Metabolism of Barbital by *Streptomyces mediterranei*

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**ABSTRACT**

KLUEPFEL, D. (Lepetit SpA, Milan, Italy), G. C. LANCINI, AND G. SARTORI. Metabolism of barbital by *Streptomyces mediterranei*. Appl. Microbiol. 13:600–604. 1965.—The metabolism of barbital in cultures of *Streptomyces mediterranei* was studied. Small quantities were transformed during the fermentation. Four compounds were isolated and identified as 5-ethyldialuric acid, 5-ethylbarbituric acid, 5-(5-hydroxyethyl)-5-ethylbarbituric acid, and diethylmalonic acid monoureid. The influence of these compounds on the production of rifamycin was of minor importance. With the aid of C14-barbital, it was established that neither barbital nor any of its metabolic products can be considered as precursors of the rifamycins B.

*Streptomyces mediterranei* produces in liquid culture at least five distinct antibiotic substances, called the rifamycin complex (Sensi, Greco, and Ballotta, 1959), which show antimicrobial activity in vitro against gram-positive bacteria and *Myco- bacterium tuberculosis* (Sensi, Margalith, and Timbal, 1959). (The name “rifamycin” has been used instead of “rifomycin,” as in some previous papers, to avoid confusion with other antibiotics.) Rifamycin B, a component of this complex, was easily isolated on the basis of its acidic nature. During studies to improve the fermentation conditions, Margalith and Pagani (1961) found a number of barbituric acid derivatives which changed the pattern of antibiotic production. Thus, it was observed that the addition of 0.2% 5,5-diethylbarbituric acid (barbital) to the fermentation medium shifted the antibiotic formation in such a manner that only one compound was produced, i.e., rifamycin B. A somewhat similar influence was observed when 5-phenyl-5-ethylbarbituric acid, 5-ethyl-5-methylbarbituric acid, or 5-ethyl-5-isopropyl-barbituric acid was added, but these compounds did not bring about such marked and complete response. Margalith and Pagani (1961) proposed as possible explanations of the action of barbital either that a shift occurred in the biosynthetic pathways (metabolic shunt) by intervening as activator or inhibitor of enzyme systems, or that barbital was a precursor of the antibiotic and was incorporated in part or as a whole into the rifamycin B molecule. Oppolzer, Prelog, and Sensi (1964) disclosed the structure of this compound, and it appeared unlikely that the entire barbital molecule could be considered a precursor. However, preliminary experiments had indicated that small quantities of barbital had undergone metabolic transformations, and it was thought possible that some metabolites might act as precursors of rifamycin B. The present paper reports the isolation of some of these metabolic products from liquid cultures of *S. mediterranei* and their effect on the production of rifamycin B.

**MATERIALS AND METHODS**

The cultures of *S. mediterranei* ME/156 were grown in the form of frozen vegetative mycelium. The vegetative culture medium V6 (g per liter: meat extract, 5; peptone, 5; yeast extract, 5; enzymatically hydrolyzed casein, 3; glucose, 20; NaCl, 1.5; CuSO4·5H2O, 0.003; KH2PO4, 2; MgSO4·7H2O, 0.013; ZnSO4·7H2O, 0.05; MnSO4·4H2O, 0.004; CoCl2·6H2O, 0.002; (NH4)2MoO4·4H2O, 0.001; and distilled water, 1,000 ml; final pH was 6.8 (after sterilization).

Fermentations were carried out in 500-ml Erlenmeyer flasks containing 75 ml of medium. This volume was reduced to 10 ml in 100-ml Erlenmeyer flasks when radioactive barbital was used. The cultures were agitated on a rotary shaker at 300 rev/min, and the incubation period lasted 120 hr at 28°C. To isolate possible metabolic products of barbital, it was necessary to find a suitable extraction procedure which permitted the separation from rifamycin B and the nonmetabolized barbital. The following method was designed. Fermentation broth was filtered and extensively washed with 0.033 M phosphate buffer (pH 7.3). The culture filtrate was acidified with HCl to pH 2.3 and was evaporated to dryness. The residue was extracted with methanol and the methanolic extract was then fractionated. Fractionation yielded fractions containing the rifamycins B. (The isolation of rifamycin B was carried out in the Research Laboratories of Lepetit SpA, Milan, Italy.)

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2 and was extracted exhaustively with ethyl acetate. The organic phase was subsequently re-extracted with phosphate buffer (pH 7.3). The exhausted ethyl acetate contained large quantities of barbital. The buffer solution was adjusted to pH 6.5 and washed extensively with ethyl ether to remove the remaining barbital. After acidification to pH 2, this buffer solution was extracted with chloroform for removal of rifamycin B. The remaining aqueous phase contained the various metabolic compounds of barbital (for a more detailed description of the extraction procedure, see Fig. 1).

The separation and purification of these compounds was achieved by countercurrent distribution with either ethyl acetate-0.01 N HCl or an ethyl acetate-petroleum ether-methanol-0.01 N HCl (2:1:1:2) solvent system. The 5,5-diethylbarbituric acid-5-C\textsuperscript{14} with a specific radioactivity of 2.0 μc/mmol was kindly supplied by V. Prelog, Organisch-Chemisches Laboratorium, Swiss Institute of Technology, Zurich, Switzerland)

The 5,5-diethylbarbituric acid-5-C\textsuperscript{14} with a specific activity of 1.2 μc/mmol was prepared in our laboratory by the method of Goldschmidt and Wehr (1955). The purity of both compounds was confirmed by countercurrent distribution and paper chromatography. The radioactivity was determined with a Selo superscaler with flow counter (Societa Elettronica Lombarda-Milano).

The ultraviolet spectra were recorded on a Beckman model DK2 spectrophotometer. The nuclear magnetic resonance (n.m.r.) spectra were obtained by use of a Varian A-60 spectrometer (60 mc/sec) in pyridine-d\textsubscript{4} with tetramethylsilane as internal reference.

**RESULTS**

Preliminary experiments on the recovery of barbital from the culture broth indicated that a certain small percentage was metabolized by the microorganism. Since only small quantities of metabolites could be expected to occur, experiments were conducted with radioactive-labeled barbital. For this purpose, the barbital in the fermentative medium was substituted either by barbital-2-C\textsuperscript{14} or barbital-5-C\textsuperscript{14}. After fermentation with *S. mediterranei*, the radioactive compounds were extracted together with the rifamycin B. Preliminary separation, achieved by countercurrent distribution with ethyl acetate-0.01 N HCl as the solvent system, confirmed the presence of several radioactive metabolites. The rifamycin B isolated by this method from fermentations with either barbital-2-C\textsuperscript{14} or barbital-5-C\textsuperscript{14} was nonradioactive, thus excluding the possibility that barbital or one of its metabolites was a direct precursor of the antibiotic. As had been expected, the major portion of the radioactivity was recovered in its original form of barbital. However, 7 to 8% of the radioactivity was found to be metabolized into other compounds.

To isolate the unknown radioactive metabolites, it was necessary to find an appropriate extraction procedure which allowed their separation from unaltered barbital and rifamycin B. A suitable procedure for this purpose is given in Fig. 1.

![Diagram](http://aem.asm.org/)

**Fig. 1. Extraction method for culture filtrates of* Streptomyces mediterranei**

Fermentation broth of *S. mediterranei* → Filtration (pH 7.3) → Mycelium discarded → Culture filtrate → Acidification to pH 2.0 and extraction with ethyl acetate

- Ethyl acetate containing barbital, rifamycin B, and II, III, and IV → Exhausted broth containing I
- Extraction with phosphate buffer (pH 7.3) → Phosphate buffer containing rifamycin B and II, III, and IV
- Ethyl acetate containing major quantity of barbital

- Adjusted to pH 6.5 buffer solution; washed extensively with ethyl ether → Ethyl ether containing remaining barbital

Acidification to pH 2.0; extraction with chloroform

- Chloroform extract containing rifamycin B

Aqueous phase extracted exhaustively with ethyl acetate

- Ethyl acetate containing II, III, IV, and small quantities of I → Aqueous phase exhausted
The mixture of radioactive metabolites was fractionated by countercurrent distribution with ethyl acetate-0.01 N HCl as solvent system. After 100 transfers, each tube was analyzed for radioactivity, and the data were plotted in a graph. Four well-separated maxima were found, indicating the presence of four distinct radioactive compounds which had the following partition coefficients: (I) maximum at tube 14, partition coefficient 0.16; (II) maximum at tube 31, partition coefficient 0.45; (III) maximum at tube 59, partition coefficient 1.44; (IV) maximum at tube 86, partition coefficient 6.14. Checking the various extracts given in Fig. 1, it was found that major quantities of I had remained in the original exhausted culture filtrate. For its isolation, broth was evaporated to dryness, and the residue was extracted extensively with ethyl acetate. From this extract, compound I was obtained in fairly pure form.

For the isolation and characterization of these products, it was necessary to repeat the fermentations in larger scale, substituting radioactive barbital for the nonlabeled compound.

The same extraction procedure was used. The various radioactive metabolic products were added to the final ethyl acetate extract to serve as tracers. Thus, it was possible to isolate compounds I, II, and III in sufficient quantities to allow their characterization by different analytical methods, such as microanalysis, infrared and ultraviolet spectrophotometry, and nuclear magnetic resonance. Compound IV had to be separated from nonradioactive impurities via another countercurrent distribution with a different solvent system consisting of ethyl acetate-petrol ether-methanol-0.01 N HCl (2:1:1:2). All compounds were purified by repeated crystallization from acetone and had the following characteristics (structures are given in Fig. 2).

(I) Ultraviolet spectrum (in borate buffer, pH 10), $\lambda_{max}$ 243 m$\mu$; $E_{1%}^{m}$ = 437; mp 220-221 C. Analysis: C$_6$H$_8$N$_2$O$_4$; calculated: C, 41.86; H, 4.68; N, 16.30, N, 16.90, O, 37.18; found: C, 41.82, H, 4.64, N, 16.30, O, 37.18. From these data and from the n.m.r. spectrum, this compound was identified as 5-ethyldialuric acid. Confirmation was obtained by comparison with a synthetic sample prepared using the method of Marberg and Stanger (1939).

(II) Ultraviolet spectrum (borate buffer, pH 10), $\lambda_{max}$ 238 m$\mu$; $E_{1%}^{m}$ = 508; mp 172-175 C. Analysis: C$_6$H$_8$N$_2$O$_4$; calculated: C, 47.99; H, 6.04, N, 13.99, O, 31.97; found: C, 48.02, H, 6.30, N, 13.51, O, 30.17. From these data and from the n.m.r. spectrum, this compound was identified as 5-(beta-hydroxylethyl)-5-ethylbarbituric acid. Confirmation was obtained by comparison with a synthetic sample prepared according to Goldschmidt and Wehr (1957).

(III) Ultraviolet spectrum (borate buffer, pH 10), $\lambda_{max}$ 268 m$\mu$, $E_{1%}^{m}$ = 635; mp 189-190 C. Analysis: C$_6$H$_8$N$_2$O$_4$; calculated: C, 46.1, H, 5.22; N, 17.95, O, 30.8; found: C, 45.88, H, 5.55, N, 18.21, O, 31.16. From these data and from the n.m.r. spectrum, this compound was identified as 5-ethylbarbituric acid. Confirmation was obtained by comparison with a synthetic sample prepared according to Merkatz (1919).

(IV) Ultraviolet spectrum (borate buffer, pH 10, and phosphate buffer, pH 7.38) not well defined, shoulders at 240 and 280 m$\mu$; mp 161-163 C. Analysis: C$_6$H$_8$N$_2$O$_4$; calculated: C, 47.5, H, 6.97, N, 13.95, O, 31.6; found: C, 47.51, H, 7.15, N, 13.70, O, 31.64. From these data and the n.m.r. spectrum, this compound was identified as diethylmalonic acid monoureid. Confirmation was obtained with a synthetic sample prepared according to Aspelund and Stolt (1955).

**Table 1. Average percentage of barbital-2-C$^14$ and barbital-5-C$^14$ converted into the four metabolites by Streptomyces mediterranei**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Barbital-2-C$^14$</th>
<th>Barbital-5-C$^14$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Ethyliduralic acid</td>
<td>1.76</td>
<td>1.70</td>
</tr>
<tr>
<td>5-(beta-hydroxyethyl)-5-ethylbarbituric acid</td>
<td>2.19</td>
<td>2.09</td>
</tr>
<tr>
<td>5-Ethylbarbituric acid</td>
<td>0.51</td>
<td>0.70</td>
</tr>
<tr>
<td>Diethylmalonic acid monoureid</td>
<td>3.09</td>
<td>3.30</td>
</tr>
<tr>
<td>Total barbital converted</td>
<td>7.55</td>
<td>7.79</td>
</tr>
</tbody>
</table>

**Fig. 2. I, 5-ethyliduralic acid; II, 5-(beta-hydroxyethyl)-5-ethylbarbituric acid; III, 5-ethylbarbituric acid; IV, diethyl-malonic acid monoureid; V, barbital.**
The quantity of each of the four metabolic compounds was determined by measuring the total radioactivity of the individual fractions of the countercurrent distribution (Table 1).

As had been expected, the distribution of the four metabolic products was essentially the same for the barbital-2-C\textsuperscript{14} and for the barbital-5-C\textsuperscript{14} These results were confirmed by the determination of the radioactivity of the nonutilized barbital, which amounted to 88 to 90\%. The difference from 100\% is attributed to experimental error.

In contrast to compounds I, II, and III, which appeared to be true metabolic products, IV could have arisen as an artifact of the isolation procedure. [Aspelund and Stolt (1955) have demonstrated that the diethylmalonic acid monoureid is a product of the degradation of barbital under mild alkaline conditions.] To investigate this hypothesis, the normal quantity of radioactive barbital was added to the RFB/159 medium and incubated in the absence of the microorganism for 120 hr. Extracts and analyses were carried out in the usual manner. No traces of compound were found.

The influence of the four metabolites on the production of rifamycin was tested by carrying out fermentations with \textit{S. mediterranei}, substituting barbital in the RFB/159 medium by varying the amounts of each of these compounds. Culture filtrates were analyzed by ultraviolet spectrophotometry and paper chromatography by the methods of Gallo, Sensi, and Radaelli (1960) and Sensi et al. (1960). Although none of the compounds favored the production of rifamycin B, it was observed that both 5-ethyl-dialuric acid and 5-ethylbarbituric acid markedly inhibited the total antibiotic formation at concentrations of 0.5 and 1 g per liter of culture medium, respectively. However, these same compounds at lower concentrations each enhanced the formation of one of the still badly defined antibiotic fractions of the rifamycin complex.

**Discussion**

The use of radioactively labeled barbital permitted the detection of several metabolic products of this compound. The rifamycin B isolated from the fermentations did not contain any radioactivity; thus, it can be concluded that neither barbital nor any of its metabolic products act as precursors of this antibiotic. The action of barbital must, therefore, be an inhibition of the normal metabolic pathway of \textit{S. mediterranei}. The level on which this interference takes place remains to be established. An important requirement for the inhibitory action appears to be the presence, in position five, of substituents of lipophilic nature, such as alkyl and aryl groups. In addition, double substitution is required. Thus 5,5-diethylbarbituric acid is active, whereas 5-ethylbarbituric acid does not stimulate the production of rifamycin B. The latter compound, however, possesses the ability at lower concentrations to stimulate the production of rifamycins C and D. A similar effect had previously been noted by Margalith and Pagani (1961) for 5,5-diethylbarbituric acid, a compound doubly substituted in position five but of definitely lower lipophilic nature. Hence, hydroxyl groups in the side chains, as in 5-(beta-hydroxyethyl)-5-ethylbarbituric acid and in 5-ethylidialuric acid, reduce even further the lipophilic character and cannot be expected to shift the antibiotic production toward rifamycin B. It may be of interest to note that the hypnotic action of the barbituric acid derivatives in human organisms has been correlated with the lipophilic nature of the side chains (Burger, 1960).

The mechanism of this metabolism of barbital for compounds I, II, and III must be considered as oxidative degradation of the side chain, whereas the formation of compound IV could be attributed to hydrolysis during fermentation. Similar results were described by Goldschmidt and Wehr (1957) in their study on the metabolism of barbital in rats. In fact, these authors identified by radioautography small quantities of 5-(beta-hydroxyethyl)-5-ethylbarbituric acid (II), 5-ethylbarbituric acid (III), and a conjugated product in the urine of the animals. The latter was considered a complex between II and gluconic acid, based upon a similar complex discovered by Titus and Weiss (1955) in their studies on the biodegradation of pentobarbital.

It appears that the metabolic degradation of barbital by culture of \textit{S. mediterranei} follows patterns similar to those established in animals. Only the formation of 5-ethylidialuric acid has to be considered a variation of this established reaction.

**Acknowledgments**

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**Literature Cited**


