BIOTRANSFORMATION OF ANTIBIOTICS

II. INVESTIGATION OF THE CHLORAMPHENICOL ACETYLTRANSFERASE IN *STREPTOMYCES GRISEUS**

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Additional parameters for the chloramphenicol acetyltransferase (CAT) activity in spores of *S. griseus* are substantiated. A linear increase in activity was observed with increasing spore number up to a concentration of 5×10^{10} spores/ml. Similarly an increase of the chloramphenicol concentration up to 500 μg/ml increased the activity. However, a drastic decrease in activity was noted above this level suggesting inhibition of the enzyme by the substrate. The CAT activity in the spores was highly influenced by the pH of the medium reaching a maximum at pH 6.5. This may suggest that CAT is apparently located to the outer surface of the spores and therefore very sensitive to variations in pH of the medium. The CAT showed a marked specificity for D-*threo* and D-*erythro* chloramphenicol, while no activity was observed with L-*isomers*. The enzyme acetylates D,L-*erythro* dechlor-chloramphenicol with a yield of 45% as compared to the D-*threo* parent antibiotic.

While the tyrosinase characteristic (melanin formation) of *S. griseus* was eliminated by acriflavine or ethidium bromide treatment the CAT characteristic was persistent. The melanin negative variants retained all other properties of the parent strain including the production of antimicrobial agents; and revertants were not detected. The results suggest that the tyrosinase determinant gene is apparently located on an extrachromosomal element (plasmid). On the other hand, the location of the gene for CAT is not assigned yet. The nature of CAT in growing cells and the spores of *S. griseus* was investigated. The results show that CAT accumulated during the sporulation phase or the vegetative growth is inducible in nature; therefore the morphogenetic sequence in the strain bears no influence on CAT induction.

Before 1960, antibiotic resistance in bacteria was believed to emerge by mutation and selection. This view originated from the fact that bacteria *in vitro* could easily acquire resistance to antibiotics. Thereafter, investigations have changed this view and most antibiotic resistance in bacteria appears to be plasmid-determined.[1] Resistance to at least 15 antibiotics is known to be associated with resistance plasmids (R factors). In some instances the mechanism may involve impermeability to the antibiotic (*e.g.* tetracycline), but in most cases the resistance phenotype is usually mediated by the presence of different specific enzymes that modify and thereby inactivate the antibiotic[2,3]. The recognition of these enzymes and of their reaction mechanisms contributed significantly to the preparation of new semi-synthetic antibiotics, as well as to the advancement of biological sciences[4,5]. These enzymes may be highly useful as analytical reagents in the identification and determination of specified antibiotics as well as in their effective removal by inactivation from reaction mixtures[6,7].

In a previous communication[8] we showed that acylation is the mechanism of chloramphenicol

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inactivation by the spores of *Streptomyces griseus* (NRRL B-8078). It was also observed that the chloramphenicol acetyltransferase (CAT) in the spores shares the same preference for short-chain acyl radicals as CAT from *Staphylococcus aureus, Staphylococcus epidermidis,* or enteric bacteria[9]. However addition of certain carbon sources to the spores showed a pronounced effect on the chloramphenicol conversion process and on the relative concentration of the inactivated products. The present work describes additional factors influencing the CAT activity of the spores, the enzyme substrate specificity, effect of acriflavine or ethidium bromide on the enzyme synthesis, and finally, the nature of CAT during the life cycle of *S. griseus*.

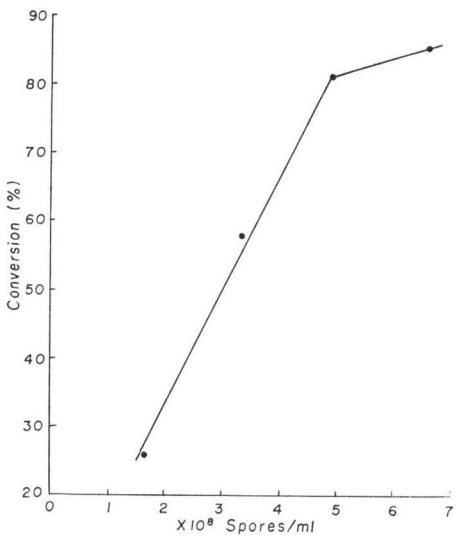
Material and Methods

1. Organism: The strain of *Streptomyces griseus* (NRRL B-8078) used was previously isolated from an Egyptian soil. Culture media, spore collection, and methods for the inactivation of chloramphenicol by spores or resting cells of the strain are described in a previous paper[8].

2. Chemicals: Chloramphenicol was obtained from Sigma Chemical Co., Inc., St. Louis, Missouri. The chloramphenicol isomers: L-*threo* chloramphenicol, L-*erythro* chloramphenicol, and D-*erythro* chloramphenicol were obtained through the courtesy of Dr. M. C. REBSTOCK, Parke Davies & Co., Ltd. Ann Arbour, Michigan; D,L-*erythro*-2-acetamido-1-*p*-nitrophenyl-1,3-propanediol (dechlor-chloramphenicol) and D-*threo*-2-amino-1-*p*-nitrophenyl-1,3-propanediol (dedichloroacetyl-chloramphenicol) were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. All other chemicals used were also of reagent grade.

3. Acriflavine or Ethidium Bromide Treatment of *S. griseus*: The influence of acriflavine or ethidium bromide on the CAT characteristic and/or on the phenotype tyrosinase characteristic of *S. griseus* was investigated as follows: a fresh stock culture of the strain (about 10^{10} spores) was used to inoculate minimal medium[10] with or without acriflavine (1 μg/ml) or ethidium bromide (4 μg/ml) in 500-ml Erlenmeyer flasks. The dyes were added to the culture media immediately prior to inoculation. The flasks were incubated on a rotary shaker (200 r.p.m.) at 30°C for 7 days. The collected mycelial pellets were washed twice with sterile distilled water by centrifugation and resuspended in 50 ml 0.02% Tween 20. The mycelial suspension was then fragmented in a Waring Blendor for 30 seconds. The relative proportion of tyrosinase-negative (tye$^-$) mycelial fragments was determined by spreading appropriate dilutions of the suspensions on the surface of complete medium which is composed of 1.9% corn meal agar with dextrose (Difco), 0.3% yeast extract, and 0.02% L-tyrosine. After 2~3 days' incubation at 30°C, all tyrosinase-positive (tye$^+$) colonies were surrounded by dark-brown to black pigments (melanin), while the tye$^-$ colonies were not. The tye$^-$ colonies were purified by subcultures on the same medium and tested for the chloramphenicol transformation activity.

Fig. 1. The influence of spore concentration of *S. griseus* on the conversion of chloramphenicol (estimated as chloramphenicol monoacetate). Spores (as indicated) in 0.1 M phosphate buffer (pH 6.5); chloramphenicol, 0.5 mg/ml; incubation 12 hours at 30°C.



4. Measurement of the Chloramphenicol Acetylating Activity of *S. griseus*. Gas chromatography

Fig. 2. The influence of chloramphenicol concentration on its conversion by the spores of *S. griseus*.
Spores, 5×10^8/ml; chloramphenicol, 0.5 mg/ml; incubation 12 hours at 30°C.

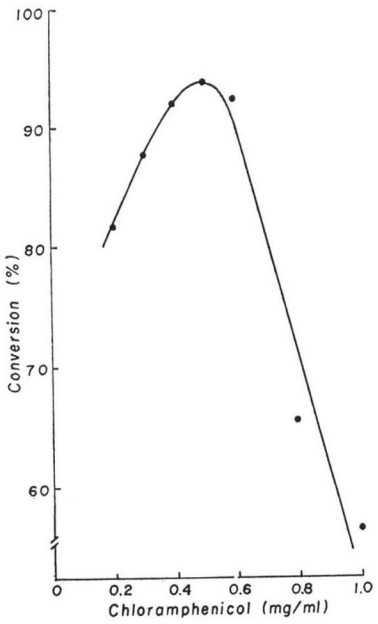
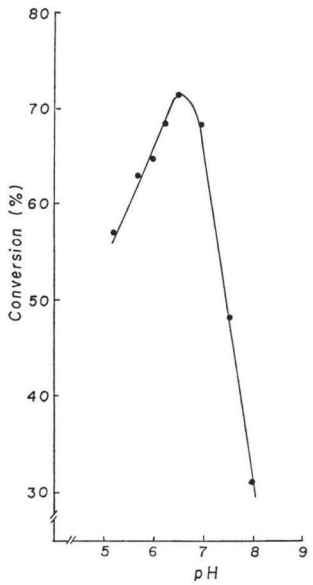


Fig. 3. The influence of pH on the conversion of chloramphenicol by the spores of *S. griseus*.



was used to identify and determine the residual chloramphenicol and its transformation products as previously described[8].

Results and Discussion

I. Factors Influencing the CAT Activity of Spores of *S. griseus*

In previous work[8] we found that maximum CAT activity in the spores of *S. griseus* occurred after 12-hour incubation with the regeneration of the antibiotic thereafter. The influence of the following additional parameters on CAT activity of spores were investigated as follows:

(1) Effect of Spore Concentration:

In microbial transformations by the spores, the rate of the conversion processes usually increases with increasing ratio of spores/initial substrate concentration[11]. The results from this study are presented in Fig. 1 which show a linear increase in chloramphenicol conversion with an increase in the spore number up to a concentration of 5×10^8 spore/ml. Above this level the increase in spore concentration was not proportional with the increase in activity. This may be attributed to an increase of viscosity of the medium affecting the accessibility of the chloramphenicol substrate to the enzyme activity in the spores.

(2) Effect of Chloramphenicol Concentration:

The optimal chloramphenicol conversion by the spores of *S. griseus* was investigated at different concentrations of the antibiotic. The results presented in Fig. 2 show a linear increase in activity up to the concentration of 500 μg/ml. A drastic decrease of activity was observed above this level suggesting the substrate inhibition of the enzyme. In all chloramphenicol concentrations studied, chloramphenicol-3-acetate was always the main transformation product, while other chloramphenicol esters were found in negligible amounts.

Table 1. Substrate specificity of CAT in spores of *S. griseus*

Chloramphenicol stereo-isomers	Relative activity (%) to the D-*threo* isomer	
	Intact spore barriers	Disrupted spore barriers
D-*threo* chloramphenicol	100	100
D-*erythro* chloramphenicol	70	71
L-*threo* chloramphenicol	1	1
L-*erythro* chloramphenicol	0	0
D,L-*erythro* dechlorchloramphenicol	45	45

Table 2. Frequency of tyrosinase-deficient mutants in the fragmented mycelia of *S. griseus* grown in minimal medium with and without 1 μg of acriflavine or 4 μgs of ethidium bromide per ml.

Acriflavine-treated	Ethidium bromide-treated	Non-treated
3.49±0.18*%	2.26±0.07*%	0.0%

* Standard error.

Fig. 4. Structure formulas of chloramphenicol and its isomers.

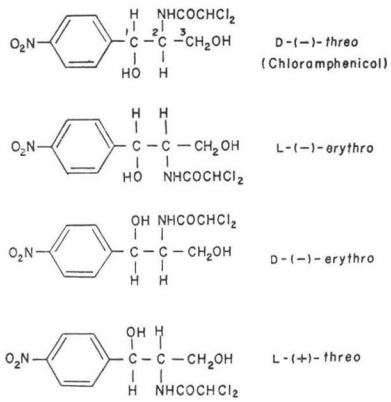
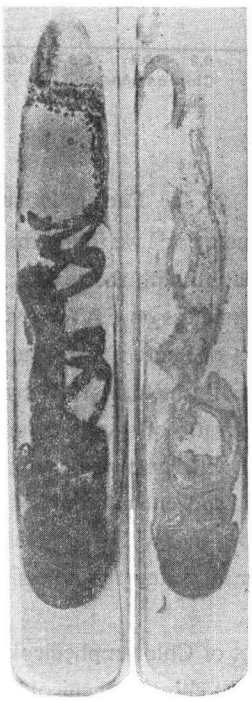


Fig. 5. Elimination of the phenotype tyrosinase characteristic (melanin) of *S. griseus* by acriflavine or ethidium bromide treatment.



(3) Effect of pH:

Previous reports[8,9] on CAT's from Gram-positive and Gram-negative bacteria, either controlled by the chromosomes or by R-factors have similar optimal activity at pH 7.8. Our results (Fig. 3) on the chloramphenicol acetylation by spores of *S. griseus* showed an optimum activity at pH 6.5. Since our study used intact spores (*in vivo*) and not the isolated enzyme (*in vitro*), this optimum may not represent the real pH optimum for the isolated enzyme. Rather, this pH of 6.5 may be an overall optimum pH for:

1) The maximum activity and stability of CAT in the spores during the transformation process.
2) The maximum stability of the transformation products.

3) The maximum accessibility of the chloramphenicol substrate-enzyme active site(s) in the spores.

Whether the above mentioned pH (6.5) is real or not for CAT in the spores of *S. griseus* must await the preparation of active cell-free extract. It must be pointed out that a broad pH optimum with a peak at 7.8 was reported for the CAT of *Escherichia coli*[12] for the formation of chloramphenicol monoacetate. It may also be noted that microbial transformations of steroids by the spores of certain microorganisms are not significantly influenced by pH, ranging from 4.0 to 8.5[11,13]. This may be explained by the fact that the spores generally tolerate a fairly wide range of pH and some of the enzyme(s) involved are located away from the spore surface[14]. On the other hand, the spore-chloramphenicol acetylation activity is highly influenced by the pH of the medium; the optimum value being 6.5 (Fig. 3). This suggests that the CAT is located to the outer surface of the spore and therefore, is very sensitive to variations in pH of the medium.

II. Substrate Specificity of CAT in the Spores

In addition to our previous report[8] on the specificity of CAT for various acyl species, we investigated the steric configuration of chloramphenicol isomers (Fig. 4) and of a closely related compound on the substrate specificity of the enzyme in the spore of *S. griseus*. To ensure that the spores were permeable to the chloramphenicol congeners, spores with disrupted permeability barriers were used as compared to intact spores. The permeability barriers of the spores were disrupted by 0.1% phenethyl alcohol[14]. Results (Table 1) are expressed as percentage of that obtained with D-*threo* chloramphenicol. The CAT in the spore showed a marked specificity for the biologically active D-*threo* chloramphenicol. The D-*erythro* isomer also presented a comparable affinity for the enzyme. This affinity may be explained by the presence of the $-NHCOCHCl_2$ group in the same position as in D-*threo* chloramphenicol. In contrast, no activity was observed towards L-*threo* or L-*erythro* isomers, such structures show the $-NHCOCHCl_2$ group in opposite position to that of chloramphenicol (Fig. 4). The same pattern has been reported with CAT of *Staphylococcus aureus, S. epidermidis* or *E. coli*[6,9]. However, fluctuating results were observed for the D-*erythro* isomer with yields of 1.0~32.0% to that of D-*threo* chloramphenicol[9,15]. Moreover, SUZUKI and OKAMOTO[12] reported that D,L-*erythro* chloramphenicol was acetylated by CAT of *E. coli* at a rate of 55% as compared to the D-*threo* chloramphenicol. This may explain the high activity we obtained with the D-*erythro* isomer. This variation in results may be attributed to the particular assay procedure they used.

We also found (Table 1) that the CAT in the spores of *S. griseus* acetylates the D,L-erythro-2-acetamido-1-*p*-nitrophenyl-1,3-propanediol (dechlorchloramphenicol) with a yield of 45% as compared to the D-*threo* parent antibiotic. This low activity may be explained by the hindrance of the 2-acetamido group of L-*erythro* portion on the conformation of the enzyme. Thus, the activity observed may be attributed to almost complete activity on the D-*erythro* portion of the racemic mixture. These results suggest that the CAT in the spores of *S. griseus* requires acyl group on the 2-amino position of the propane moiety, but not necessarily the chlorine atoms. Similar results were also obtained with the CAT enzyme which is controlled by extrachromosomal plasmid(s) or R-factor(s) in different Gram-positive and Gram-negative bacteria[6,15,16].

III. Influence of Acriflavine and Ethidium Bromide on the CAT of *S. griseus*

Our results revealed a striking similarity between chloramphenicol inactivation by our strain of

S. griseus and by other bacteria carrying R factors (or plasmids), as far as the mechanism of inactivation and the specificity of CAT are concerned. Consequently, an experiment was undertaken to investigate the possibility that CAT is episomally-controlled in *S. griseus*. Fortunately, our strain produces melanin which has been shown to be controlled by an episomic element in several *Streptomyces* species[10]. For convenience we used this phenotype tyrosinase characteristic (melanin) as a marker in our investigation.

Acriflavine and ethidium bromide are organic dyes known to eliminate or cure R factors and other episomal elements[8,10]. They were used to investigate their effect on the synthesis of tyrosinase and of chloramphenicol acetyltransferase in *S. griseus*. The results presented in Table 2 show that both acriflavine or ethidium bromide are effective in the elimination of the tyrosinase characteristic (melanin) in the treated *S. griseus*. As could be seen from the non-treated culture, spontaneous loss of this marker was not observed. The fairly high frequency of the tyrosinase-deficient variants among the fragmented mycelia of the acriflavine or ethidium bromide treated *S. griseus* strongly suggests that the tyrosinase-determinant gene appears to be located on an extrachromosomal element (plasmid). Similar findings have been observed for many other *Streptomyces* species[10,17].

Although ethidium bromide is reported[1,8,18] to be more effective in curing bacterial cytoplasmic episomes, our results (Table 2) show that acriflavine is more effective in curing the tyrosinase-determinant

Fig. 6. Persistance of the CAT characteristic of *S. griseus* after acriflavine or ethidium bromide treatment.
A, conversion of chloramphenicol by resting cells of the non-treated *S. griseus*.
B, conversion of chloramphenicol by resting cells of the treated *S. griseus* (melanin-negative).
Resting cells 0.7 mg/ml (wet weight) in 0.1 M phosphate buffer (pH 6.5); chloramphenicol, 0.5 mg/ml; incubation 12 hours at 30°C.

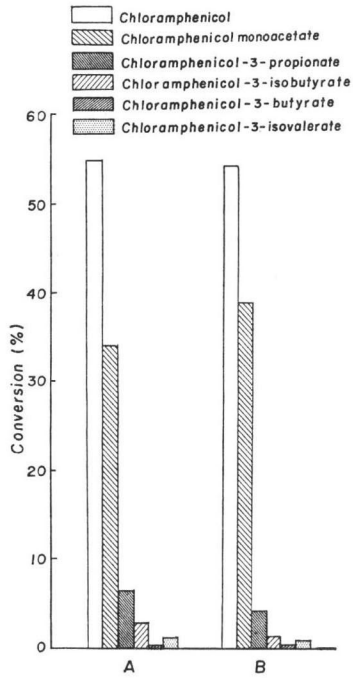
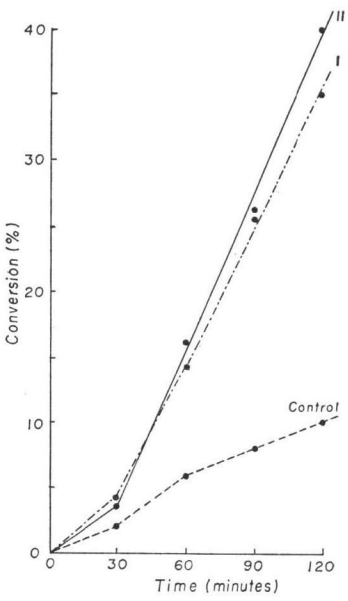
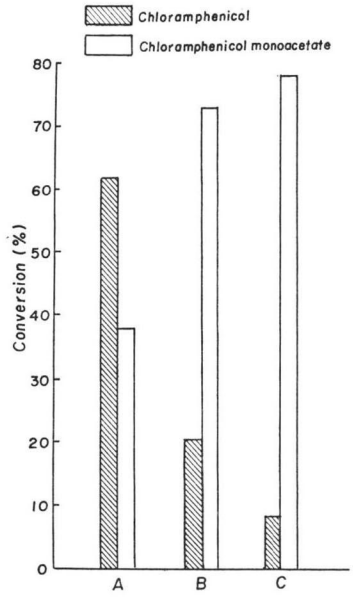


Fig. 7. Induction of CAT in growing cells of *S. griseus*.
Cultures (50 ml) were induced in the early logarithmic phase of growth by the addition of either inducer (I) or (II) (0.5 mM); Aliquot samples (5 ml) withdrawn at the indicated time intervals were centrifuged, washed, and resuspended in the half of the original volume in 0.1 M phosphate buffer (pH 6.5) before assay for the recovered CAT activity.



plasmid in *S. griseus*. The *S. griseus* melanin-negative variants, as seen in Fig. 5, were found to be stable and revertants were not detected. They retained all the other properties of the non-treated *S. griseus* including the production of the antimicrobial agents[8]. However, the growth rate of the latter was slightly lower than that of the parent organism. In addition the *S. griseus* tyrosinase-deficient variants retained the chloramphenicol acetyltransferase characteristic of the parent strain with the same inactivation pattern as seen in Fig. 6. This does not necessarily mean that the CAT synthesis in *S. griseus* is not controlled by a plasmid since it is known that several types of plasmids are refractory to the effect of the curing agent(s).[1,3,17,18]

Fig. 8. Induction of CAT during the sporulation phase of *S. griseus*.
A, spores produced in the absence of an inducer. B, spores produced in the presence of dedichloroacetyl-chloramphenicol; C, spores produced in the presence of dechlorchloramphenicol. Spores, 1.5×10^8/ml in 0.1 M phosphate buffer (pH 6.5); chloramphenicol, 0.5 mg/ml; incubation 12 hours at 30°C.



The existence of plasmids in streptomycetes has recently been clarified. HOPWOOD *et al.*[19] in their investigation on the genetic map of *Streptomyces coelicolor* A3(2) demonstrated that the fertility in this strain is coded by a plasmid. Later on, in 1975, HOPWOOD and his collaborators[20] were first to isolate the plasmid(s) (closed supercoiled DNA) from *S. coelicolor* A3(2). In addition, AKAGAWA *et al.*[21] showed by genetic evidence as well as by other tests the existence of a plasmid involved in chloramphenicol production in *Streptomyces venezuelae*. Other phenotypic characters in streptomycetes have also been claimed to be controlled by extrachromosomal elements[17,21].

Thus, at the moment, additional experiment is needed to determine whether CAT synthesis in *S. griseus* is episomally mediated or chromosomally directed.

IV. Induction of CAT in the Growing Cells of *S. griseus*

The most important distinction between the R-mediated enteric system and that of plasmid-linked chloramphenicol resistance in staphylococci is that the acetylating enzyme in the latter species is induced by the presence of the parent antibiotic and certain related analogues[6]. In consideration of this distinction, we observed that the addition of chloramphenicol (100 μg/ml) to *S. griseus* at the beginning of the logarithmic phase of growth resulted in almost complete acetylation of the antibiotic. Conversely, the addition of chloramphenicol at the late logarithmic phase of growth resulted in significantly lower yield of acetylated antibiotic. A similar observation was reported by STEINMAN[22] who found that growing staphylococcal cells yielded induced penicillinase more rapidly than the stationary phase-cells. Our observations strongly suggest that CAT synthesis in *S. griseus* appears to be inducible in nature. Previous studies on CAT induction in *Staphylococcus aureus* established[5,9,15] that chloramphenicol is not a good inducer for its acetylating enzyme; since the antibiotic is not only the inducer but is a potent

specific inhibitor of protein synthesis and enzyme induction and is also the substrate for the enzyme in question. To circumvent these problems, D-*erythro*-chloramphenicol (**I**) and D,L-*erythro*-dechlorchloramphenicol (**II**) analogues were used in the study of CAT induction in growing cells of *S. griseus*. As both compounds showed a measurable affinity for the acetylating enzyme in *S. griseus* (Table 1) a relatively high concentration of 0.5 mM of these inducers was used in the study of induction.

Cultures of *S. griseus* in the fermentation medium[8] (50 ml) were induced in the early logarithmic phase of growth (2 hours after inoculation from an overnight culture on the same medium) by the addition of either inducer **I** or **II** at a final concentration of 0.5 mM. Samples (5 ml) withdrawn at different time intervals were centrifuged, and the pellets washed twice with cold sterile distilled water, and resuspended in half of the original volume in 1% phosphate buffer (pH 6.5) before assay for the recovered chloramphenicol acetylating activity as usual[8]. The results presented in Fig. 7 show a linear increase in CAT activity with time after the addition of either inducer I or II to the growing culture of *S. griseus*. They showed a very similar pattern of kinetic induction.

The CAT activity of the controls (non-induced *S. griseus* cultures) was significantly lower than with either inducer. It is interesting to note that, although neither I nor II is a "gratuitous" inducer (a compound which is neither acetylated nor serves as inhibitor of protein synthesis), both are effective in increasing the basal CAT level in the growing cultures of *S. griseus*.

V. Nature of CAT in Spores of *S. griseus*

The complex and specialized structure of spores result from a morphogenic change during vegetative growth. This differentiation is not only of a morphological, but sometimes, also of a physiological nature. Thus, while the enzymes involved in steroid transformations by the mycelium of certain microorganisms were found to be adaptive, the same enzymes in the spores of the same cultures were constitutive in nature[11,18]. The importance of these findings prompted us to determine the nature of CAT in spores of *S. griseus*. Washed spores of *S. griseus* previously produced in the sporulating medium[8] in the absence or presence of 0.5 mM of dedichloroacetyl-chloramphenicol or D,L-*erythro*-dechlorchloramphenicol as inducers were tested for their chloramphenicol conversion activities. A submaximal concentration of spores (1.5×10^8 spores/ml) was used to assess the influence of the inducer. The results obtained (Fig. 8) show that the chloramphenicol-acetylating activity was significantly increased in spores previously produced in the presence of either inducer as compared to that of non-induced spores. The results also show that the spores produced in the presence of dechlorchloramphenicol as inducer gave highest activity (92% conversion), while the spores produced in the presence of dedichloroacetyl-chloramphenicol gave 78%, and the spores produced in the absence of any inducer gave only 38% transformation. These results indicate that the presence of an electronegative acyl substituent on the 2 amino group is of greater importance than the absolute D-*threo* configuration for CAT induction, at least for *S. griseus*.

These results demonstrate also that the CAT accumulated during the sporulation phase of *S. griseus* is of the same inducible nature as that found during the vegetative growth. Accordingly, the morphogenetic sequence in *S. griseus* bears no influence on CAT induction. Of particular interest, HOPWOOD *et al.*[23] found that the spore differentiation in *Streptomyces coelicolor* is controlled by only two genes in contrast to other protokaryotic system of sporogenesis where at least 15~100 genes are being involved[24,25]. On the other hand, HAFEZ-ZEDAN and PLOURDE[18] found that the morphogenetic sequence

in *Fusarium solani* strikingly influenced the inductive nature of steroid transforming enzymes. They found that the hyphal enzymes are inducible in nature whereas the spore enzymes are constitutive. In addition, VANDAMME and his collaborators[26,27] found that penicillin V acylase activity was only detectable in the spores of *Fusarium moniliforme*. They demonstrated, however, this enzyme activity in the mycelia of *Aspergillus, Penicillium* and *Botrytis* species, but not in their spores.

Although our results demonstrated the inductive nature of CAT during vegetative growth and sporulation phase of *S. griseus,* the induction was not absolute. Thus, either morphological form of *S. griseus* previously grown in absence of a chloramphenicol analogue as inducer still shows a measurable amount of CAT activity (Fig. 7 & Fig. 8). This activity may be attributed to a basal level of enzyme formation. This parallels the observation of OKAMOTO *et al.*[28] who studied the occurrence of chloramphenicol-acetylating enzymes in various Gram-negative bacilli. They observed that only the chloramphenicol-sensitive strains containing the enzyme could easily produce chloramphenicol-resistant strains with higher enzyme activity. An exceptional case was reported by RICHMOND *et al.*[29] for a type of β-lactamase in *Pseudomonas aeruginosa*. Without induction, no enzyme was detected in cultures whether they have been disrupted or not. They specified that the synthesis of this enzyme differs from other inducible β-lactamases since, elsewhere, induction always leads to an increase in a basal level of enzyme formation which is the case with CAT in our strain of *S. griseus*.

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