MICROBIAL ACETYLATION OF M FACTOR OF VIRGINIAMYCIN

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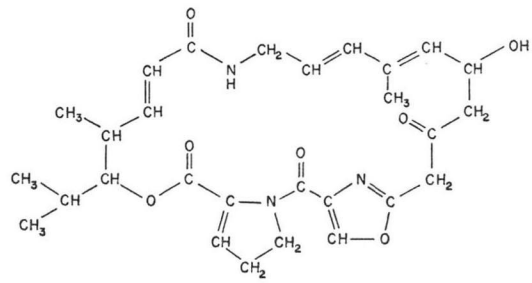
The M component of virginiamycin was found to be modified by whole cells or cell-free enzyme preparations of a *Staphylococcus aureus* strain. It was shown that this reaction proceeds by enzymatic acetylation of the secondary alcoholic function of the molecule, followed by a rapid chemical degradation of the O-acetylated product.

Virginiamycin is a mixture of two antibiotic substances, the M and S factors, which are strongly synergistic both *in vivo* and *in vitro*[1]. This marked synergism is a property common to all antibiotics of the streptogramin group, together with a very low level of bacterial resistance[2]. Their structure and nomenclature have been reviewed[3] (Fig. 1). As previously reported[4], an M factor transformation has been observed in broth seeded with an *S. aureus* strain, particularly resistant to the M component of virginiamycin. Five reaction products have been detected after thin-layer chromatography by a fluorescence quenching method.

Fig. 1. Structure of M factor of virginiamycin.



In this same work, a close relationship between M factor disappearance and its transformation into several reaction products has been firmly established using tritium-labeled M factor; the transformation was shown to proceed according to first order kinetics. The major reaction product, called P_1, was found to be rather unstable and gradually transformed to the other reaction products. No S factor inactivation or transformation could be detected under similar conditions[4].

This paper provides additional experimental evidence for a microbial inactivation of the M factor of virginiamycin into an O-acetyl derivative, identified as the reaction product P_1. It describes under which conditions the transformation best occurs and explains how further chemical modification leads to the other observed products.

Materials and Methods

Bacterial strains:

S. aureus ATCC 6538P, sensitive to 0.35 mcg/ml virginiamycin, was used for assaying the biological activity of this antibiotic.

S. aureus 71, a coagulase-negative isolate showing an unusually high degree of resistance toward the M component of virginiamycin, was exclusively used for the transformation experiments. This particular strain was isolated in the virginiamycin production plant from a workman in continuous contact with this antibiotic. Although resistant to concentrations of M factor higher than 100 mcg/ml and to concentrations of S factor higher than 200 mcg/ml, this strain remained relatively sensitive to

the combination of both the M and S components of virginiamycin, exhibiting a MIC value of 12.5 mcg/ml for virginiamycin.

Chemicals:

Pure isopropanol-crystallized M factor was received from R.I.T. Laboratories (Genval, Belgium). Stock solutions at 10 mg/ml were prepared in pure ethanol, kept at 4°C and discarded after 8 days. Sodium acetate 1-^{14}C (49.5 mCi per mmole) was purchased from The Radiochemical Center, Amersham, England.

Lysostaphin (205 U/mg) was obtained from Swarz-Mann, New York.

Acetyl coenzyme A and DNase (1.000 U/mg) were purchased from Boehringer - Mannheim.

Experimental:

Preparation of the cell suspension: Strains were maintained on slants of Antibiotic medium #1 Difco (PSA). The cell suspensions used for the M factor transformation experiments were prepared by inoculating 200 ml Tryptic soy broth, Difco (TSB) in a 500-ml conical flask, with a loopfull from a slant culture and incubating at 37°C, on a rotary shaker (90 rpm), to late log phase. The cells were collected after 5-minute centrifugation at $1,500 \times g$ and suspended again in broth of the same composition, or in a synthetic medium as given in the text.

Transformation by intact cell suspension: The initial cell number was adjusted with broth to the desired turbidity (A 522 nm). The cell suspension (100 ml) was introduced in a small glass reactor, equipped with pH electrodes and a magnetic stirrer. The pH value was adjusted with 6 N HCl and an adequate amount of the ethanolic M factor solution was added.

Temperature and pH were kept constant during the reaction by means of water bath and automatic titrator (Radiometer ABU 1b) supplied with 0.02 N NaOH.

Most experiments were performed in TSB broth, at 37°C and pH 5.0, supplemented with M factor to an initial concentration of 100 mcg/ml and containing 6×10^7 viable cells per ml, corresponding to a culture absorbance at 522 nm of 0.54. Samples were taken at regular intervals for controling turbidity at 522 nm and residual M factor content during a 6-hour period.

Transformation by cell-free extracts: Cell-free extracts were obtained according to SHAW and BRODSKY[5]. The pellet from centrifugation of a 100-ml 18-hour culture was washed with 50 ml of 0.05 M tris - 0.15 M NaCl buffer, pH 7.2 and resuspended in 10 ml of the same buffered saline solution. Lysostaphin was added to a final concentration of 10 U/ml and the suspension incubated for 15 minutes at 37°C. DNase was then added (50 U/ml) and the suspension was further incubated for another 15 minutes at 37°C. The supernatant obtained by centrifuging at $120,000 \times g$ for 15 minutes constituted the cell-free enzymatic preparation; it was adjusted to pH 5.0 and supplemented with glucose (to 100 mcg/ml) for the transformation experiments.

Analytical:

Chromatographic identification: The residual M factor together with the reaction products were easily extracted from the broth by chloroform (v/v). The solvent phase was evaporated to dryness *in vacuo*. The dry residue was dissolved in 1 ml chloroform and spotted on a thin-layer silica gel-coated plastic sheet, with fluorescence indicator (Schleicher and Schüll F 1500 LS 254, W. Germany). Development was carried out with chloroform - methanol (95: 5) for about 45 minutes. In addition to their UV absorbance at 254 nm, M factor and several reaction products could be detected as well-defined pink-coloured spots, after spraying with acidic EHRLICH reagent and heating the plates at 100°C for 5 minutes[6]. For bidimensional chromatography, the same thin-layer sheets were first developed with pure methyl-isobutyl ketone and thereafter with a chloroform - methanol mixture (95: 5).

This system was used for a comparative mapping of the microbial transformation products with those obtained after acid or basic hydrolysis of M factor, as described by ROLLMANN and RONDELET[7,8]. It was also useful for checking the purity of isolated products.

Radiochromatography: The radioactivity of the labeled substance was scanned on the thin-layer chromatogram, with a windowless gas flow scanner (Packard - Model 7.201). For more accurate recording of the radioactivity, the surface corresponding to the P_1 spot was scraped, eluted into a

chloroform - methanol mixture (50: 50) and counted in a liquid scintillation counter (Packard Model 3.380) with BRAY's naphtalene-dioxane counting fluid.

Fluorescence quenching assay method for M factor: The M factor concentration was assayed by a fluorescence quenching method developed by ROLLMANN[9] and adapted to thin-layer chromatography. According to this method, the 254 nm absorbance area at Rf 0.32, was recorded and the concentration was calculated from a standard curve established under the same operative conditions from reference solutions of M factor.

Microbiological assay method for M factor: The residual biological activity was assayed by a cylinder plate agar diffusion method, according to GROVE and RANDALL[10]. The cups contained the properly diluted samples, supplemented with S factor at a concentration roughly corresponding to half the estimated M factor content. *Staphylococcus aureus* ATCC 6538 P was used as the test organism with PSA broth.

Extraction and purification of the P_1 substance: A 1-liter, 18-hour culture in TSB at 37°C, of *Staphylococcus aureus* 71, was adjusted to pH 5.0 with 6 N HCl, supplemented with M factor(200 mcg/ml) and further incubated on a rotary shaker for 60 minutes. The fermentation broth was then twice extracted with CCl_4 and the solvent phase evaporated to dryness *in vacuo*. The dry residue which contained about 75% P_1 substance, was dissolved in 5 ml chloroform and chromatographed on a silica gel (Kieselgel 60, Merck, W. Germany) column (4×10 cm) equilibrated with chloroform. A chloroform - methanol mixture (98: 2) was used as eluting solvent. The fractions were checked for P_1 substance by fluorescence quenching. The positive fractions were collected, evaporated to dryness and dissolved again in 15 ml acetone. Distilled water (85 ml) was slowly added to the acetonic solution and P_1 substance crystallized after an 8-hour standing period at 4°C, as fine white needles.

The crystals were removed by filtration, washed with distilled water and twice recrystallized from identical solvent mixtures. The final P_1 substance yield reached a weight value of 47% of the initial M factor concentration. The purity was controlled by bidimensional thin-layer chromatography.

Purification of M factor: M Factor was further purified according to the same column chromatographic procedure and recovered after crystallization from isopropyl alcohol. Its purity was controlled by bidimensional thin-layer chromatography.

Spectral methods: UV spectra were recorded with a Beckman spectrophotometer (Model 25); IR spectra were taken on a Unicam SP 1,000 infrared spectrophotometer and NMR spectra were measured with a 300 MHz Varian apparatus with TMS as internal standard. Mass spectra were determined on a LKB 9,000S mass spectrometer with 20 eV ionising energy and a source pressure of 10^{-7} mm/Hg. ^{13}C Spectra were recorded on a XL-100 Varian apparatus in 10 mm outside diameter tubes.

Results

M Factor Transformation into P_1 Substance

The microbial transformation of M factor into several unidentified reaction products by a suspension of *S. aureus* 71, has been described elsewhere[4]. Five new UV-absorbing spots, called P_1 to P_5, could be detected in the chloroform extract by chromatography, simultaneously with M factor disappearance.

The close similarity between the M factor concentration decrease, as shown by fluorescence quenching, and its inactivation, as shown by the microbiological assay, suggest that this M factor transformation contributes to the inactivation of the antibiotic (Fig. 2).

The effect of pH on the M factor microbial transformation has been studied over the range of pH 4.0~7.0, by maintaining the cell suspension at a constant pH value and assaying for residual M factor, after 3-hour incubation time. A typical pH - reaction rate dependance has been observed, with a maximum M factor transformation in the pH range of 5.0~5.5 (Fig. 3). Above pH 7.0, the M factor becomes sensitive to alkaline degradation[8].

Fig. 2. Relationship between M factor content and biological activity of the chloroform extract.

M factor (100 mcg/ml) was modified by a late log phase TSB culture of *Staphylococcus aureus* 71, containing 6×10^7 initial viable cells per ml, at 37 °C and pH 5.0. Residual M factor content of the chloroform extracts was followed by fluorescence quenching after thin-layer chromatography and biological activity was determined with S factor supplement, using *S. aureus* ATCC 6538P as test organism.

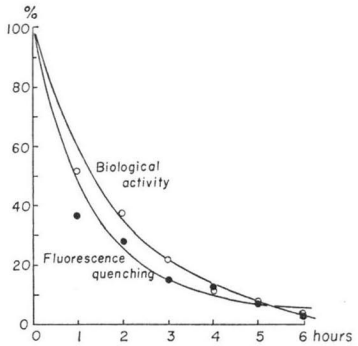


Fig. 3. Effect of pH on M factor modification.

The percent M factor modified was determined after 3-hour incubation in TSB broth at 37°C, with 6×10^7 initial cell number and 100 mcg/ml initial M factor concentration.

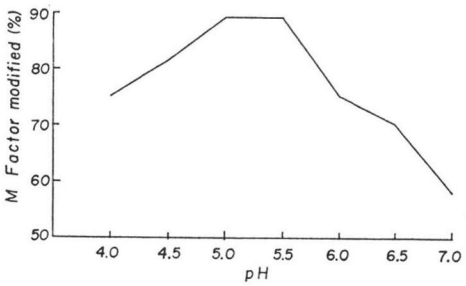
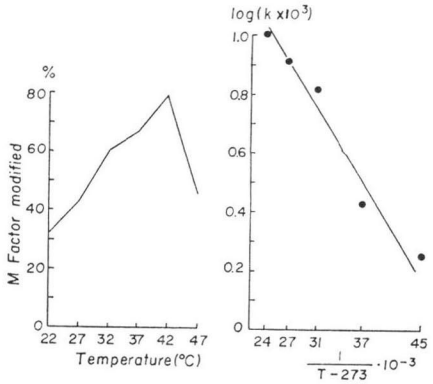


Fig. 4. Effect of temperature on M factor modification.

M factor modification was determined after 4-hour incubation in TSB broth at pH 5.0, with 6×10^7 initial cell number and 100 mcg/ml initial M factor concentration. The ARRHENIUS plot was calculated from the disappearance rate data (k).



Two groups of reaction products could be observed, according to the pH of the cell suspension: at pH 5.0 or lower the so-called P_1 compound was the most abundant derivative; whereas at neutral pH, the so-called P_2 compound was predominant.

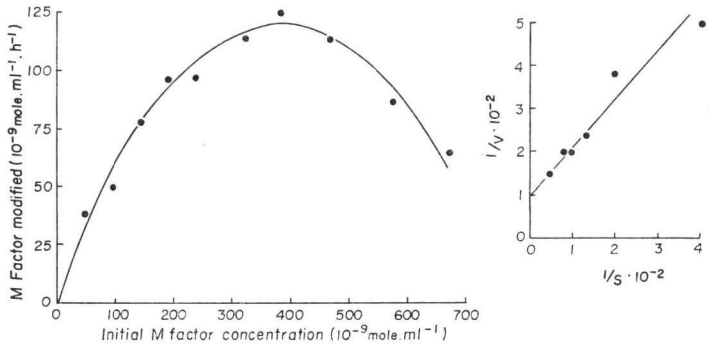
This work was more particularly concerned with the reaction occurring under acidic conditions leading to the accumulation of P_1.

The maximum reaction rate, at pH 5.0, occurred at 42°C (Fig. 4). The activation energy for M factor disappearance was calculated from an ARRHENIUS plot of these data as 15.9 k cal/mole. Heating the cell suspension at 60°C for 10 minutes suppressed all transformation activity. Growing cells collected after 12- or 18-hour incubation, had the same efficiency, while late stationary cells, obtained from a 24-hour culture, lost more than 75% of their initial inactivating capacity.

A typical substrate reaction velocity relationship according to the MICHAELIS-MENTEN formulation was observed for M factor concentrations varying from 5×10^{-8} to 4×10^{-7} mole/ml (Fig. 5). Beyond this level, the M factor solubility was exceeded and the results were not reliable. The MICHAELIS constant (Km) calculated from the LINEWEAVER-BURK plot was 1.7×10^{-4} M, with a V_{max} value of 1.6×10^{-7} mole/ml/hour.

A similar reaction has been observed in synthetic media containing glucose. A 0.03 M glucose solution containing 0.3 M NaCl was particularly effective: 46% of the M factor was converted into P_1, after 5-hour incubation. A significant improvement was obtained when supplementing this formulation with NaOAc (0.01 M): 69% P_1 formation, suggesting that this product might proceed by enzymatic

Fig. 5. Effect of substrate concentration on initial M factor modification rate.
The amount of M factor modified was estimated after 1-hour incubation in TSB broth at 37°C and pH 5.0, with 6×10^7 initial cell number. The LINEWEAVER-BURK plot of the data is shown at the right.

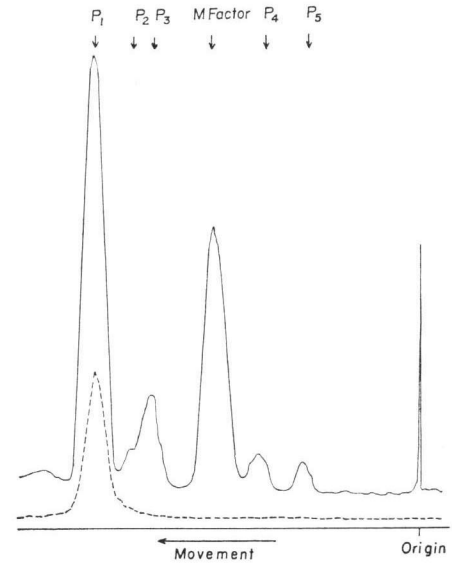


acetylation of the M factor: In order to test the acetylation hypothesis, Na acetate-1-^{14}C was added to the glucose-NaCl suspension: the thin-layer chromatographic separation displayed a radioactivity profile located only in P_1; this scanning was in perfect agreement with the fluorescence quenching recording (Fig. 6). Nevertheless the incorporation of this labeled molecule into P_1 did not exceed 2.2% of the total radioactivity, while 45% of the initial M factor content had been converted into P_1 substance.

Lysis of the staphylococcal cells with lysostaphin caused a dramatic loss of transformation power. This could be prevented by adding sucrose (0.5 M final) to the cell suspension, prior to lysis. The extracts from lysostaphin-treated cells were thus prepared from a more important cell number and concentrated in a small volume, because to poor activity was recovered in the experiments with acetyl coenzyme A when extracts were prepared from the usual cell concentration (6×10^7 viable cells per ml). The experiments performed on cell-free extracts, prepared in saline-glucose medium, showed that acetyl coenzyme A (100 mcg/ml) was essential for P_1 formation: 64% of the initial M factor content was converted into P_1, after 1-hour incubation while no acetylation was observed in media lacking that coenzyme (Fig. 7). This also strongly supported the view of an acetylation process.

The P_1 substance has been extracted from the reaction mixture and purified according to the procedure given under "Materials and Methods". The identification of this substance as an O-acetyl derivative of M factor, was convincing

Fig. 6. Labeled Na acetate (1-^{14}C) incorporation during M factor transformation.
M factor (100 mcg/ml) was incorporated in a 0.03 M glucose solution containing 0.3 M NaCl and 0.01 M Na acetate with 0.5 mc mole labeled Na acetate (25 mCi) in a total volume of 100 ml, seeded with 6×10^7 viable cells and incubated at 37°C, pH 5.0 for 3 hours. The solid line shows the absorbance at 254 nm, as recorded by the fluorescence quenching method, after thin-layer chromatography. The dotted line displays the radioactivity profile of the same chromatogram.



proof that the microbial M factor inactivation, observed under acidic conditions, proceeds by O-acetylation.

Identification of the P_1 Substance

The comparative melting points (uncorrected) were: 197~200°C (M factor) and 126~129 °C (P_1).

$[\alpha]_D^{20}$ --198° (M factor) and −231° (P_1)

(*c* 0.2, 95% ethanol).

The UV absorption spectra in 95% ethanol showed no changes in the λ_{max} value (227 nm) but only in the extinction coefficients:

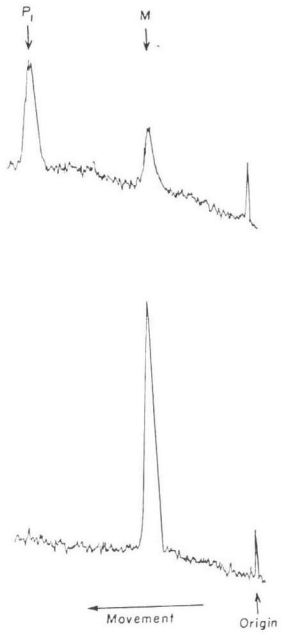
$E_{1cm}^{1\%}$A(M)=610 and $E_{1cm}^{1\%}$A(P_1)=540.

The bands in the infrared (50 mg in 2 ml chloroform) were roughly at the same values, except a new one for P_1 at 1250 cm^{-1}, typical for acetic esters. The mass spectrometry of M factor showed a "molecular ion" at *m/e* 525 (0.4 % of base peak)[11] with a more intense peak at *m/e* 507. With P_1, the peak at highest mass occurred at *m/e* 567 (0.5% of base peak). This increase of 42 mass units supports the view of an acetylation of the secondary alcoholic function of M factor. The *m/e* 507 peak reached a value of 26% of the base peak, suggesting acetic acid release during analysis.

The NMR spectra contained an additional signal for P_1 at δ 1.97 ppm, as singlet accounting for 3 hydrogens. One additional proof for the acetylation hypothesis was the shift from δ 4.7 ppm to δ 5.8 ppm for the hydrogen atom adjacent to the esterified function (Fig. 8).

Fig. 7. Effect of acetyl coenzyme A on P_1 formation by cell-free extracts.

The pellet from centrifugation of a 100-ml 18-hour culture was washed with 50 ml 0.05 M Tris, 0.15 M NaCl buffer, pH 7.2 and resuspended in 10 ml of the same buffered saline solution. Lysostaphin was added to a final concentration of 10 U/ml and the suspension incubated for 15 minutes at 37 °C. DNase was then added (50 U/ml) and the suspension was further incubated for another 15 minutes at 37°C. The cell-free preparation obtained by centrifuging at 120,000×*g* for 15 minutes was adjusted to pH 5.0, supplemented with glucose (to 0.03 M) and M factor (to 100 mcg/ml) and divided in two parts. To one preparation 500 mcg acetyl coenzyme A was added. The fluorescence quenching method after thin-layer chromatography shows the profile when the mixtures are incubated for 1 hour at 37°C, with (upper line) and without (under line) acetyl coenzyme A.



In the ^{13}C spectra of P_1 and M factor, two further peaks appeared for the acetylated product at 21.14 ppm (CH_3) and 171 ppm (C=O).

Acidic and Basic Degradation of the P_1 Compound

Chromatographic spots similar to the P_3, P_4 and P_5 compounds were detected in the acid hydrolysate of P_1 substance. The acid hydrolysis of the M factor also gives reaction products of similar characteristics: their Rf, EHRLICH staining and UV spectra were found very similar to those of the P_3~P_4 and P_5 compounds.

The P_1 compound was easily coloured yellow by adding NaOH to its ethanolic solution; while the same treatment was much less effective with the M factor. The chromatographic analysis of the

Fig. 8. Nuclear magnetic resonance spectra of M factor (A) and its O-acetyl derivative (B).

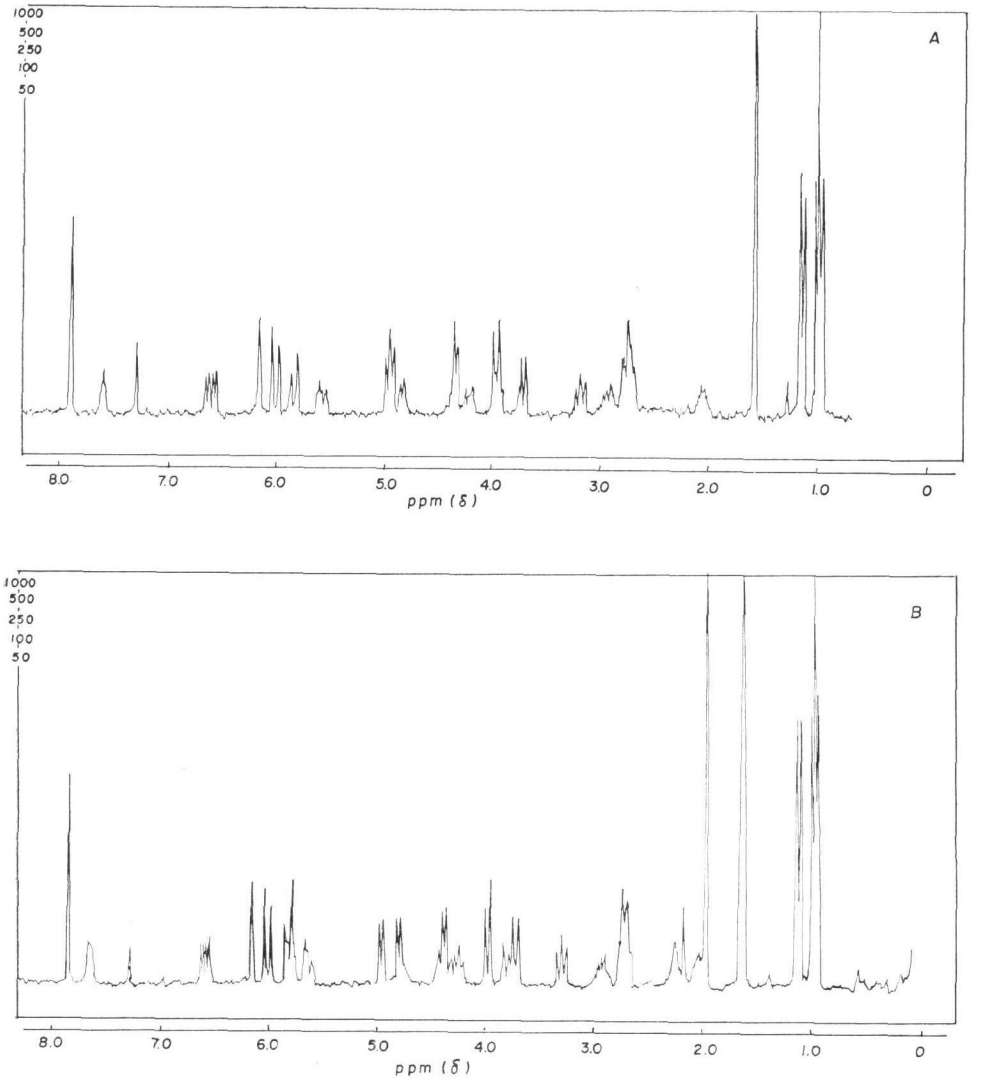


Table 1. Effect of pH on the comparative stability of M factor and P_1 product.
M factor and P_1 product (100 mcg/ml) were incubated in sterile TSB for 6 hours at 37°C and pH 5.0. Disappearance rates were calculated from the linear regression data. Transformation products were observed after UV exposure (254 nm) of the chromatograms.

		pH 4.0		pH 5.0		pH 6.0		pH 7.0	
		M	P_1	M	P_1	M	P_1	M	P_1
Transformation products observed	Disappearance rate $\times 10^{-4}$ (min^{-1})	1	15	0	8	3	11	1	32
	P_1	−	+	−	+	−	+	−	+
	P_2	−	+	−	+	+	+	+	+
	P_3	−	+	−	+	−	+	−	−
	M	+	+	+	+	+	+	+	−
	P_4	−	+	−	+	−	+	−	−
	P_5	−	+	−	+	−	+	−	−

NaOH treated P_1 allowed the detection of a main reaction product with the same Rf value, UV and staining characteristics as the product P_2. Similarly, the properties of P_2 were also found to be identical to those of the major product of alkaline degradation of the M factor: the substance D_1 previously reported[8].

The rates of P_1 disappearance were in every case much higher than for the M factor, indicating a higher chemical instability for this acetylated derivative.

Some degree of deacetylation with production of M factor was also observed in sterile broth at acidic or neutral pH values. Nevertheless, this nonenzymatic deacetylation, which was confirmed by both chromatographic and biological assay methods, did not exceed 10% of the initial P_1 concentration, even under the most favourable conditions (3 hours at pH 5.0)

Discussion

The M factor of virginiamycin was shown to be rapidly inactivated in the presence of cells of a particular strain of *Staphylococcus aureus* resistant toward this antibiotic component. The main reaction product, described as the P_1 substance, has been isolated and analysed using the usual physicochemical methods.

Decisive arguments for a M factor acetylation process were obtained from experiments with ^{14}C-labelled acetate, while mass spectrometry and ^{13}C-NMR spectroscopy data for the P_1 compound clearly corresponded to an O-acetyl derivative of the M factor. Moreover, it was possible to locate the site of the reaction on the secondary alcoholic function of the molecule, by interpretation of the ^1H-NMR spectroscopic data.

The stability of the P_1 substance is much lower than that of the M factor and it was demonstrated that its rapid disappearance from the medium was due to pH-dependant chemical reactions leading to the production of the other described reaction products: the P_2 to P_5 compounds.

No structural information could be obtained for the chemical modification products of the O-acetyl derivative: all attempts to purify these products failed.

Pure P_1 substance in acidic or alkaline aqueous solutions undergoes a rapid transformation into several distinct substances showing identical chromatographic properties to the P_2 or P_3 to P_5 compounds. It has been reported earlier that the M factor is similarly hydrolysed, but at a lower rate, to give similar reaction products.

It seems reasonable to propose the following sequence of events:

$$\left[\text{M factor}\right] \xrightarrow[\text{transferase}]{\text{O-acetyl-}} \left[P_1\right] \begin{cases} \xrightarrow{H_3O\oplus} P_3 + P_4 + P_5 \\ \xrightarrow[OH\ominus]{} P_2 \end{cases}$$

The microbial acetylation of the M factor was shown to proceed according to MICHAELIS-MENTEN kinetics. The amount of M factor inactivated after 3 hours' incubation time, was proportional to the initial cell number in the broth suspension according to the equation:

$$\log N_0 = 0.012\,[\%\text{ M factor inactivated}] + 7.1$$

The linear regression data were put into the formula $y = A + BX$ where B is the slope of the line and A the intercept. The O-acetyltransferase involved in this reaction seems at least partly constitutive since its activity was detected independently of any cell preincubation with the M factor and without any significant lag phase. The experiments performed on the cell-free system demonstrated that the acetylating enzyme can be solubilized, at least in part and that acetyl CoA is a necessary co-factor. No investigations were further done in order to detect the physical state of the acetylating enzyme. The modification of antibiotic substances by acetylating enzymes is a very common inactivating mechanism since it has been reported for numerous unrelated antibiotics including aminoglycosides, macrolides,

chloramphenicol and cycloheximide[12]. As reported with chloramphenicol[13], a higher level of acetylating activity was found when the cells were previously grown in presence of M factor. The chloramphenicol O-acetyltransferase of *Staphylococcus aureus,* which has been most extensively investigated, presents several properties common to the enzyme here studied. It was not found in sensitive strains nor in *in vitro* induced resistant isolates. Nevertheless this enzyme differs in its inductive character and its substrate specificity. Attempts to inactivate chloramphenicol with *Staphylococcus aureus* 71 were unsuccessful. This strain exhibited an exceptional property in its unusually high degree of M factor resistance. All attempts to find other strains naturally resistant to M factor or to virginiamycin failed; no one showed the acetylating activity of strain 71[14]. Owing to the rarity of this kind of antibiotic resistance, it was impossible to evaluate the significance of this inactivation reaction in spite of an extensive screening on several hundreds of clinical and animal isolates of pathogenic *Staphylococcus aureus* strains.

Acknowledgements

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