METABOLIC PRODUCTS OF MICROORGANISMS 137*

RINAMYCIN, A NEW INHIBITOR OF RNA SYNTHESIS

K. UCHIDA and H. ZÄHNER

Institut für Biologie II, Lehrstuhl Mikrobiologie I,
Universität Tübingen, D-74 Tübingen,
Auf der Morgenstelle 28, Germany

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A new antibiotic rinamycin was isolated from the culture mycelium of a streptomycete designated as *Streptomyces venezuelae* Tü 1102. The antibiotic was obtained as a honey-coloured powder having the tentative molecular formula $C_{20}H_{55}NO_8$. It exhibits a significant inhibition of fungi, yeasts, gram-positive and some gram-negative bacteria. The primary site of action of the antibiotic was suggested to be the RNA metabolism of rinamycin-sensitive cells.

In our course of screening for new antibiotics, we found a new substance named rinamycin, produced by the strain Tü 1102 which was isolated from a soil sample collected in India and identified to be *Streptomyces venezuelae*.

The antibiotic possesses an ultraviolet spectrum similar to musarin,[1] antiprozoin,[2] hygrostatin[3] and azalomycin F,[4] and somewhat resembles those antibiotics in some other physico-chemical properties. However it could be differentiated from the former two by its chemical and biological characteristics and from the latter two by its infrared spectrum and paper-chromatographic and electrophoretic behaviour. In liquid media, rinamycin inhibits the growth of fungi, gram-positive and some gram-negative becteria. The inhibition is weak by the agar diffusion method, apparently due to the poor diffusibility of the antibiotic.

Studies on the influence of rinamycin on macromolecular synthesis *in vivo* using *Bacillus subtilis* indicated that RNA synthesis is inhibited prior to DNA and protein syntheses.

In this paper the production, isolation, physico-chemical and biological properties of rinamycin as well as its effect on macromolecular synthesis of *Bacillus subtilis* are described.

General Methods

Paper disc (6-mm diameter) assays were used to determine the antibiotic activity. Sterilization where needed was carried out for 20 minutes at 120°C unless otherwise mentioned. All evaporations were conducted *in vacuo* below 40°C. Counter-current distribution was performed under following conditions; Volume of upper and lower phases, 50 ml each; reciprocal shakings, 30 times; separation time, 15 minutes. Silica gel used was DC-Alufolien Kieselgel (Merck, Darmstadt) for qualitative test and Kieselgel 60, 70~230 mesh (Merck) for column preparation. Paper chromatography was done on Whatman No. 1 by the ascending method.

An ultraviolet absorptiometer (LKB-Uvicord) was used to detect the antibiotic in the effluent of a column of Sephadex LH 20. Liquid culture for minimal inhibitory concentration and radioactive incorporation was carried out in a Jouan Biophotometer. Radioactivity was

* Stoffwechselprodukte von Mikroorganismen, 136; W. HEBERLE, W. A. KÖNIG und W. LÖFFLER: Asposterol, ein Antibiotikum aus *Aspergillus microcysticus*. Arch. Microbiol. 100: 73~95, 1974

measured by a Nuclear Chicago (Mark II) Liquid Scintillation Counter.

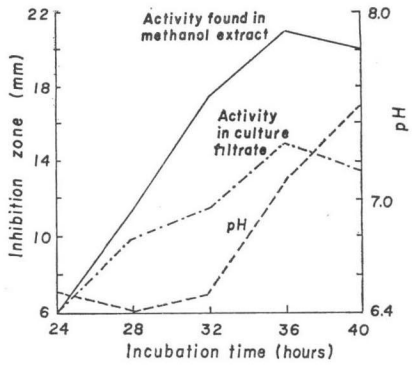
Production

The medium for inoculum preparation and production culture was composed of: Brennerei-Solubles (SITOS-Werk, Minden) 2.0%, glucose 2.0%, NaCl 0.5%, $NaNO_3$ 0.1%. The pH was adjusted to 7.2.

Streptomyces Tü 1102 was inoculated into 60 flasks, each containing 100 ml medium, and cultivated for one day at 27°C on a reciprocal shaker.

The production culture was prepared in six jar fermentors each of which contained 9 liters medium. The medium was sterilized for 30 minutes at 134°C. After cooling, and the addition of ten flasks of inoculum culture to each jar, fermentation was carried out under the following conditions: temperature, 26~28°C; aeration, 2 liters/minute; agitation, 220~250 r.p.m. The antibiotic activity was determined by the paper disc method, separately for culture filtrate and the methanol extract of mycelium during the fermentation. *Streptomyces viridochromogenes* was used as the test organism on a medium composed of malt extract 1%, yeast extract 0.4%, glucose 0.4% and agar 2% (pH 7.3): An aliquot (100 ml) of fermentation broth was removed at intervals of 4 hours and filtered with Celite. The activity and the pH of subsequent filtrate (*ca* 90 ml) was assayed. A mixture of mycelium and Celite was extracted twice with methanol (50 ml each) and the activity of total methanol solution was determined.

Fig. 1. Rinamycin production (bioassay against *Streptomyces viridochromogenes*).



One example of results is shown in Fig. 1. As can be seen, the activity, which appeared after 24 hours of incubation both in culture filtrate and in mycelium, reached a maximum after about 36 hours and disappeared rapidly on further incubation. The maximal activity observed in methanol extract was usually obtained at pH 7.1~7.5 of the culture filtrate.

Isolation

Fermentation was stopped when the pH of the fermentation broth reached 7.3 and the broth was filtered with Celite. The resultant filter cake was extracted with methanol (3 times, 5 liters each) and the combined extracts were concentrated to about 1.5 liters; ethanol (4.5 liters) was added to this concentrate under vigourous stirring.

After one night at 4°C, the resulting supernatant solution was collected by decantation and the syrup at the bottom was washed twice with methanol (500 ml each). The supernatant solution and the methanol washings were combined and concentrated to about 500 ml, which then subjected to a second precipitation procedure and the concentration of solvent gave a viscous solution (200 ml).

The solution thus obtained was subjected to a countercurrent distribution (one fourth

i. e. 50 ml at a time) using 105 steps in a solvent system, *n*-butanol - water. Antibiotic activity was usually found between the 57 th and the 105 th step. Active fractions were combined and concentrated to 50 ml, which then were applied to a second distribution using 91 steps in the solvent system; ethyl acetate - *n*-butanol (1 : 1)-water. Active fractions, found between the 20 th and the 91 st steps, were combined and concentrated to a syrup (28 g).

The syrup was chromatographed on a column of Kieselgel (450 g) in methanol from which 100-ml fractions were collected. The antibiotic activity was recovered between Fr. 9 and 26. Concentration of these fractions gave a syrup (18.5 g), which applied to a second chromatography on a column of Kieselgel (700 g) in the solvent system, methanol-ethylacetate (1 : 1). Fractions of 200 ml were collected. The antibiotic was recovered between Fr. 25 and 36 which gave a brown residue (1.94 g) after evaporation of the

Fig. 2. Ultraviolet spectra of rinamycin.
(a) measured in methanol; (b) measured in water; (c) measured in water after 15 minutes at 37°C.

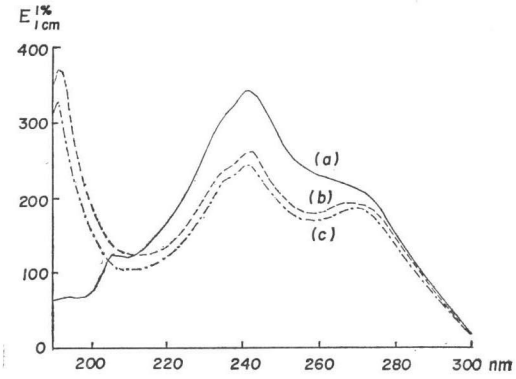


Chart 1. Purification

Mycelium
↓ extracted with MeOH
MeOH extract
↓ concentrated *in vacuo*
↓ added EtOH
↓ allowed to stand at 4°C
Supernatant solution
↓ concentrated *in vacuo*
↓ added EtOH
↓ allowed to stand at 4°C
Supernatant solution
↓ evaporated *in vacuo*
Syrup 200 ml
↓ counter-current distribution
↓ with *n*-BuOH and H_2O
Active fractions
↓ evaporated *in vacuo*
Syrup 50 ml
↓ counter-current distribution
↓ with AcOEt - *n*-BuOH (1:1) and H_2O
Active fractions
↓ evaporated *in vacuo*
Syrup 28 g
↓ Silicagel chromatography in MeOH
Active fractions
↓ evaporated *in vacuo*
Syrup 18.5 g
↓ Silicagel chromatography in
↓ AcOEt - MeOH (1 : 1)
Active fractions
↓ evaporated *in vacuo*
Brown powder 1.94 g
↓ Dowex 2×4 (Cl^-) adsorption in MeOH
MeOH effluent
↓ evaporated *in vacuo*
Light brown powder 1.85 g
↓ adsorbed on Amberlite IRC-50 (Pyr^+)
↓ in MeOH - H_2O (4 : 6)
↓ eluted with MeOH
MeOH eluate
↓ evaporated *in vacuo*
Honey-coloured powder 792 mg
↓ Gel filtration on Sephadex LH 20
↓ eluted with MeOH
Active eluate
↓ evaporated *in vacuo*
Honey-coloured powder 708 mg
↓ Gel filtration on Sephadex LH 20
↓ eluted with MeOH
Active eluate
↓ evaporated *in vacuo*
Rinamycin 674 mg

Fig. 3. Infrared spectrum of rinamycin in KBr.

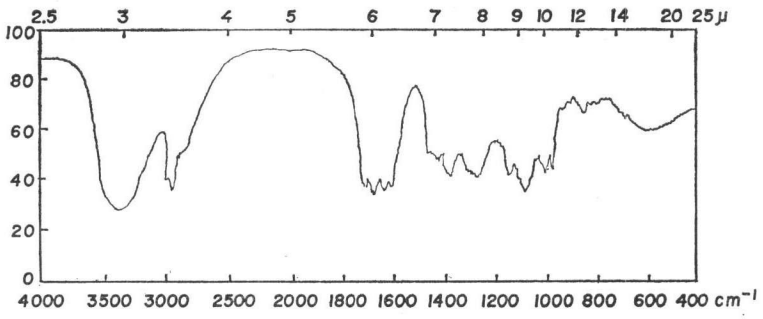


Fig. 4. p.m.r. spectrum of rinamycin in methanol-d_4 (100 MHz).

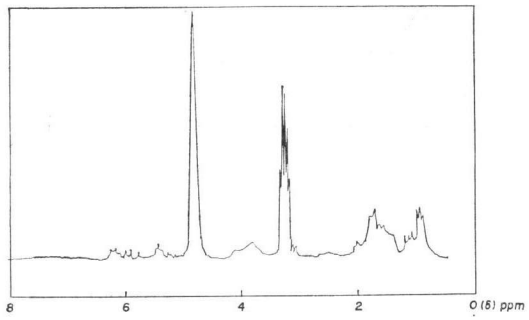


Fig. 5. Comparison of the infrared spectra of rinamycin, hygrostatin and azalomycin F in KBr.

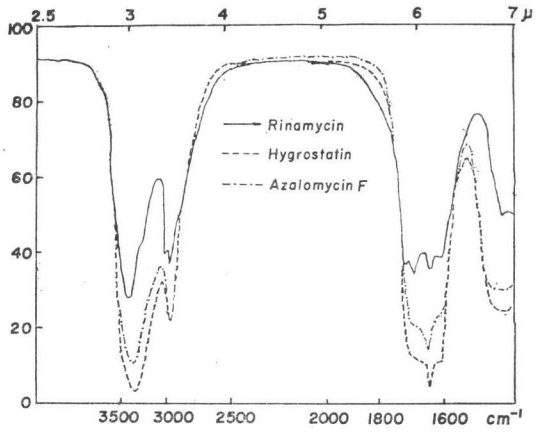


Fig. 6. Paper chromatograms of rinamycin, hygrostatin and azalomycin F (bioautograph of rinamycin against *Pseudomonas saccharophila*). A *n*-Butanol satd. with water; B 20% NH_4Cl; C Acetone-water (1 : 1); D Benzene-methanol (4 : 1); E *n*-Butanol-methanol-water (4 : 1 : 2); F Ethyl acetate-pyridine-water (2:1:2) (upper phase); G *n*-Butanol-acetic acid-water (4:1:2).

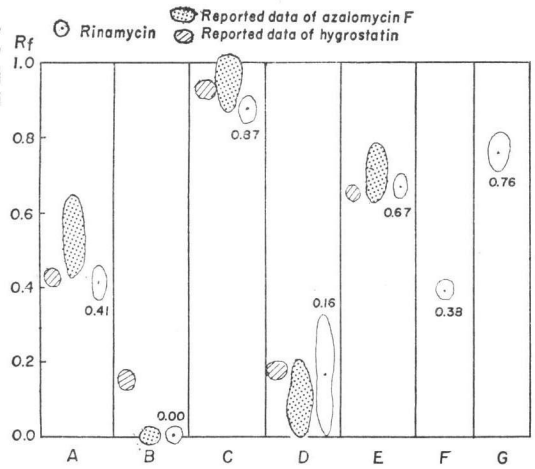


Fig. 7. Silicagel chromatograms of rinamycin (bioautograph against *Pseudomonas saccharophila*). a *n*-Butanol. b Ethylacetate-pyridine-water (2 : 1 : 2) (upper phase). c Ethyl acetate-methanol (1 : 1).

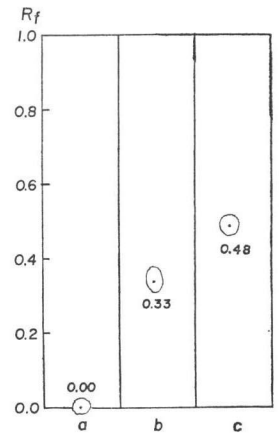


Fig. 8. Paper electrophoresis of rinamycin (250 V/19 cm, 5 cm, 3 hours). Bioautograph against *Pseudomonas saccharophila*. R rinamycin; G glucose; * run for 3 and 10 hours.

1) Acetate buffer (pH 4.1, 1/150 M).
2) Phosphate buffer (pH 7.0, 1/10 M).
3) Veronal buffer (pH 8.6, 1/10 M).

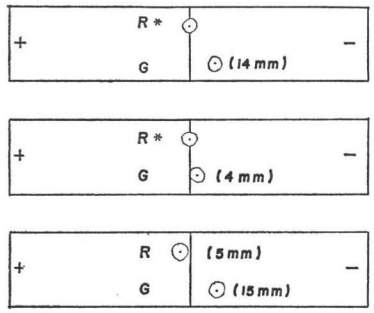
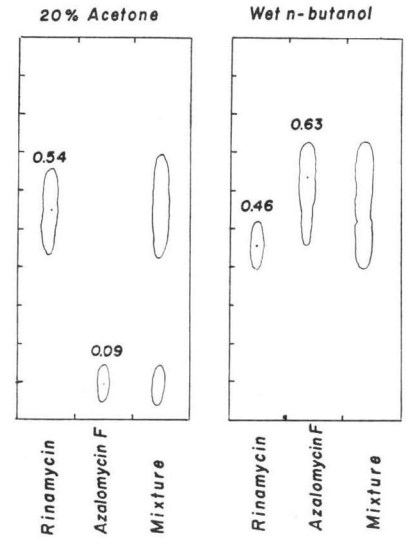


Fig. 9. Comparative paperchromatographies of rinamycin and azalomycin F. (Bioautograph against *B. subtilis*)



solvent.

The residue was dissolved in the minimal amount of methanol and poured into a column of Dowex 2×4 (Cl^- form, 50~100 mesh, 50 ml) previously saturated with methanol. The column was washed with methanol until the concentrate of a washing fraction (each 50 ml) showed no more activity.

Concentration of the total methanol washing gave a lightly coloured powder (1.85 g) which still contained several ninhydrin-positive impurities according to paper chromatography in the solvent system, ethyl acetate-pyridine-water (2 : 1 : 2).

This powder was dissolved in methanol-water (4 : 6), and applied on a column of Amberlite IRC-50 (50 ml, previously washed with pyridine-water (1 : 4) and then with water), from which ninhydrin-positive substances (354 mg) could be washed off with the same solvent (250 ml) before the antibiotic was eluted with methanol (200 ml). Evaporation of the methanol eluate gave a honey-coloured powder (792 mg) which was subsequently chromatographed on a column of Sephadex LH 20 (800 ml) in methanol. The antibiotic-containing fraction, which appeared after the effluence of 330 ml methanol, was detected by ultraviolet absorption (wave length 260 nm) as well as by an activity assay. Evaporation of the solvent of the antibiotic-containing fraction yielded a honey-coloured powder (708 mg). A second column chromatography on Sephadex LH 20 gave the antibiotic (674 mg) which was chromatographically homogeneous when tested by iodine, silver nitrate and ninhydrin on a paper chromatogram and by 50% sulfuric acid on a Kieselgel chromatogram. The solvent systems are described in Figs. 6 and 7, and the purification procedure is summarized in Chart 1.

Physico-chemical Properties

Rinamycin is soluble in methanol and to some extent in ethanol and *n*-butanol. The solubility in ethanol is about 0.5% at room temperature. The antibiotic is practically insoluble in water or acetone and in other less-polar solvents. Although soluble in pyridine and acetic acid at room temperature, it decomposes gradually, yielding a red-brown solution.

Rinamycin was obtained as a honey-coloured powder which decomposes at about 132°C. Its microanalysis data are as follows:

Found:	C 57.69,	H 8.57,	N 3.38	
Calcd. for $C_{20}H_{35}NO_8$:	C 57.55,	H 8.39,	N 3.36.	MW 417.

The molecular weight corresponded to about 400 according to molecular sieving column of Sephadex LH 20 in methanol, with several polyethyleneglycols as standard agents.

The specific rotation of rinamycin is $[\alpha]_D^{20}+30.8°$ (c 0.555, methanol).

The ultraviolet spectra are shown in Fig. 2. In methanol it has characteristic maxima at λ_{max} 242 nm ($E_{1cm}^{1\%}$ 340), 206 nm ($E_{1cm}^{1\%}$ 122) and a shoulder λ_{sh} 265 nm ($E_{1cm}^{1\%}$ 222), whereas 3 peaks are observed in water at λ_{max} 195 ($E_{1cm}^{1\%}$ 369), 240 nm ($E_{1cm}^{1\%}$ 258) and 265 nm ($E_{1cm}^{1\%}$ 192), the intensities of which significantly decrease after 15 minutes at 37°C.

The infrared spectrum in KBr pellet (Fig. 3) indicates the presence of OH ($\sim$3400 cm^{-1}), carbonyl (1650$\sim$1730 cm^{-1}) and double bond (1580$\sim$1680 cm^{-1}). However the NH absorption ($\sim$1530 cm^{-1}) of mono-N-substituted amide which is usually found in peptides is absent.

The p. m. r. spectrum measured in methanol-d_4 (Fig. 4) shows characteristic absorptions at δ 0.9$\sim$2.0, 3.3, 3.9 and 5.3$\sim$6.3 ppm, which suggest the presence of vinyl protons (δ 5.3$\sim$ 6.3 ppm) but the absence of aromatic protons in the rinamycin molecule.

Attempts to acetylate rinamycin (in pyridine-acetic anhydride, 1 : 1, at room temperature for 0.5, 5, 20 hours) led to several compounds, apparently caused by decomposition.

Hydrolysis (6 N HCl, 110°C, 20 hours) and methanolysis (1 N HCl, 70°C, 20 hours) of the antibiotic liberated neither ninhydrin-positive compounds nor sugars detectable by silver nitrate or aniline hydrogen phthalate.

Among known antibiotics, the ultraviolet spectrum of rinamycin is very similar to those of musarin, antiprozoin and hygrostatin in that they have a characteristic maximum at 240$\sim$ 242 nm and an inflexion at 260$\sim$270 nm.

Musarin, reported to be unstable even at room temperature, is an acid, the sodium salt of which is soluble in water. The free acid is soluble in 30% aqueous acetone. Musarin gives no coloration with sulfuric acid or iodine. Antiprozoin is an acid which forms an acetate in pyridine and acetic anhydride. Hydrolysis yields ninhydrin-positive substances and inositol. The characteristics of the two antibiotics are thus sharply contrasted to those of rinamycin.

Hygrostatin is a basic antibiotic which has a similar infrared absorption to rinamycin except that found in the field of carbonyl absorption (Fig. 5). Although the authentic sample of hygrostatin was not available, paper chromatography of rinamycin compared with reported data on hygrostatin (Fig. 6) offered a significant difference when 20% NH_4Cl and acetone-water (1 : 1) were used as solvents.

In addition, rinamycin seems to have neutral properties when paper electrophoresis is performed in acetate buffer (pH 4.1) (Fig. 8), whereas hygrostatin is reported to be basic, moving to the cathode faster than glucose in the same buffer.[4]

Azalomycin F resembles rinamycin in its ultraviolet spectrum in methanol; the former exhibits, in addition to a maximal absorption at 241 nm, another maximum at 268 nm instead of an inflexion observed on the rinamycin spectrum. However comparison of infrared spectra (Fig. 5) and paper chromatographic behaviour in n-butanol saturated with water and in 50%

acetone (Fig. 6) allowed us to distinguish these antibiotics. Furthermore azalomycin F is described to be moderately soluble in 20% aqueous acetone and to give a ninhydrin-positive solution after hydrolysis, which is not the case with rinamycin.

Rinamycin was differentiated from an authentic sample of azalomycin F* by comparisons of i.r. spectra and paperchromatographic behaviours.

It is therefore appropriate to recognize rinamycin as a new antibiotic.

Biological Properties

Antimicrobial characteristics of rinamycin tested by the agar diffusion method and the dilution method in liquid media are summarized in Table 1. The test medium used for most bacteria contained; meat extract 0.3%, peptone 0.5%, NaCl 0.25%, agar 1.5% (pH 7.2);

Table 1. Antimicrobial spectra

Strain	M.I.C. by liquid dilution method* (μg/ml)	Inhibition zone on agar plate 1 mg/ml concn. (mm)	Strain	M.I.C. by liquid dilution method* (μg/ml)	Inhibition zone on agar plate 1 mg/ml concn. (mm)
Eubacteriales			*Streptomyces griseoviridis* Tü 8		11.0
Achromobacter geminiani	3	19.0	*Streptomyces griseus* Tü 17		11.5
Agrobacterium tumefaciens	100	–	*Streptomyces prasinus* Tü 30		10.5
Arthrobacter aurescens	2	12.5	*Streptomyces ramulosus* Tü 34	5	13.5
Arthrobacter pascens	1	15.0	*Streptomyces viridochromogenes*		
Arthrobacter simplex		11.5	Tü 57	5	8.0
Bacillus brevis		9.5	*Streptosporangium roseum* Tü 74	3	14.0
Bacillus subtilis	3	10.5	*Streptosporangium roseum* Tü 78		19.5
Chromobacterium violaceum	50	13.5	Fungi		
Corynebacterium rathayi	10	16.5	*Aspergillus fumigatus* Tü 143		–
Escherichia coli K 12	100	–	*Aspergillus niger* Tü 503		10.5
Lactobacillus casei	100	–	*Botrytis cinerea*		22.5
Micrococcus luteus	5	14.5	*Fusarium larvarum* Tü 257		14.0
Propionibacterium freudenreichii		9.0	*Fusarium solani* Tü 156		16.0
Propionibacterium shermanii		11.0	*Mucor miehei* Tü 284	1	19.0
Staphylococcus aureus	25	11.0	*Mucor mucedo*		17.0
Pseudomonadales			*Paecilomyces varioti* Tü 137		14.0
Pseudomonas saccharophiia	5	16.0	*Penicillium* sp. Tü 288		16.0
Actinomycetales			*Trichophyton mentagrophytes*		
Nocardia brasiliensis Tü 69	25	8.0	Tü 295		19.5
Streptomyces antibioticus Tü 4		14.0	Yeasts		
Streptomyces collinus Tü 105		8.0	*Candida albicans*	10	10.0
Streptomyces fradiae Tü 29		6.5	*Candida lipolytica*		10.0
Streptomyces glaucescens Tü 49		–	*Saccharomyces cerevisiae*		7.5

* Minimal inhibitory concentrations (M.I.C.) examined by agar diffusion method for all the microorganisms on the table were more than 100 μg/ml except for *Botrytis cinerea* (50 μg/ml).

* We thank Dr. M. ARAI, Sankyo Co., Tokyo, for the azalomycin sample.

medium for *Nocardia*, *Streptosporangium*, fungi and yeasts contained; yeast extract 0.4%, malt extract 1%, glucose 0.4%, agar 2% (pH 7.3). *Arthrobacter* and *Corynebacterium* were grown in nutrient broth and *Propionibacteria* in the medium containing; meat extract 0.3%, malt extract 0.3%, yeast extract 0.3%, peptone 2%, glucose 0.5%, ascorbic acid 0.02%, agar 0.1% (pH 7.0). Appropriate liquid media for these organisms were prepared without agar.

These antimicrobial tests were carried out at 27°C except for *Bacilli*, *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Streptomyces viridochromogenes*, which were incubated at 37°C.

As can be seen in Table 1, rinamycin is active against fungi, yeasts, gram-positive and some gram-negative bacteria when tested by the liquid dilution method, though agar-diffusion plates showed only weak activity. The latter effect is apparently due to slow diffusion of rinamycin. In this respect rinamycin resembles musarin.

Effect on Macromolecular Synthesis

To determine the antibiotic action on DNA, RNA and protein syntheses, the effect of rinamycin on the incorporation of ^{14}C-thymidine, ^{14}C-uracil and ^{14}C-isoleucine which represent constituents of each macromolecule was examined using logarithmically growing cells of *Bacillus subtilis* in the presence and absence of rinamycin in minimal inhibitory concentration.

Bacillus subtilis ATCC 6051 was cultivated at 37°C is the synthetic medium containing KH_2PO_4 0.3%, K_2HPO_4 0.7%, sodium citrate 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, $(NH_4)_2SO_4$ 0.1%, glucose 0.2%. After 18 hours the seed culture was inoculated to a fresh medium of the same composition to allow a transmission of 95% at 578 nm. The cell suspension was incubated at 37°C until the transmission reached 80% and placed in cuvettes. The cuvette cultures were further incubated.

When the transmission approached 70%, ^{14}C-thymidine* (17.0 mμ moles, 1.0 μCi), ^{14}C-uracil* (24.2 mμ moles, 1.5 μCi) or ^{14}C-isoleucine* (7.4 mμ moles, 2.5 μCi), in this last case along with non-labeled isoleucine (3.5 μg) to reduce rapid consumption of radioactive isoleucine, was added to a cuvette. After 10 minutes, rinamycin in 3 μl of methanol was added to make the final concentration of the antibiotic 3 μg/ml. The antibiotics were omitted in the control cuvette.

Two hundred μl of the cuvette culture were assayed at the indicated times on the Figs. 10, 11 and 12 and added to cold trichloroacetic acid (1 ml, 7%). The precipitate which formed was collected on a membrane filter (pore size, 0.45 μ) and washed (4 times, 5 ml 5% trichloroacetic acid each wash). The washed precipitate and filter were allowed to stand overnight, placed in a scintillation vial and mixed with toluene (5 ml) containing 0.4% 2,5-diphenoloxazole before radioactivity was measured.

The results show that the incorporation of ^{14}C-uracil was stopped immediately after the addition of inhibitor, while those of ^{14}C-thymidine and ^{14}C-isoleucine continued to increase during one hour. This observation led us to the suggestion that inhibition of the RNA metabolism of rinamycin-sensitive cells contributes to cell damage.

* Purchased from The Radiochemical Center, Amersham, England.

Fig. 10. Effect of rinamycin on the incorporation of [^{14}C]-thymidine (test organism: *Bacillus subtilis*).

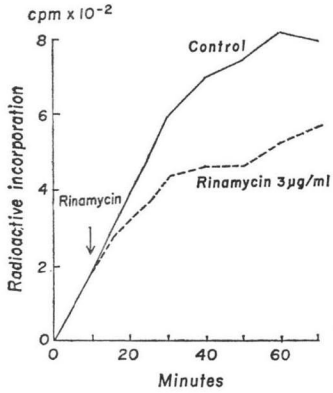


Fig. 11. Effect of rinamycin on the incorporation of [^{14}C]-uracil (test organism: *Bacillus subtilis*).

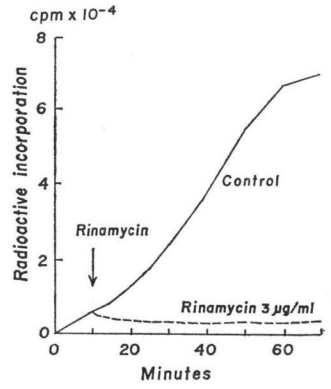
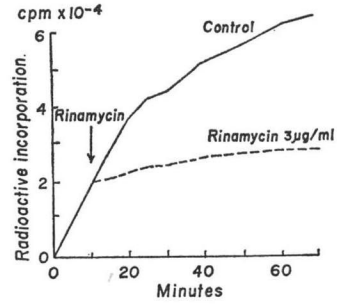


Fig. 12. Effect of rinamycin on the incorporation of [^{14}C]-isoleucine (test organism: *Bacillus subtilis*).



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