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Received March 4, 1963. P.S.E.B.M., 1963, v113.

Observations on Mechanism of Action of the Antifungal Peptide, Ro2-7758.* (28312)

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The antifungal sulfur-containing peptide, Ro2-7758 (formerly X-5079C), described recently by Grunberg *et al.*(1), has certain properties not shared by other antibiotics. Besides being the first systemically useful antifungal peptide to be described, the wide discrepancy in its apparent *in vitro* and *in vivo* activities and its limited antimicrobial spectrum make an investigation of its *in vitro* activity somewhat difficult. Its apparently enhanced activity *in vivo* has not been explained on the basis of conversion to a more active form by mammalian tissues(2). A previous report from this laboratory(3) showed a markedly thickened cell wall of *Mucor corymbifera* when grown on Sabouraud agar in the presence of the drug. The present report deals with further studies attempting to demonstrate the biochemical mechanism of action.

Materials and methods. Mucor corymbifera (Duke Fungus Registry no. 2902) was used as test organism in reversal experiments. Of several organisms tested, this one had been found previously to give the most marked inhibitory pattern(3). Fleischmann's wet yeast was obtained from Standard Brands, Inc. Starch-free baker's yeast from Anheuser-Busch was used for preparation of the cell-free sulfate reductase system(4). All chemicals used were reagent grade and were used without further purification. The Ro2-7758 was obtained from Hoffmann-LaRoche.

Attempts to overcome inhibition of *M. corymbifera* with various substances were done on Sabouraud agar plates using a crossed filter paper strip technique similar to the one described by Weinberg *et al.*(5). In this procedure, a filter paper strip impregnated with a solution of the compound being tested for possible reversal of inhibition was placed on a Sabouraud agar plate uniformly streaked with a spore suspension of the test organism. Molar concentrations of these compounds were 1 × and 10 × that of the antibiotic, which has a molecular weight of about 3000. After 10 minutes, another filter paper strip soaked in a solution of 1.0 mg/ml Ro2-7758 was placed on the plate at an angle of 90° to the first strip. Plates were observed at 48 hours for presence or absence of continued growth at the intersection of the two strips.

Measurements of uptake of $S^{35}O_4$ by intact yeast cells were done in 0.1 M potassium phosphate buffer at pH 7.0, containing 1% glucose. Aliquots were removed periodically, clarified by centrifugation, and the activity remaining in the supernatant solution was measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.). Calculations of sulfate uptake were made after determining packed cell volume by centrifugation of an aliquot in a Wintrobe hematocrit tube at 2000 × g for 15 minutes. Protein content of each sulfate reductase preparation was estimated by absorption of the solution at 280 λ and 260 λ.

Results. Gross appearance of cultures of *M. corymbifera* on Sabouraud agar plates inhibited by Ro2-7758 was as described previously(3). When a filter paper strip impregnated with the drug was placed on a plate

* Aided by grant from Nat. Inst. Health, U.S.P.H.S.

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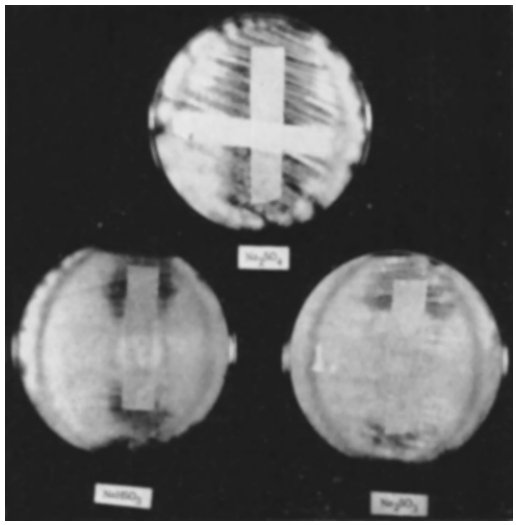

FIG. 1. Effect of sulfate and sulfite on inhibition of *M. corymbifera* by Ro2-7758 on Sabouraud agar. The vertically viewed filter paper strip on each plate was impregnated with Ro2-7758, 1.0 mg/ml. Each horizontally viewed strip was impregnated with a 10.0 mg/ml solution of the compound designated on label. Plates were incubated 48 hr at 37°C. Note growth of aerial mycelium at intersection of strips on 2 lower plates.

streaked with the test organism, appearance of the culture over the entire surface of the plate was undifferentiated up to 24-30 hours. After this period, however, growth adjacent to the drug-impregnated strip ceased, while that at the periphery of the plate continued (Fig. 1, top). There was no absolutely clear zone of inhibition as is seen with agents such as penicillin when tested against susceptible bacteria.

The following were tested by the crossed filter paper strip method for reversal of inhibitory action: amino acids, 29; vitamins, 3; mono-, di-, and trivalent inorganic cations, 16; sugars, 6; inorganic anions, 16; organic anions, 16; other organic compounds, 13. Of these, inhibition was consistently overcome by sulfite and cysteine, but not by sulfate (Fig. 1). The possibility that this reversal was an apparent phenomenon due to creation of a reducing environment in the agar was ruled out when it was shown that nitrite, thioglycollate, glutathione, or ascorbic acid was without effect.

To determine if Ro2-7758 was destroyed or inactivated by sulfite, a solution of the antibiotic (2.0 mg/ml) was added to an equal volume of sulfite (20 mg/ml), pH 6.0, and incubated at room temperature for one hour. Following this, the solution was dialyzed 18 hours against 2 changes of distilled water at 5°C to remove sulfite. Assay of the solution against *M. corymbifera* showed no loss of activity as compared with a solution treated identically but with sulfite omitted. This apparent metabolic specificity of sulfite in overcoming inhibition suggested an effect of the antibiotic on either uptake of sulfate, or its metabolism after uptake by a noncompetitive inhibition of the sulfate reductase system.

Experiments on uptake of $S^{35}O_4$ by intact yeast showed that sulfate was taken up in 2 phases. The first, with a steep slope, was complete in 6-8 minutes; sulfate space, calculated on the basis of the packed cell volume, was approximately 45% of total cell volume. Following this period of rapid uptake, the slope of the uptake curve abruptly decreased, indicating a slow phase of sulfate uptake probably associated with sulfate metabolism. The initial rapid equilibration of sulfate with the cell space was totally unaffected by Ro2-7758 at 10 mg/ml, indicating that the drug had no effect on the passage of sulfate into the cell. In the presence of 10 mg/ml drug, however, the slope of the slower uptake curve was reduced to 0.37 from a control value of 3.95, or 91% inhibition.

To determine if this block of metabolism occurred at the sulfate reduction step, as was indicated by the reversal experiments, Ro2-7758 was added to an unfractionated cell-free sulfate reductase system and the $S^{35}O_3$ formed from $S^{35}O_4$ was determined by the method of Wilson *et al.*(4). Results are shown in Table I. Following a marked stimulation of the rate of sulfate reduction at the lowest drug concentration used, inhibition became apparent at higher concentrations and reached 98% at 10.0 mg/ml (Table I, Exp. C). When sulfate was added to the enzyme system prior to addition of the drug, substrate concentration had virtually no influence on the percent inhibition obtained at the highest drug concentration (Table I, Exp.

TABLE I. Effect of Ro2-7758 on an Unfractionated Cell-Free Sulfate Reductase System from Yeast.

	Exp A		Exp B		Exp C	
	CPM	% change	CPM	% change	CPM	% change
Control	455	—	1036	—	15499	—
Ro2-7758, 1.0 mg/ml	1010	+122	1759	+70	17977	+16
" 5.0 "	not done		394	−62	1861	−88
" 10.0 "	73	−84	121	−88	303	−98

Values in CPM columns are averages of at least 2 determinations and represent the activity of $S^{35}O_3$ formed from $S^{35}O_4$. In Exp A, carrier sulfate was omitted and $S^{35}O_4$ was added simultaneously with the drug. In Exp B, conditions were the same except 5 μmoles carrier sulfate were added. In Exp C, the drug was added to the reaction vessel 7 min before $S^{35}O_4$ and carrier sulfate; carrier sulfite was added 5 min after addition of substrate. Total protein in each reaction vessel was 2.7, 2.3, and 2.9 mg/ml in Exp A, B, and C, respectively. See Wilson *et al.* (4) for detailed description of method.

A and B). In Experiment C, however, in which the drug was added to the enzyme system 7 minutes before addition of substrate, percent inhibition obtained was considerably greater than when substrate was added first. This is in spite of the fact that the activity of the control preparation in Experiment C was much higher than in Experiment B, possibly due to the delay in addition of carrier sulfite.

Discussion. Most fungi can synthesize all organic sulfur compounds required for normal growth and function from inorganic sulfate(6). Organic sulfur compounds of general distribution among fungi include cysteine, cystine, methionine, thiamine, and biotin. At moderate carbohydrate concentrations on chemically-defined media, the sulfur requirement is about 0.0001-0.0006 M for most fungi(6). Blocking the metabolism of sulfate would seem to be an effective method of antibiosis if the organism either were incapable of taking up and utilizing pre-formed organic sulfur compounds from the environment, or were in an environment which did not furnish these compounds pre-formed. A compound with a specific inhibitory effect on reduction of sulfate to sulfite would also be expected to have low toxicity in higher animals, since mammalian tissue is devoid of such an enzyme system.

The extent to which the observed inhibition by Ro2-7758 of reduction of sulfate to sulfite may be related to the actual therapeutic efficacy of the drug must await more studies when adequate supplies of the drug become available for such work. Suggested approaches to the study should include reconciling the apparent discrepancy in *in vitro* and *in vivo* activities. Although this may be related to inactivation of the drug when assayed against such relatively slowly growing fungi as *Histoplasma capsulatum* and *Blastomyces dermatitidis,* the possibility that organisms *in vivo* may selectively accumulate the drug should not be ignored. A maintained blood level of only 2 μg/ml in a patient would provide such a reservoir of available drug to the fungus cells that amounts may be accumulated far greater than those in the *in vitro* situation. Similarly, the unavailability of compounds *in vivo* capable of reversing the inhibition, such as sulfite, may play a role.

If the inhibition of sulfate reduction is invoked as a possible mechanism of antibiotic action, the matter of the limited *in vitro* antifungal spectrum must be explained, since the sulfate reduction system is of fairly general distribution among fungi. Even though the cytologic location of the sulfate reductase system is not known, the procedure required to extract it from yeast cells does not suggest an extracellular location. An intracellular position would infer the necessity for passage of the drug through a membrane to reach the enzyme system; the high molecular weight of Ro2-7758 and its peptide nature suggest that membranes through which it could pass may be rare. Another possibility is that enzymes involved in sulfate reduction have different protein struc-

tures, similar to the well-known differences in mammalian lactic dehydrogenases. Electrophoretic and sedimentation patterns of these enzymes from different organisms should be examined to determine if such differences exist, and, if so, how the different forms respond to the drug. A limited activity spectrum could also be the result of an ability of actively dividing cells to utilize sulfite and preformed organic sulfur compounds from autolyzing cells in a crowded *in vitro* population.

Of considerable interest would be an analysis of the kinetics of inhibition of the reductase system by Ro2-7758. The difficulty of such a study is manifest by the complexity of the reaction sequence. Wilson *et al.*(4) showed that adenosine triphosphate and magnesium are required, and glucose 6-phosphate plus triphosphopyridine nucleotide, or reduced triphosphopyridine nucleotide, serves as the hydrogen donor. After fractionation of the cell-free extract, adenosine triphosphate sulfurylase plus adenosinephosphosulfate kinase must be added to activate sulfate. Reduction of the resulting 3′-phosphoadenosine-5′-phosphosulfate involves at least 2 enzymes and one heat-stable protein of low molecular weight.

No conclusions can be drawn regarding the kinetics of inhibition from the data presented here. However, two facts may be pertinent. First, when sulfate was added to the reaction vessel prior to addition of Ro2-7758, the amount of substrate added, within the limits tested, did not influence the percent inhibition obtained at the highest drug concentration (Table I, Exp. A and B). This is the pattern usually obtained when dealing with a non-competitive type of inhibition. Second, when the drug was added to the reaction vessel prior to addition of substrate, percent inhibition was considerably greater than that obtained when substrate was added prior to addition of the drug (Table I, Exp. B and C). Such partial protection by substrate from inhibition is suggestive of the situation in which the inhibitor combines with the enzyme at its active site, or substrate-binding site.

Regardless of the final mechanism of inhibition, the data reveal that the sulfate reductase system may be inhibited by a compound with relatively low mammalian toxicity. Such inhibition may also play a role in its therapeutic antibiotic action. The availability of a relatively easy method of assay for this reaction sequence unique in some microorganisms clearly points out the need for attempts to synthesize compounds with high inhibitory action against this system, with a view toward development of more effective antifungal chemotherapeutic agents.

Summary. Inhibitory action of the antifungal sulfur-containing peptide, Ro2-7758 (formerly X-5079C), against the growth of *Mucor corymbifera* was annulled by sulfite and cysteine, but not by sulfate or 96 other substances tested. Experiments with $S^{35}O_4$ showed that the drug had no effect on permeability of yeast cells to sulfate. However, production of $S^{35}O_3$ from $S^{35}O_4$ by a cell-free sulfate reductase system from yeast was first stimulated at low drug concentrations and then inhibited at higher concentrations. It was concluded that this action of the drug on sulfate reductase may be involved in its antibiotic activity.

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Received March 7, 1963. P.S.E.B.M., 1963, v113.