Biogenesis of Rifamycins. The Conversion of Rifamycin B into Rifamycin Y[1]

Streptomyces mediterranei (MARGALITH and BERETTA, 1960) produces in liquid cultures at least 5 antibiotic substances[2]. However, on culturing the microorganism in the presence of diethylbarbituric acid (barbital), essentially only 2 compounds are obtained[3,4], rifamycin B and in lesser amounts rifamycin Y.

Since rifamycin Y and derivatives obtained therefrom are antibiotically inactive, studies were undertaken to clarify the biogenesis of this compound in order, eventually, to be able to suppress its formation. Strain selection work as well as the study of some fermentation variables (pH, agitation and aeration, temperature etc.), did not result in significant modification of the amount of rifamycin Y formed. On the other hand, while studying the effect of media composition on antibiotic production, it was found that the level of inorganic phosphate (as KH_2PO_4) of the culture media had a drastic effect on rifamycin Y synthesis. The rifamycin B and Y contents of fermentation media containing increasing amounts of KH_2PO_4 were determined after 120 h of fermentations. As can be seen from Figure 1, a straight correlation between the concentration of inorganic phosphate added to the medium and of rifamycin Y synthesized is evident. In the absence of added inorganic phosphate, or at very low additions of it (0.2–0.4 g/l) no significant amounts of rifamycin Y were formed. It is also apparent from Figure 1 that the increase in rifamycin Y coincides with a parallel decrease in rifamycin B production, in agreement with the hypothesis that one of the compounds might arise from the other.

The structure of rifamycin Y recently elucidated by chemical degradation[4] and X-ray diffraction studies[6] shows that this compound differs from rifamycin B in the degree of oxidation of the aliphatic ring (Figure 2).

As in the case of other macrolide antibiotics, this ring appears to derive from condensation of propionate and acetate units. Preliminary experimental data, supporting this hypothesis have been discussed by CORCORAN and CHICK[7]. This suggests that rifamycin Y may arise from rifamycin B, since it can be assumed that the hydroxyl group on the carbon atom 20 of rifamycin Y is introduced on a preformed chain, possibly that of rifamycin B.

To test this hypothesis, [^{14}C] labelled rifamycin B (12.100 counts/min/mg corresponding to 13.1 μC/mM) obtained from fermentations containing [^{14}C] inverted sugar, was added to cultures of *S. mediterranei* grown in the presence of diethylbarbituric acid. At the end of the fermentation period, the rifamycins produced were isolated and their specific radioactivity determined. As shown by the results reported in Table I, when labelled rifamycin B was added to 48-h-old cultures, that is just when antibiotic production starts, the specific radioactivity of rifamycin Y was about twice that of rifamycin B. When [^{14}C] rifamycin B was added to 72-h-old cultures, the rifamycin B and Y were about equally radioactive. This demonstrates that the microorganism possesses an enzymatic system capable of converting rifamycin B into rifamycin Y. Moreover this system appears to be active from the very beginning of the antibiotic production.

The possibility that the strain could also perform the reverse reaction of transforming rifamycin Y in rifamycin B was ruled out by a similar experiment in which [^{14}C] labelled rifamycin Y was added to *S. mediterranei* cultures.

Labelled rifamycin Y was prepared by running the fermentation in presence of [^{14}C] inverted sugar, and the radioactive mixture of B and Y thus obtained was separated by paper chromatography (solvent system, see Figure 1). The area containing rifamycin Y (Rf $\sim$ 0.2) was cut out, the antibiotic eluted with phosphate buffer pH 7.38, diluted with unlabelled rifamycin Y and

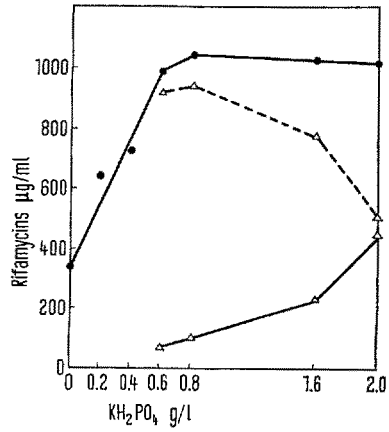

Fig. 1. Influence of K_2HPO_4 concentration in the medium on rifamycins production. ●——● total rifamycins (B + Y); △——△ rifamycin B; △---△ rifamycin Y. Culture conditions: 500 ml Erlenmeyer flasks containing 100 ml of fermentation medium (composition in g/l: peanut meal, 25; soybean meal, 10; glucose, 115; barbital, 2%; ammonium sulphate, 9.6; calcium carbonate, 9.5; trace amounts of Mg^{++}, Cu^{++}, Fe^{++}, Mn^{++}, Co^{++}, Mo_7O_{24}) agitated on rotary shaker at 28 °C for 120 h. The total amount of rifamycins B + Y was determined spectrophotometrically as described[5] for rifamycin B (the 2 rifamycins have identical ε at 425 nm). The relative amounts were determined by quantitative paper chromatography in a solvent system composed of 0.07 M phosphate buffer (pH 8.6) containing 0.1 g/l of ascorbic acid as a stationary phase, and amyl alcohol, butyl alcohol, 9:1, as mobile phase.

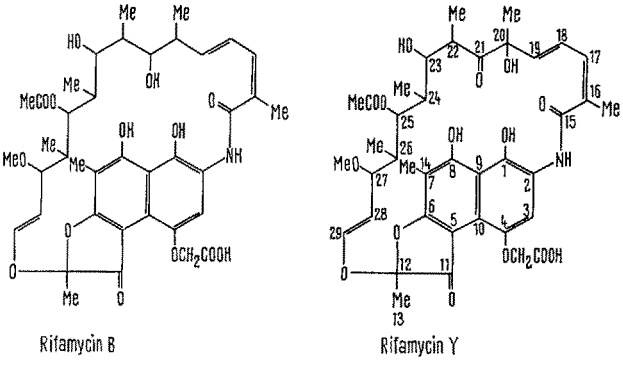

Fig. 2

[1] Presented in part at the Vth International Congress of Chemotherapy, Wien, 26 June 1967.

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crystallized. 70 mg of [C^{14}] rifamycin Y (1200 counts/min/mg, corresponding to 1.3 μC/mM) were added to 5 flasks containing 48-h-old cultures of *S. mediterranei*. After 120 h of fermentation, spectrophotometric and chromatographic analysis of the culture filtrates showed the presence of 310 mg of rifamycin B and 145 mg of rifamycin Y. After separation of the 2 antibiotics by countercurrent distribution of the extracts, it was found that only rifamycin Y was labelled (360 counts/min/mg) thus demonstrating that labelled rifamycin Y was not converted into rifamycin B.

No definite conclusion can be drawn so far on the mechanism by which phosphates influence the oxidation. Inorganic phosphate determination performed at various time intervals indicated that rifamycin Y was formed mainly after all the inorganic phosphate had disappeared (Figure 3). It is interesting to note that most of the inorganic phosphate and glucose are metabolized from the 50th to the 80th h and that during the same period the organic phosphate present in the culture filtrates does increase considerably. As previously shown[9] also the oxygen demand is highest during that part of the fermentation.

The conversion of rifamycin B into rifamycin Y is performed also by washed mycelium of *S. mediterranei*. This technique has been utilized to ascertain whether the presence of barbital in the medium is essential for this reaction. Experiments have been performed by adding [^{14}C] rifamycin B to washed mycelium suspended in phosphate buffer and in phosphate buffer containing 0.2% of barbital (Table II). After 20 h of incubation, the rifamycins were extracted and analyzed. The amount of rifamycin Y obtained was respectively 16 and 18.5% of the total rifamycins recovered. The specific radioactivity values clearly indicate that this compound is not produced by 'ex novo' synthesis but originates in both cases from rifamycin B. Furthermore, the results demonstrate that diethyl barbituric acid is not required for the transformation of rifamycin B to rifamycin Y.

The experiments provide strong evidence that rifamycin B is the natural precursor of rifamycin Y. This is also a further indication that the ansa chain of rifamycins originates from propionate units since the hydroxyl group in position 20 of rifamycin Y appears to be introduced after the completion of rifamycin B molecule.

Table I. Conversion of rifamycin B into rifamycin Y by growing cultures of *Streptomyces mediterranei*

[C^{14}] Rifamycin B added			[C^{14}] Rifamycins produced			
			B		Y	
(h after inoculum)	mg	(counts per min/mg)	mg	(counts per min/mg)	mg	(counts per min/mg)
48	28.5	12.100	253	448	84	923
72	28.5	12.100	232	581	65	550

Culture conditions as in Figure 1 (KH_2PO_4 concentration = 2 g/l). In each experiment [^{14}C] rifamycin B was added at the time indicated in 3 flasks containing 75 ml of culture. The specific radioactivity of the produced rifamycins was determined on samples separated by countercourrent distribution in a solvent system *n*-butanol/0.07 *M* phosphate buffer pH 7.38.

Table II. Conversion of rifamycin B into rifamycin Y by washed mycelium of *Streptomyces mediterranei*

Medium	[C^{14}] rifamycin B added		[C^{14}] rifamycins recovered B		Y	
	mg	counts per min/mg	mg	counts per min/mg	mg	counts per min/mg
Phosphate buffer	90	825	63	730	12	619
Phosphate buffer + 0.2% barbital	90	825	66	621	15	606

The mycelium from 225 ml of a 48 h old culture was harvested, washed, starved for 5 h in 0.7 *M* phosphate buffer (pH 6.5) and suspended in 225 ml of the same buffer (with and without barbital) distributed in 3 flasks. [C^{14}] Rifamycin B was then added and the flask incubated at 28 °C on a rotary shaker for 20 h. Analyses were performed as indicated in Table I.

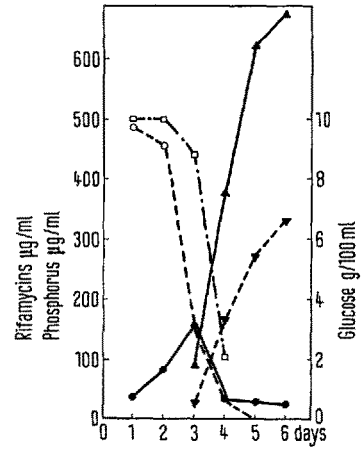

Fig. 3. Phosphate metabolism during rifamycin fermentation. ▲——▲ rifamycin B, ▲---▲ rifamycin Y, ●——● organic phosphorus, o---o inorganic phosphorus, □-●-□ glucose. Culture conditions: 4 l jar fermentors (medium as in Figure 1 plus 2 g/l of K_2HPO_4); temperature 28 °C, aeration 1 l air/min/l, agitation 800 r.p.m. Analyses were performed on the supernatant of the centrifuged broth. Phosphorus was determined according to [8]; rifamycins were determined as indicated in Figure 1.

Riassunto. È stata studiata la biogenesi della rifamicina Y, prodotta accanto alla rifamicina B da culture di *S. mediterranei*. I dati riportati dimostrano che la rifamicina Y deriva dalla rifamicina B e che la sua produzione dipende dalla concentrazione di fosfati nel terreno di cultura.

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