BIOTRANSFORMATION OF ANTIBIOTICS

I. ACYLATION OF CHLORAMPHENICOL BY SPORES OF *STREPTOMYCES GRISEUS* ISOLATED FROM THE EGYPTIAN SOIL*

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Incubation of spores, washed mycelium or whole cultures of a *Streptomyces* sp. with chloramphenicol (**I**) resulted in the loss of *in vitro* bioactivity of the antibiotic. Gas chromatographic estimation of an appropriate extract revealed that more than 95% of the antibiotic was inactivated under the specified conditions. The spores inactivated chloramphenicol in an inorganic buffer solution, or in distilled water, without the addition of carbohydrate or external co-factor. However, addition of certain carbon sources to the spores showed a pronounced effect on the chloramphenicol transformation process and on the relative concentration of the inactivated products. Time-course studies on the spore-catalyzed chloramphenicol transformation activity showed a maximum activity at 12-hour incubation. Addition of glucose or acetate at this point maintained maximum activity. The transformation products were identified as: chloramphenicol-1-acetate (**IIa**); chloramphenicol-3-acetate (**IIb**); chloramphenicol-3-propionate (**III**); chloramphenicol-3-isobutyrate (**IV**); chloramphenicol-3-butyrate (**V**); and chloramphenicol-3-isovalerate (**VI**), by techniques of TLC, CPC, GC, UV, IR, MS and NMR. The microbial characteristics of the isolated strain include the formation of flexuous gray aerial mycelium with smooth to rough spores, irregular in size. It is an H_2S and melanin former, non-chromogenic, and was inhibited by a streptomycin-producing strain of *Streptomyces griseus*. The organism was identified as a strain of subspecies of *Streptomyces griseus* (KRAINSKY 1914) WAKSMAN and HENRICI (1948).

Recent reports have described the presence of different antibiotic-inactivating enzymes in actinomycetes similar to those found in clinical isolates of antibiotic-resistant bacteria carrying R factors. BENVENISTE and DAVIES[1] reported the presence of an enzyme in *Streptomyces kanamyceticus* that acetylates kanamycin, gentamicin, and neomycin. The same authors also reported the acetylation of gentamicin by *Streptomyces spectabilis*. In addition, *Streptomyces coelicolor* contains enzymes that acetylate chloramphenicol, adenylate clindamycin, and phosphorylate lincomycin and clindamycin[2,3,4]. Furthermore, *Streptomyces bikiniensis* produces enzymes that phosphorylate streptomycin.[5]

As a part of our studies on the transformation activities of microbial spores[6,7,8,9] we have examined the potential activities of fungal and/or actinomycetes spores on the biomodification of antibiotics, and the possible relationship to antibiotic-resistant bacteria. The work presented here includes: inactivation of chloramphenicol by the spores of a *Streptomyces* sp. isolated from Egyptian soil; separation and identification of the chloramphenicol inactivated products. The latter was in agreement with the results obtained by ARGOUDELIS and COATS[2] using *Streptomyces*

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coelicolor. Time-course studies on the chloramphenicol transformation by the spores and the effect of different carbon sources were investigated. Finaly the isolated *Streptomyces* sp. was taxonomically classified.

Material and Methods

1. Microbial properties

(1) *Streptomyces* sp: The organism was isolated from a soil sample collected in 1972 at Aswan City in Egypt. During the isolation and purification procedures (Faculty of Pharmacy, University of Cairo, Cairo, Egypt) the strain was not in contact with any known antibiotic.

(2) Morphological observation: The isolated strain, incubated on glucose nutrient agar at 28°C for 14 days, was examined morphologically with an electron microscope (Phillips E.M. 300).

(3) Cultural characteristics: The strain was grown on a variety of standard media according to the description of SHIRLING and GOTTLIEB[10] and WAKSMAN.[11]

(4) Utilization of carbon sources: Utilization of carbon sources was investigated by the method of PRIDHAM and GOTTLIEB.[12]

2. Sporulation

The spores were produced on a medium consisting of corn meal agar with dextrose (Difco) 19 g, glycerol 5 ml, yeast extract 5 g, and tap water to 1 litre. Abundant sporulation was attained after incubation for 4~6 days at 28~30°C. Spores were harvested in 1 % phosphate buffer (pH 6.5) containing 0.005 % Tween 80 and spore suspension was filtered through sterile cheesecloth, washed with distilled water 5 times by centrifugation, resuspended in sterile 1 % phosphate buffer (pH 6.5) and stored at 4°C. Microscopical examination of the spores prepared by this method showed no mycelium fragments.[13]

3. Inactivation of chloramphenicol by spores, washed mycelium or whole-cell cultures of the isolated *Streptomyces* sp.

The spores, suspended in phosphate buffer were counted in a hemocytometer and suspension was diluted to give 5×10^8 spores per ml. Conversion were carried out in 50-ml Erlenmeyer flasks, each containing 10 ml of the spore suspension. Chloramphenicol solution (5 % in ethanol) was added to each flask to give a final concentration of 500 μg/ml of medium. The flasks were incubated on a rotary shaker (225 r.p.m.) at 30°C for 12 hours (unless otherwise specified).

Inactivation of chloramphenicol by growing cells of the isolated strain was carried out in a fermentation medium consisting of 1 % glucose, 2 % peptone, 1 % yeast extract, and 0.25 % calcium carbonate. A fresh stock culture was used to inoculate 50 ml of the sterile medium in 250-ml Erlenmeyer flasks; the inoculated flasks were incubated on a rotary shaker (225 r.p.m.) at 30°C for 48 hours. A 2 % inoculum from the resulting growth was used to inoculate a fresh medium, which was further incubated for 24 hours. A flask containing fresh medium was inoculated with a 2 % inoculum of the resulting growth and incubated for 10 hours; chloramphenicol was added (100 μg/ml of the medium), and the culture medium was further incubated for 12 hours. Alternatively, the cells after 10-hour incubation was collected by filtration through cheesecloth, resuspended in cold phosphate buffer (pH 6.5), washed twice with distilled water by centrifugation and finally the wet mycelia were suspended in the ratio 7 to 10 volumes of the same buffer. Chloramphenicol was added (500 μg/ml, (w/v) and the transformation process was carried out on a shaker at 30°C for 12 hours.

4. Separation and purification of the chloramphenicol inactivation products

At the end of the incubation period, the whole reaction mixture was centrifuged and the supernatant liquid was extracted twice with equal volume of ethyl acetate. The extract was dried over anhydrous sodium sulfate and concentrated under vacuum. The crude chloramphe-

nicol transformation products in the ethyl acetate extract, were separated by preparative T.L.C. on fluorescent silica gel G_{254} with acetic acid-chloroform mixture (1:4). For localization, the chromatoplates were examined under short uv 254 light. Alternatively, the inactivated products were separated from the residual chloramphenicol by partition column chromatography using $Celite_{545}$ and phosphate buffer (pH 6.0) as stationary phase. Upon elution with 25 % $CHCl_3$ in CCl_4, chloramphenicol was completely retained on the column, while the inactivated products were eluted. The inactivated products were tentatively purified by preparative T.L.C. Chloramphenicol could be eluted from the column with pure chloroform and directly determined at 278 nm in a spectrophotometer.

5. Gas chromatographic determination of chloramphenicol and its inactivated products

Qualitative and quantitative estimation of residual chloramphenicol and its transformation products in the crude ethyl acetate extract was carried out as follows: To 0.5 or 1.0 ml aliquots of the crude ethyl acetate extract, a measured amount of N-acetyl para nitrophenyl serinol (dechlorochloramphenicol as internal standard) was added to give a final concentration of 0.25 mg/ml. The solutions were evaporated to dryness in centrifuge tubes using vaccum desiccator or a stream of nitrogen, 0.2~0.5 ml TRI-SIL reagent (Pierce, Chem. Co; Illinois, U.S.A.) was added to each test tube, glass stoppered and stirred vigorously with a Vortex mixer. The uniform phase obtained was kept at room temperature for 5~10 minutes to allow complete derivatization of the products.[14] After brief centrifugation of the reaction mixture, an aliquot of the supernatant liquid was injected into the gas chromatograph equipped with a 2.5 % SE 30 column, a flame ionisation detector, and an electronic integrator. The relative concentrations of chloramphenicol and its transformation products were evaluated using the relative surface area as compared to a chloramphenicol calibration curve by the internal standard method (Fig. 4). For convenience the results were expressed as percentage of the starting chloramphenicol.

Results and Discussion

I. Taxonomical characteristics of the isolated *Streptomyces* sp.

(1) Morphological characteristics: As shown in Fig. 1 the aerial mycelium is open branched, showing flexuous sporophores. The chains contain an average of 14 spores. The spores are smooth to rough, usually cylindrical and irregular in size.

(2) Cultural characteristics: Cultural characteristics of the isolated strain on the media for taxonomical study are presented in Table 1. The vegetative mycelium is colorless to light yellow on most media.

Fig. 1. Scaning reflection electron micrograph of the isolate *Streptomyces* sp.

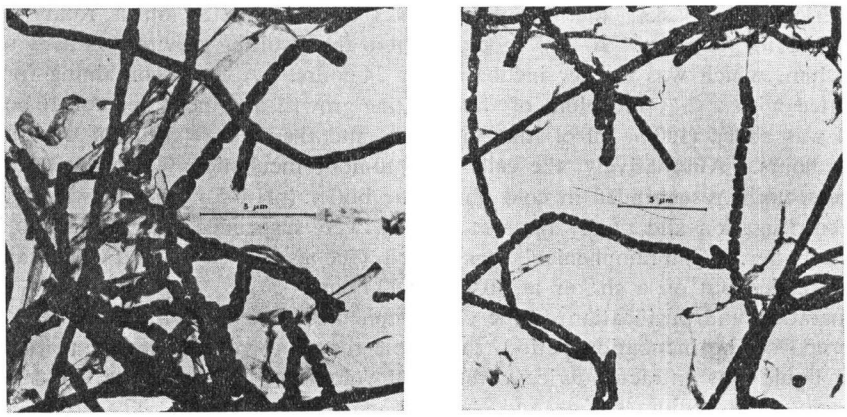


Table 1. Cultural characteristics of the isolated *Streptomyces* sp.

Culture medium	Vegetative mycelium	Aerial mycelium	Soluble pigment
CZAPEK's agar	G.* poor thin white	Whitish gray	Absent
Glucose-CZAPEK's	G. moderate white	Powdery gray	Absent
Glucose-asparagine-agar	G. colorless, slightly raised	Moderate gray	Absent
Glycerol-asparagine-agar	G. moderate, slightly raised	Powdery gray	Faint pink
Starch agar	G. colorless	Whitish gray	Absent
Nutrient agar	G. good, pale yellow	Good, silver gray	Brown
Nutrient broth	G. ring and descending	None	Light brown
Tyrosin agar	G. colorless to pale yellow	Olive green-gray	Brown black
Oat-meal agar	G. good, wrinkled, pale yellow	Abundant gray	Brown black
Corn-meal agar	G. good, smooth, colorless	Abundant gray	Brown black
Potato-malt agar	G. poor, smooth, amber	None	Absent
Peptone-iron agar	G. good, wrinkled, yellow	Poor, gray	Blue black
Egg (cultured at 37°C)	G. good, wrinkled, pale yellow	Moderate gray	Brown black
LOEFFLER's serum (cultured at 37°C)	G. poor, glistening, colorless	None	Slight black
Blood agar	G. good raised, haemolysis	Powdery	Blacking

*G: Growth

The aerial mycelium is white at first then becoming light gray to gray with time. When the strain is cultured on media containing L-tyrosine or peptone, the deep brown to black color of melanoid pigments was always observed. A faint pink diffusible pigment is sometimes observed on a few synthetic media. As there was no distinctive pigmentation on starch agar, glucose-asparagine agar, or glycerol-asparagine agar media, the isolated strain was considered to be non-chromogenic.

Table 2. Physiological properties of the isolated *Streptomyces* sp.

Temperature	growth occurs at 18~40°C
	better growth at 28~35°C
	no growth at 10°C and 50°C
pH range	growth occurs at pH 4~9
	no or poor growth at pH 4 and 10
	optimum range at pH 6~8
Oxygen requirement	no growth occurs under anaerobic condition
Starch hydrolysis	positive
Cellulose utilization	negative
Gelatin	positive
Tyrosinase reaction	positive
Litmus milk	peptonization with delayed coagulation
Blood haemolysis	positive
H_2S formation	positive
Chromogenicity	negative
Casein hydrolysis	positive
Nitrate reduction	positive

The physiological properties of the isolated strain are shown in Table 2. Hydrolysis of starch, peptonisation of milk, reduction of nitrate to nitrite, H_2S production, tyrosinase reaction, casein hydrolysis, and gelatin liquefaction were positive while chromogenicity and cellulose decomposition[15] were negative.

Various carbon sources such as starch, glycerol, D-sorbitol, D-mannitol, *i*-inositol, raffinose, sucrose, maltose, glucose, and sodium tartarate, were effective for growth. On the other hand, galactose, lactose, fructose, D-mannose, L-sorbose, D-xylose, L-arabinose, rhamnose, sodium acetate were either not or only slightly effective for growth (Table 3).

(3) Antimicrobial activity of the isolated strain

The organism, when cultured on glycerol-

Table 3. Utilization of carbon sources by the isolated *Streptomyces* sp.

Carbon sources	Growth	Carbon sources	Growth
D-Sorbitol	+++	D(+)Mannose	−
D-Mannitol	+++	(+)Rhammose	±
Starch	+++	Glycerol	+++
i-Inositol	++	Sucrose	+++
D-Xylose	±	Maltose	+++
(+)Arabinose	±	Glucose	+++
Galactose	−	L(−)Sorbose	−
Lactose	−	Sodium acetate	+
Fructose	−	Sodium tartrate	+++
Raffinose	+++	None (control)	−

+++: Abundant growth ++: Good growth
+: Moderate growth ±: Poor growth
−: No growth

Table 4. Rf values of the different antimicrobial agents produced by the isolated *Streptomyces* sp.; as observed under ultraviolet light; after development in the solvent system chloroform-acetic acid (24:1) on labeled Silica gel G_{254} thin-layer chromatography

Antimicrobial agent	Rf
A	0.0
B	0.15
C	0.21
D	0.44
E	0.56
F	0.88

lactate-serine medium[16,17] produced six components as separated by T. L. C. (Table 4). Preliminary investigations showed that these components are biologically active mainly against Gram-positive bacteria including spore-forming bacilli, but none of them showed activity against yeast. The mixture of these components gave an intense violet color in alkaline solution changing to yellow in acidic medium. They were not further investigated.

According to the above data T. G. PRIDHAM (personal communication) classified the isolated organism as a subspecies of *Streptomyces griseus* (KRAINSKY 1914) WAKSMAN and HENRICI (1948). He also suggested that the antibiotics produced by the strain might be of the rhodomycin type, the nogalamycin type, or other anthracycline antibiotics. The precise identification of each of the antibiotics produced by the strain would aid in determining whether it could be related to other anthracycline-type antibiotic producers, or would justify being named as a new subspecies.

A culture has been deposited in the Agricultural Research Service Culture Collection, (Northern Regional Research Laboratory) Peoria, Illinois, U.S.A. where it is registered as NRRL B-8078.

II. Inactivation of chloramphenicol by the spores, resting cells, and whole-cultures of *Streptomyces griseus*

After a 12-hour incubation period of chloramphenicol (500 μg/ml) with the spores of *Streptomyces griseus*, the ethyl acetate extract yielded seven distinct components (Fig. 2) as viewed under U. $V_{254\,nm}$ light after separation by T.L.C. The first spot (**I**) on the plate was chloramphenicol as compared to an authentic sample, and showed antibiotic activity. The transformation products **IIa**, **IIb**, **III**, **IV**, **V**, and **VI** showed no antibiotic activity. (Fig. 2).

Fig. 2. Typical thin-layer chromatogram of chloramphenicol transformation products by the spores of *Streptomyces griseus*

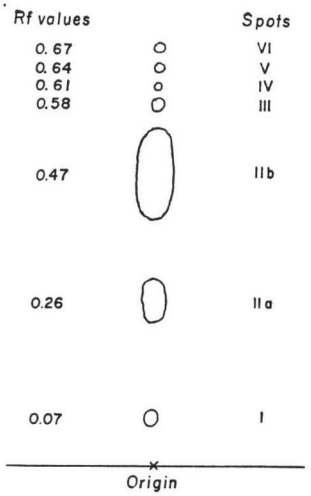


Fig. 4. Calibration curve of chloramphenicol using D, L-*erythro*-2-acetamido-1-(para-nitrophenyl)-1, 3-propanediol (dechlorochloramphenicol) as internal standard
Scm, surface area of chloramphenicol
Sis, surface area of the internal standard

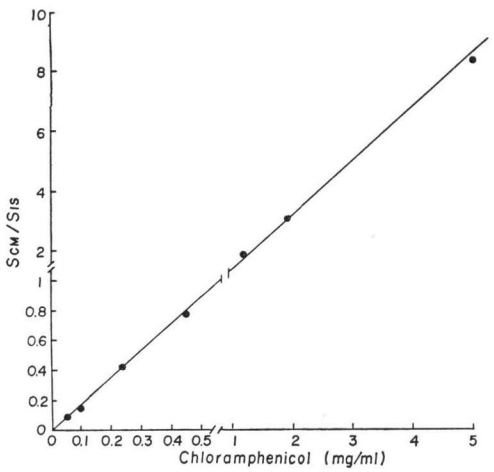
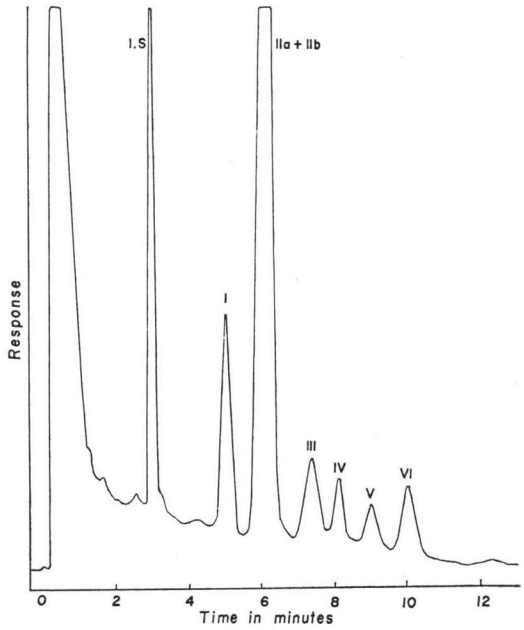


Fig. 3. Gas-chromatogram of the chloramphenicol transformation products by the spores of *Streptomyces griseus*
I.S. (internal standard) dechlorochloramphenicol; **I**, chloramphenicol; **IIa**, chloramphenicol-1-acetate; **IIb**, chloramphenicol-3-acetate; **III**, chloramphenicol-3-propionate; **IV**, chloramphenicol-3-isobutyrate; **V**, chloramphenicol-3-butyrate; **VI**, chloramphenicol-3-isovalerate



Gas chromatography of the ethyl acetate extract (Fig. 3) showed that component **IIb** with its isomer **IIa** in the same peak are the major transformation products. We observed that the relative concentration of the transformation products (**III** → **VI**) varied with different spore batches. This might be attributed to a variation of the content of internal reserved materials and co-factors in the spores.

Reports from our laboratory, as well as from others, have shown that the spores of certain micro-organisms sometimes exert more selective enzymatic activities as compared to corresponding growing cultures.[7,8,18] Consequently, an investigation on the ability of the whole cultures or the resting cells of the *Streptomyces griseus* to inactivate chloramphenicol was undertaken. The same pattern of inactivation products was obtained in both cases as compared to the transformation by the spores. Accordingly, the enzymatic inactivation of chloramphenicol by the spores of *S. griseus* appears to be an inherited character in this strain and not an acquired one associated with the sporulation process.

III. Separation and identification of the chloramphenicol inactivated products

The chloramphenicol inactivated products in the ethyl acetate extract of the spore reaction mixture were separated from residual chloramphenicol by partition column chromatography. Using this method residual chloramphenicol was completely separated from its inactivated products; we could not obtain the same separation with the reported selective solvent extraction.[19] The pooled inactivated products from the column were then separated by preparative T.L.C. It is interesting to note that the great advantage of spore-mediated reaction, as compared to that of the corresponding growing cultures, is the ease of recovery of the transformation products in the absence of the growth metabolites and the complex growing media.

During the purification of compound **IIb** (major chloramphenicol inactivated product) by T.L.C., the presence of compound **IIa** (about 5~20 %) could not be eliminated. Similarly compound **IIa** showed the same phenomenon in reverse. Two dimensional T.L.C. showed that there is no chemical decomposition during the development, since the second run gave only one corresponding spot for each compound. However, when the above plate was kept for a few days and redeveloped in the same solvent mixture, each individual spot gave two spots as observed during the purification process. We assumed that this phenomenon might be attributed to a non-enzymatic equilibrium between chloramphenicol-3-acetate and chloramphenicol-1-acetate.[19,20] To support this assumption, mild alkaline treatment of the inactivated compounds, as described by SHAW[21], yielded chloramphenicol. Using TLC, CPC, UV, IR, MS, and NMR, chloramphenicol and its transformation products were identified as: chloramphenicol (**I**); chloramphenicol-1-acetate (**IIa**); chloramphenicol-3-acetate (**IIb**); chloramphenicol-3-propionate (**III**); chloramphenicol-3-isobutyrate (**IV**); chloramphenicol-3-butyrate (**V**); and chloramphenicol-3-isovalerate (**VI**) (Fig. 5). Our data for these compounds were identical to those reported by ARGOUDELIS and COATS[2] using *S. coelicolor* MÜLLER.

IV. The influence of the incubation period on the conversion of chloramphenicol by the spores of the *S. griseus*

The time-course of chloramphenicol conversion by the spores is illustrated in Fig. 6.

Fig. 5. Transformation products of chloramphenicol (**1**) by *Streptomyces griseus*. **IIa**, chloramphenicol-1-acetate; **IIb**, chloramphenicol-3-acetae; **III**, chloramphenicol-3-propionate; **IV**, chloramphenicol-3-isobutyrate; **V**, chloramphenicol-3-butyrate; **VI**, chloramphenicol-3-isovalerate

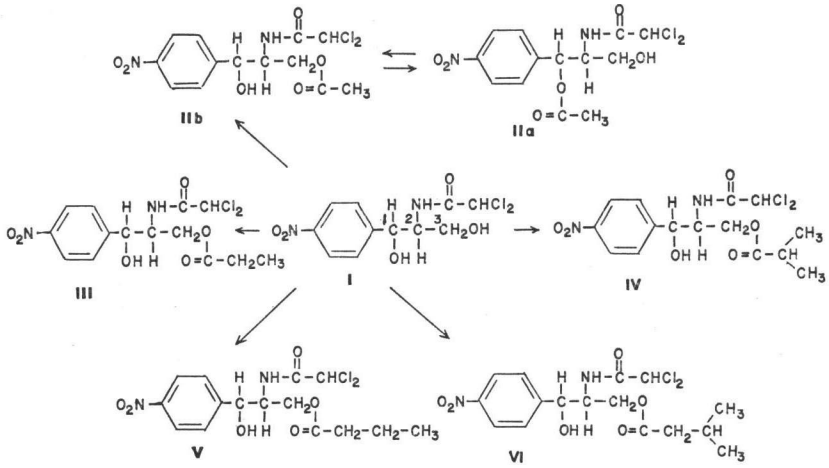
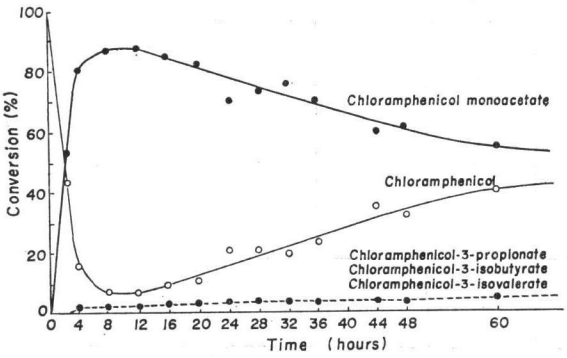


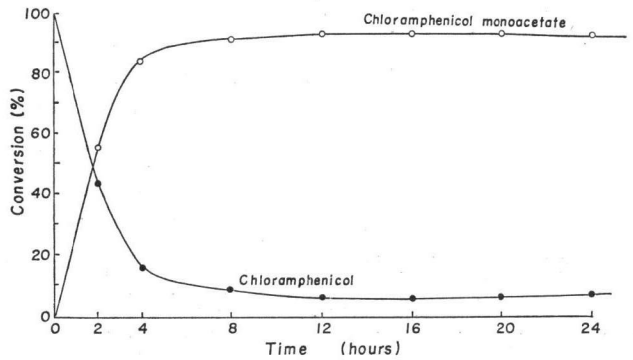
Fig. 6. Time course of the conversion of chloramphenicol by the spores of *Streptomyces griseus*. Spores 5×10^8/ml in 0.1 M phosphate buffer (pH 6.5); chloramphenicol, 0.5 mg/ml; incubation at 30°C;



Chloramphenicol (**I**) is rapidly transformed into its 3-acetoxy ester (**II**) reaching a maximum after 12-hour incubation. Other esters (**III** → **VI**) of chloramphenicol were formed non-sequentially and their sum never exceeded 5~10 % of the initial chloramphenicol. After 12-hour incubation chloramphenicol is slowly regenerated, at the expense of the 3-acetoxy ester. The other chloramphenicol esters remain constant or show a slight increase.

At that time (12 hours) the spores appear to have reached a depletion stage or a shortage of energy sources.[22] As a result, a preferential reversible reaction of the enzyme chloramphenicol-acetyltransferase (CAT) may take place to restore part of the spores energy requirement. If our assumption is correct, the addition of an energy source in the form of glucose or acetate would restore the chloramphenicol transformation activity in the spores. The results obtained from such additions are shown in Fig. 7. Both glucose and acetate were effective in maintaining the chloramphenicol conversion activity in the spores. The graph does not include the other chloramphenicol esters (**III** → **VI**) as they are involved in negligible amounts as side reactions in the chloramphenicol-3-acetate formation.

Fig. 7. Time course of the conversion of chloramphenicol by the spores of *Streptomyces griseus* in the presence of glucose or sodium acetate. Spores 5×10^8/ml in 0.1 M phosphate buffer (pH 6.5); chloramphenicol, 0.5 mg/ml; glucose, 2 mg/ml; incubation at 30°C



V. Specificity of the chloramphenicol transformation reaction by the spores of *S. griseus*

SHAW *et al.*[23] and others[19,24] demonstrated that the enzyme chloramphenicol acetyltransferase (CAT) from multiple-drug resistant bacteria carrying R-factors, requires ATP, Mg^{++}, co-enzyme A and actate or acetyl co-enzyme A to inactivate chloramphenicol. In addition, SHAW *et al.*[23] proved that the enzyme CAT could also transfer the propionyl or butyryl group to chloramphenicol. In our investigation, the spores of the *Streptomyces griseus* strain acetylate

Table 5. Effect of external carbon sources additives on the conversion of chloramphenicol by the spores of *Streptomyces griseus* and on the specificity of the reaction

Added carbon sources 0.03 M	Conversion (%)						
	I	II	III	IV	V	VI	VII
None (control)	28	66	3	1	0	2	0
Glucose	2	95	2	0	0	1	0
Acetate	1	91	8	0	0	0	0
Propionate	1	8	91	0	0	0	0
Butyrate	73	24	1	0	2	0	0
Isobutyrate	71	25	2	1	0.5	0.5	0
Valerate*	71	16	5	0	1	4	3
Relative retention time to internal standard	1.66	2.00	2.40	2.63	2.92	3.27	3.64

I. chloramphenicol; **II.** chloramphenicol-3-acetate; **III.** chloramphenicol-3-propionate; **IV.** chloramphenicol-3-isobutyrate; **V.** chloramphenicol-3-butyrate; **VI.** chloramphenicol-3-isovalerate; **VII.** chloramphenicol-3-valerate

* In the absence of sodium valerate chloramphenicol-3-valerate (**VII**) could not be detected.

chloramphenicol in 1 % phosphate buffer or even in distilled water. These results suggest that the acetyl group either exists in the internal metabolic pool of the spores in a pre-active form or that the enzymatic machinery of the spores is capable of activating the reserved materials to acetyl co-enzyme A or both.[22] Consequently, experiments were designed to test the specificity of the enzyme CAT in the spores and its ability to transfer other acyl groups to chloramphenicol in quantitative terms. Table 5 summarized these experiments in which a submaximal concentration of the spores (3×10^8/ml) was used to assess the influence of the additives. Glucose or the sodium salt of the fatty acids was added to the chloramphenicol-spore reaction mixture before the usual incubation. Glucose showed a marked increase in the spores-chloramphenicol conversion activity as did the acetate. The table also showed that although, in the presence of propionate, the enzyme CAT in the spores inactivated chloramphenicol almost quantitatively (99 % conversion), chloramphenicol-3-propionate (**III**) was the main converted product (91 %), with little (8 %) chloramphenicol-3-acetate (**II**) being formed. These results were not surprising, since the enzyme CAT preparation obtained from *E. coli* carrying an R-factor inactivated chloramphenicol in the presence of propionyl co-enzyme A with a yield of 94 % as compared to the acetyl co-enzyme A inactivation.[23] Conversely, the overall spores-chloramphenicol transformation activity was significantly inhibited in the presence of butyrate, isobutyrate, or valerate. Our results suggest competitive inhibition of the enzyme CAT, rather than a toxic effect on viability or other biochemical activities of the spores, since addition of acetate or glucose restores completely the chloramphenicol acetyltransferase activity in the spores. However, these results did not exclude the indirect CAT enzyme inhibition due to the possible interference with other enzyme(s) involved in the generation and/or the availability of active acyl groups. It may be noted, that the enzyme CAT from *Staphylococcus aureus*, *Staphylococcus epidermidis*, or enteric bacteria shared the same preference for short-chain acyl radicals.[21,23] Other properties of the enzyme CAT in *Streptomyces griseus*

NRRL B-8078 are being investigated and will be described elsewhere.

It is also interesting to note that although the inactivation patterns of chloramphenicol by our strain of *Streptomyces griseus* (NRRL B-8078) and by *Streptomyces coelicolor* MÜLLER (NRRL 3532)[2] appear to be similar, during our preliminary screening investigation we found that *Streptomyces coelicolor* (ACTC 19832) and some other strains of *Streptomyces griseus* did not inactivate chloramphenicol. In addition, other *Streptomyces* species have been reported to inactivate chloramphenicol by completely different mechanisms.[16,17,25] Obviously, there is a remarkable strain specificity in the biochemical behavior of these organisms.

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