leo Pharmaceutical Products, Ballerup, Denmark. Anisomycin was kindly provided by Nathan Belcher of Pfizer Inc., Groton, Connecticut. Anisomycin was dissolved in water; trichodermin was dissolved in dimethyl sulfoxide (Me_2SO). The final concentration of Me_2SO in experimental media was always less than 2% (v/v). In control cultures, Me_2SO did not affect cell growth or viability at this concentration, nor did it apparently contribute to the effect of trichodermin on cell growth.[1]

Measurement of anisomycin in culture medium

The anisomycin concentration of solutions and media was determined by absorption at 224 nm (ϵ = 10,800 M^{-1}/cm). The anisomycin activity of the culture media was assayed biologically, using *Saccharomyces cerevisiae* strain D273-10B, a haploid wild type. Log phase yeast cells were spread on a plate of YPGA (1% yeast extract, 1% peptone, 2% glucose and 2% agar) to give 1×10^7 cells per 100 mm petri dish. Filter paper discs (Schleicher and Schuell, No. 740-E) were placed on the lawn of cells and spotted with 0.02-0.04 ml of antibiotic solution or medium. The diameter of the zone of inhibition was measured after 20 hours of incubation at 30°C. Relative activity was determined by comparison with a control using standard stock solutions at several concentrations. The lower limit of detection of anisomycin is 2.5 μg per disc. This bioassay was also used to standardize all trichodermin solutions, however it was not sensitive enough to establish small differences in activity in the culture medium at the concentrations employed. The lower limit of detection for trichodermin is 0.5 μg per disc.

RESULTS

The effect of trichodermin on growth

When trichodermin is added to a log phase culture of myxamoebae, there is an immediate cessation of growth, accompanied by an apparent decrease in cell number. However, on continued incubation, the cells recover from the inhibitory effects of the drug and resume growth. Figure 1 shows the response of strain DJ4-37 to three concentrations of trichodermin. The growth rate following transient inhibition is slightly lower than in the untreated controls, with the decrease being more pronounced as drug concentration increases.

A response of this nature might be accounted for in any of several ways. However, as will be discussed further, the ability to resume growth in the presence of trichodermin after a period of inhibition appears to be due to adaptation, the development of phenotypic but not genotypic drug insensitivity. Cells resuming growth in the presence of drug are referred to as adapted cells, and the period of inhibition preceding adaptive growth, the lag phase.

Stability of trichodermin in the culture medium

It appears highly unlikely that the resumption of growth is due to rapid extracellular breakdown or inactivation of the drug. If logarithmically growing adapted cells are transferred to fresh medium containing drug at the same concentration, there is no interruption of growth. Thus the entire population appears to be phenotypically drug resistant. When me-

[1] No difference in inhibition or adaptation were detected when propylene glycol was used in place of DMSO as solvent.

dium from adapted culture is tested for drug activity by examining its effect on fresh control cultures, inhibition is apparent, and the lag induced by the used drug medium is similar to that obtained with fresh drug medium. Therefore, there is no significant drug inactivation in the medium during adaptive growth. In addition, variation in the number of amoebae per culture when drug is added also has no significant effect on the length of the lag period (tested over a 5-fold range of cell densities). If the cell were able to actively degrade the drug, a shorter lag at higher cell densities would be expected.

Viability of inhibited cultures

As some cytotoxicity is evident after the addition of trichodermin, it seemed possible that the apparent correlation between drug concentration and recovery time reflected increased lethality at higher levels of drug. To assess this, viability during the lag period was examined further. Viable count declines for 8 to 12 hours following transfer to drug, then no further loss is evident (as illustrated in fig. 2 for 40 μg/ml). A comparison of the survival at different concentrations of trichodermin (calculated as described in fig. 2) is summarized in table 1. Above 1 μg/ml, viability is reduced by a remarkably constant amount, giving an average survival of 37% of the initial population, regardless of drug concentration. It is clear that viability does not continue to decrease significantly with increasing drug concentrations.

Determination of the lag phase

Due to the initial drop in viable count, it is not possible to determine the trichodermin induced lag period accurately. This difficulty is most apparent at low drug concentrations, where the cell count drops due to cytotoxicity then increases due to growth, without reaching a stable value (as illustrated for 1 μg/ml in fig. 2). To correct for the initial loss of viable cells, the lag phase has been arbitrarily defined as the time between the addition of drug and the point where the back extrapolate of the adapted growth curve (obtained from the regression line of values of $N/N_0 > 1.0$) intersects the average minimal survival, N_{min}/N_0[2] (fig. 2). The lag period estimated in this fashion is relatively constant from experiment to experiment. (The average variability is 12%).

The results of several measurements of the lag period at different levels of trichodermin are summarized in figure 3. The lag period is roughly proportional to concentration from 2.5 to 20 μg/ml.

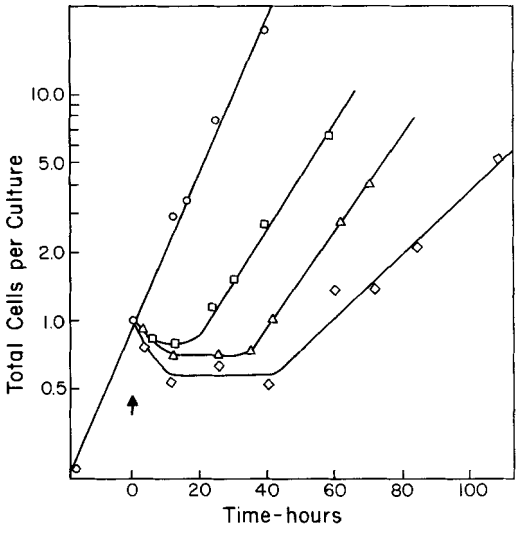

Fig. 1 The effect of trichodermin on growth of strain DJ4-37. Amoebae were grown on Millipore filters, as described. In mid log phase (arrow), cultures were transferred to media containing various concentrations of trichodermin: control (○), 2.5 μg/ml (□), 5 μg/ml (△) and 10 μg/ml (◇).

Growth of single colonies on agar plates

It was possible that the resumption of growth in the presence of trichodermin could be explained by an initial heterogeneity of the population in regard to drug sensitivity, so that, at successively higher concentrations, a smaller but more highly resistant fraction of pre-existing variants was being selected. To examine the drug sensitivity of individual cells, a cloned culture of DJ4-37 was plated at several dilutions on a series of agar plates containing trichodermin at increasing concentrations, and the rate and extent of colony formation determined. At all cell densities, the rate of appearance of colonies was proportionately decreased with increasing drug concentration. However, even at the highest drug concentration, a large percentage of the amoebae were able to form colonies, although only after prolonged incubation. Data from one dilution are shown in table 2A. The delay in colony formation indicated that the cells were

[2] This estimate is based on the assumptions that growth is arrested immediately after the addition of drug, and that all surviving amoebae are capable of resuming growth.

TABLE 1

The effect of trichodermin at several concentrations on cell viability

Trichodermin μg/ml	Average survival
1.0	0.56±0.10 (2)
2.5	0.40±0.02 (4)
5.0	0.33±0.05 (5)
10.0	0.32±0.02 (3)
20.0	0.41±0.07 (2)
40.0	0.36±0.02 (2)

The values shown are the averages of several independent determinations of N_{min}/N_0 ± S.E. (n). In any one experiment, a single value for N_{min}/N_0 was calculated from several points, as described in figure 2. At 1 μg/ml, N_{min}/N_0 is a single minimal value per experiment. The mean for concentrations of 2.5 μg/ml and higher = 0.37 ± 0.02. By analysis of variance the differences are not significant, F = 0.02.

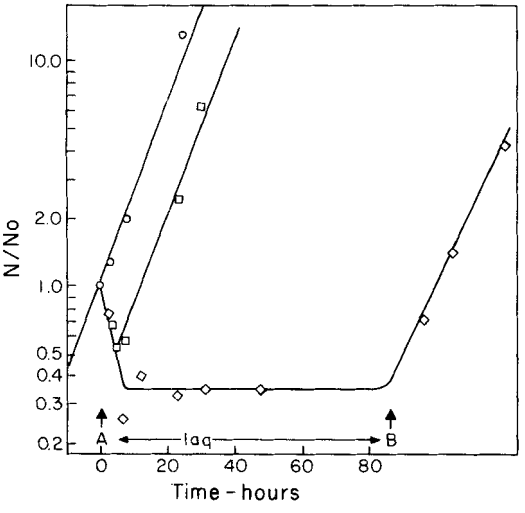

Fig. 2 Viability of trichodermin inhibited cultures. The viable cell count was determined at intervals after transfer to trichodermin at time 0. The viable count, normalized to the viable count at time 0 (N/N_0) is shown: control (○), 1 μg/ml (□) and 20 μg/ml (◇). The arrow, point B, indicates the point of resumption of growth in 20 μg/ml trichodermin. The average minimal survival (N_{min}/N_0) is estimated by averaging all points during the period of no decrease or increase in viable count after the addition of drug. The time interval A to B is used to define the corresponding lag phase (84 ± 1 hours) where N_{min}/N_0 = 0.34 ± .02.

not resistant,[3] but were adapting to the drug. To confirm this, clones from all plates were picked, subcultured on drug-free medium and restreaked on test plates. All clones, including over 100 from the initial drug-free plates, grew on trichodermin, but only after long incubation. As the amoebal streaks did not allow unambiguous scoring (resistance vs.

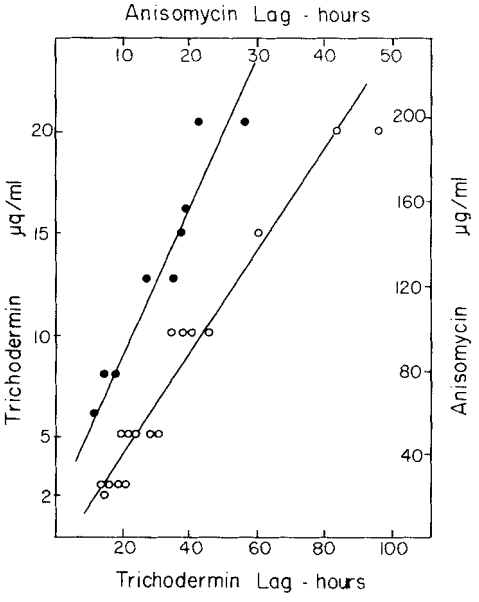

Fig. 3 The relationship between drug concentration and the drug-induced lag period. The lag phase at a given drug concentration is shown as a linear function of concentration. The trichodermin lag (○) was determined as shown in fig. 2. By regression analysis, the data best fit a linear relationship (r = 0.95 for a linear and 0.72 for a logarithmic curve). The anisomycin data (●) fit a logarithmic (r = 0.89) or linear (r = 0.88) equally well.

sensitivity with transient inhibition), several clones were retested by plating for single colony formation. By this analysis, none of the clonal sublines selected from drug plates proved to be more trichodermin resistant than control clones. Data for clones obtained from 40 μ/ml plates are shown in table 2B. These results indicate that the initial population does not contain sub-populations of stable drug-resistant variants, but rather that a majority of the cells are capable of adapting to the drug.[4]

Loss of the resistant phenotype

As the progeny of clones selected from trichodermin plates take as long as control cells to form colonies on drug plates, the adapted phenotype must be lost when the cells are subcultured in the absence of drug. Experiments were undertaken to determine how

[3] The term resistant is used here to designate a clonal subline which shows no inhibition at a drug concentration that is clearly inhibitory to the wild type.

[4] The frequency of cells unable to adapt is probably quite low, as all attempts to isolate mutants of this sort have been unsuccessful.

TABLE 2

Colony formation on trichodermin agar plates

A. Initial plating

Trichodermin μg/ml	Average number of amoebal colonies per plate			
	Day 7	Day 10	Day 14	Day 17
0	43	44	—	—
10	0	30	38	38
20	0	0	21	27
40	0	0	15	20

B. Retest of colonies from set A

Source of subclone		Colonies on trichodermin (20 μg/ml) / Colonies on drug free medium	
		Day 7	Day 14
No drug—	Original culture	0	0.47
	Clone 1	0	0.56
	Clone 2	0	0.28
Trichodermin 40 μg/ml	Clone 1	0	0.14
	Clone 2	0	0.42
	Clone 3	0	0.64
	Clone 4	0	0.39

Amoebae were plated in duplicate on LIA-trichodermin-agar plates seeded with *E. coli* and incubated at 26°C. Colonies were scored when the plaque diameter = 1 mm. The data in A are for a dilution estimated to give 50 colonies per plate. On day 14, clones were picked from each set of plates, and subcultured on drug-free medium. For retest, the cells were diluted and plated as described above. The time of appearance of colonies on drug medium was used as the criterion for scoring each clone; colony formation within seven days indicating resistance, and between 7 and 14 days indicating adaptation. Trichodermin was used at a concentration lower than the selection level to facilitate scoring (resistant cells should show little or no growth retardation). No significant difference in drug resistance between clones selected from drug and drug-free plates was detected. Variation in the number of colonies scorable on day 14 is not correlated with the source of the clone [1] (F = 0.009).

[1] The differences in the number of colonies on trichodermin plates in separate clones may reflect clonal heterogeneity in the rate of adaptation. This variability, which appears to be related to the physiological state of the cells at the time of plating rather than to genetic differences, has not been further analysed.

quickly adapted cells would regain drug sensitivity after removal of the drug. Cultures adapted to trichodermin (20 μg/ml) were transferred to drug-free medium and after various intervals of growth, tested for trichodermin resistance. The lag following rechallenge, compared to the initial lag, was used as an index of drug sensitivity. As shown in figure 4, a gradual return to full sensitivity is demonstrated. At least five to six generations are required to become fully sensitive. From this it is evident thaz the drug resistant phenotype is fully reversible.

Adaptation to anisomycin

The eucaryotic protein synthesis inhibitor anisomycin, while not structurally similar to trichodermin, has been shown to act at the same or an overlapping site on the 60s ribosomal subunit (Jiminez et al., '75). It was therefore of interest to determine if the amoebae would adapt to this structurally dissimilar but functionally similar antibiotic in the same way. *P. polycephalum* myxamoebae proved to be relatively insensitive to anisomycin, probably due to permeation barriers. However, at levels between 50 and 250 μg/ml, transient inhibition is evident (see examples in figs. 5, 7A). The response is similar to that seen for trichodermin, except that cell viability is not decreased after transfer to anisomycin. The lag, here defined as the time between addition of anisomycin and resumption of logarithmic growth (the intersection of the adapted growth curve at $N/N_0 = 1.0$) can be measured relatively accurately, and is roughly proportional to dose (fig. 3).

Measurement of anisomycin in the culture medium

The high concentration of anisomycin necessary to demonstrate growth inhibition made it possible to measure quantitatively the amount of drug remaining in the medium during the course of adaptation. The data are given in table 3. The amount of anisomycin, measured both by spectrophotometric determination and bioassay, remained relatively

TABLE 3

Determination of the amount of anisomycin in the culture media during drug adaptation

Time hours	Growth N/N_0	Anisomycin concentration $\mu g/ml$ [1]	Bioassay Relative Activity [2]
0	1.00	123	1.00
6	0.91	111	0.88
16	1.08	104	0.75
23	1.12	103	0.88
29	1.61	96	1.04
40	2.45	116	0.75
48	4.65	106	0.75
54	5.15	111	0.88
64	10.70	98	—
54	Control no amoebae	124	0.94

[1] Calculated from absorption at 224 mμ, corrected for adsorption of control media obtained from untreated cultures at same cell density.

[2] Drug concentration determined from standard curve, expressed as a ratio of concentration at the experimental point to the concentration at time 0, (C/C_0).

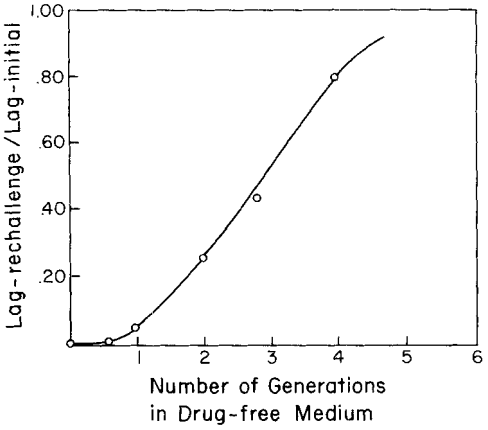

Fig. 4 Resensitization to trichodermin: Log phase cultures were transferred to trichodermin at 20 μg/ml. Following a lag (84 hours) the growing, adapted cultures were maintained on trichodermin for four generations. The cells were washed, then subcultured and transferred to drug free medium. At intervals, sets of cultures were transferred to 20 μg/ml trichodermin and the growth lag determined. The sensitivity to trichodermin is expressed as the ratio of the lag on rechallenge to the initial lag.

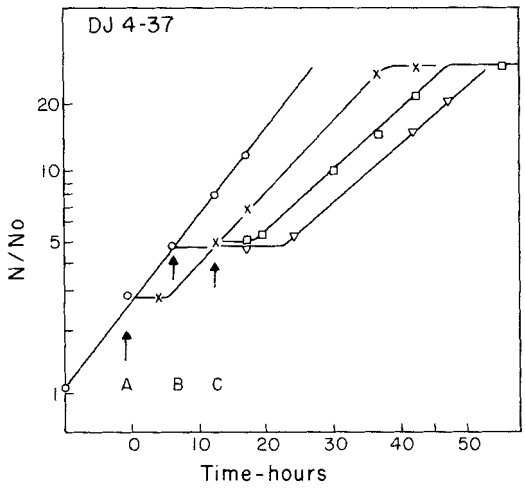

Fig. 5 The effect of rechallenge with a higher concentration of anisomycin on cells adapted to a lower concentration. Log phase cultures were transferred to medium without drug (○) or with anisomycin at 80 μg/ml (×) at zero time (point A). After resuming growth in the presence of anisomycin, the adapted cells were rechallenged with anisomycin at 160 μg/ml (□) point C. The response to 160 μg/ml without preadaptation (▽) determined by transfer of control cultures at point B, is shown for comparison. N/N_0 represents normalized viable counts per sample.

constant over a period of 64 hours, during which the culture adapted and grew tenfold. These results demonstrate that no significant exogenous drug inactivation or breakdown occurs.

Cross adaptation

If the recovery of growth involves a specific change in a cellular component directly related to the action of these antibiotics, cells adapted to one drug might be expected to be phenotypically resistant to the other. Before testing for cross resistance, the sensitivity of cells adapted to a given drug to higher levels of the same drug was examined. As illustrated in figure 5, when cells adapted to one concentration of anisomycin are challenged with a higher dose, they are immediately inhibited. However, the lag period is considerably short-

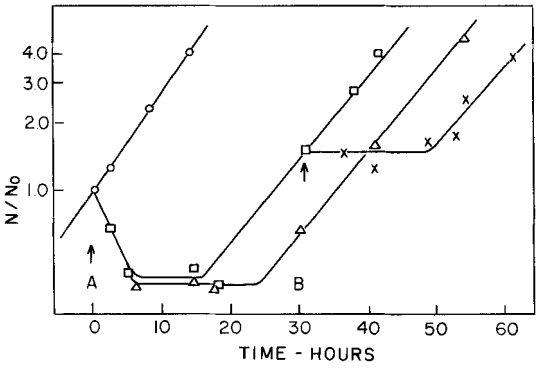

Fig. 6 The effect pretreatment on trichodermin inhibition. Control cultures (○) were transferred to trichodermin at 2.5 μg/ml (□) or 5 μg/ml (△) at point A. When the cultures in 2.5 μg/ml drug had resumed growth, several were transferred, point B, to trichodermin at 5 μg/ml (×). All points represent normalized viable counts.

er than that of a non-adapted culture given the same drug media (7 hours compared with 15 hours in the experiment shown). In all experiments of this sort, the lag is reduced by a time period roughly equal to the preadaption lag. This indicates that the total time needed to adapt to a given concentration of anisomycin is relatively constant. The level of phenotypic resistance is related to the inducing concentration, and can be progressively increased.

When cells are adapted to a low concentration of trichodermin, the most striking effect of challenge with a higher dose is the lack of cytotoxicity during the ensuing growth inhibition (as shown in fig. 6). The lag period of preadapted cultures is also somewhat shorter than that of untreated controls at the same of trichodermin level, but the differences are not pronounced. In contrast to anisomycin, the difference is not equal to the preadaptation lag, but considerably less.[5]

As the exogenous concentration of anisomycin and trichodermin required to demonstrate growth inhibition differ by 50-fold, the levels used to test cross resistance were based on similarity of effect rather than on absolute amounts. In experiments testing the sensitivity of cells preadapted to anisomycin to different levels of trichodermin, an insensitivity to toxic effects was again apparent. Regardless of the drug levels employed, cells preadapted to anisomycin are inhibited by trichodermin, but are resistant to any cytotoxic effects. The trichodermin lag is also reduced when the cells are preadapted to rela-

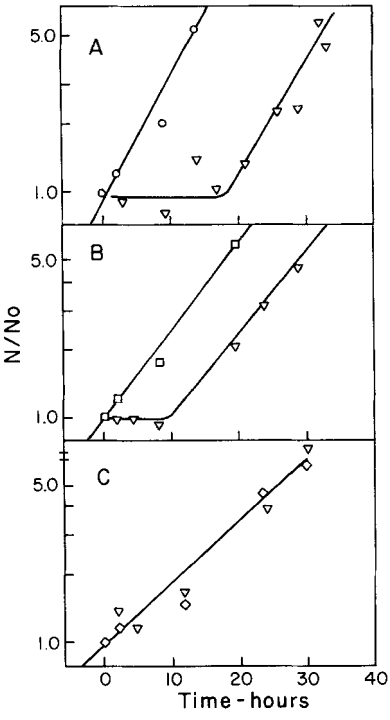

Fig. 7 Cross adaptation to anisomycin in cells adapted to trichodermin. Log phase cultures were adapted to various concentrations of trichodermin, washed and transferred to 150 μg/ml anisomycin at time 0.

A The nonadapted control (○) transferred to anisomycin (▽); 18 hour lag.

B Cells preadapted to 2.5 μg/ml trichodermin (□) transferred to anisomycin (▽); 10 hour lag.

C Cells preadapted to trichodermin at 10 μg/ml (◇) transferred to anisomycin (▽), no lag.

tively high concentrations of anisomycin (for example, the 16-hour lag induced by trichodermin at 2.5 μg/ml is reduced to 9 hours in cells preadapted to 160 μg/ml anisomycin). Complete cross resistance to trichodermin has not been demonstrated, even with preadaptation to a very high level of anisomycin. To the contrary, complete insensitivity to any toxic effects of trichodermin is established by pretreatment with even very low levels of anisomycin.

The reverse experiments, using cells preadapted to trichodermin to test sensitivity to anisomycin, were more definitive. At concentrations estimated to give approximately the same lag for each drug, the preadapted cells

[5] As the calculation of the trichodermin lag phase includes allowance for less of viability, this correction may contribute unequally to error inherent in the definition of the lag phase when cytotoxicity differs.

show a significantly shorter lag than non-adapted cells when challenged with anisomycin (figs. 7A,B). By using progressively higher levels of trichodermin, it was found that complete cross-resistance to anisomycin can be established (fig. 7C).

DISCUSSION

Of the possible mechanisms that might be invoked to explain the phenomenon of adaptation described in this study, it is clear that neither extracellular drug inactivation nor accumulation of stable drug-resistant mutant cells is involved. Instead, it is apparent that *Physarum* myxamoebae can develop a phenotypic drug resistance in the presence of certain antibiotics which is gradually lost after the drug is removed. This phenomenon could be based on any one of several different mechanisms, involving either a change at the level of the receptor molecule to which the drug binds, or the development of a system which effectively excludes drug from the cell.

The receptor molecule which is sensitive to the protein synthesis inhibitors used is most likely a ribosomal component. While there is no direct evidence that anisomycin and trichodermin act at the ribosomal level in *Physarum,* it appears likely that the growth inhibition seen is the result of a direct effect on protein synthesis. *Physarum* is sensitive to other eucaryotic inhibitors, and, in the case of cycloheximide, the site of action is at the ribosomal level (Haugli and Dove, '72). Thus, there is no reason to suspect that the primary effect of trichodermin or anisomycin is not analogous to that demonstrated in other eucaryotes.

If the alteration resulting in drug resistance occurs at the site of drug binding, a structural modification or rearrangement of the ribosomal subunit could be postulated. While the *de novo* synthesis of resistant ribosomes might be involved, this appears less likely as protein synthesis would be required. In addition, it is difficult to envision a continuous series of ribosomal changes, each resulting in a higher level of resistance. As adapted cells are transiently inhibited when challenged with higher doses of antibiotic, a mechanism allowing for progressive increase in resistance is necessary.

The development of a system effectively leading to drug exclusion seems equally probable. This could result from the induction of an internal detoxification system or a decrease in permeability (coupled with the inactivation or removal of the drug already present within the cell). Alternatively, the drug might be actively extruded from the cell by the increased formation and activity of contractile vacuoles or other membrane systems.

The induction of at least partial cross resistance (cross-adaptation) to one drug by the other implies that a common alteration is involved. As the two antibiotics tested do not act at independent ribosomal sites (Jiminez et al., '75), cross adaptation might indicate a single ribosomal change affecting sensitivity to both drugs. In yeast, mutation to trichodermin resistance due to permeation changes does not alter sensitivity to anisomycin, and vice versa (Julian Davies, personal communication), while ribosomal alteration is associated with cross resistance (Schindler, '74; Jiminez et al., '75). However, a non-specific exclusion system could equally well account for cross adaptation. In drug-resistant lines of cultured mammalian cells, cross-resistance to dissimilar agents has been associated with altered membrane permeability (Ling and Thompson, '74).

The present experiments do not exclude any of these mechanisms, and other alternatives are possible. As many types of changes would require at least limited protein synthesis, it is premature to postulate specific mechanisms until the effects of these inhibitors on cell metabolism are examined. *Physarum* plasmodia, in contrast to amoebae, can be cultivated in axenic medium. As preliminary studies indicate that plasmodia also adapt to trichodermin and anisomycin, studies of the effect of these drugs on protein synthesis have been initiated.

The initial loss of amoebal viability complicates the analysis of trichodermin adaptation. As the drug is not toxic to cells which have been pre-adapted to even low levels of either anisomycin or trichodermin, it appears that, once components conferring drug resistance are present, the cells are refractory to toxic effects even when the levels of such components are insufficient to prevent growth inhibition. The plateau in initial kill might reflect a rather constant time interval needed for induction of the minimal number of resistant components needed to prevent lethality. If so, the fact that the viability declines at a relatively constant rate at all drug concentra-

tions suggests that the rate of induction of resistance is also independent of the inducing concentration.

If resistance is due to the induction of such resistant components or "sites," with the level of resistance proportional to the number present, the lag period should reflect the time needed to form sufficient sites to confer resistance to the inducing level of drug. The linearity of the dose-response curves again suggests a concentration independent rate of synthesis. However, as the observed lag might also include a finite period of endogenous drug "inactivation," the relationship between lag and concentration may not be an accurate index of the rate of induction of resistance.

It is clear that the cells are capable of forming additional sites when exposed to higher drug concentrations, but that, at a given concentration, they remain at a steady state level just sufficient to render the cell resistant to the amount of drug present. When drug is removed, the formation of new sites ceases. The kinetics of trichodermin "deadaptation" indicate that the loss of resistance is less than would be expected by a simple dilution of resistant sites during subsequent growth, for nearly three generations are required to reduce resistance by one half. This may reflect some residual formation of resistant components, or increased efficiency of those remaining.

Inducible drug resistance has been observed in a few other organisms, where, as in the present case, there is no significant drug degradation, and phenotypic resistance is lost in the absence of drug. In certain strains of *Staphylococcus,* erythromycin induces resistance not only to itself, but to additional inhibitors of 50S ribosomal subunit function (Weisblum and Demohn, '69). This resistance is mediated by modification of the ribosomes by an erythromycin induced rRNA methylase (Lai and Weisblum, '71), which decreases drug binding (Saito et al., '71). This accounts for the cross resistance to 50S but not 30S subunit inhibitors. In this example, however, it should be noted that cross-resistance is induced by sub-inhibitory concentrations of erythromycin.

The ciliate *Tetrahymena* adapts to a wide variety of drugs, including cycloheximide, colchicine, actinomycin D and 2,4-dinitrophenol (see Frankel, '70). The widely different structure and mode of action of these inhibitors has supported the theory that mechanisms leading to active drug extrusion are involved (Frankel, '70; Heyer and Frankel, '71). However, although cycloheximide adapted cells are cross-resistant to emetine (Roberts and Orias, '74), they remain sensitive to colchicine (Frankel, '70). Thus, it is possible that more than one mechanism is involved in adaptation to different classes of drugs.

It will be of interest to determine the spectrum of antibiotics to which *Physarum* can adapt and develop cross-resistance to further assess the mechanisms involved.

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