This contrast may point out a fundamental difference in the pathogenesis of these two types of hypertension. An understanding of this difference might shed light on the still mysterious basic derangement in all types of hypertension.

Summary. Arterial hypertension was produced in a group of rats by narrowing one renal artery. These hypertensive rats ingested large doses of a thiazide drug for a period of five weeks. During this period, the drug was completely ineffective for lowering arterial pressure. This is in striking contrast to the great effectiveness of thiazide drugs in alleviating essential hypertension in man. This contrast may point out a fundamental difference in the pathogenesis of these 2 types of hypertension. Moreover, this observation might prove to be clinically useful; hypertensive patients who get a good response to thiazide drugs alone would be more likely to have essential hypertension rather than renovascular hypertension. The inability of the thiazide drugs to alleviate renovascular hypertension in the rat is paralleled by a similar ineffectiveness of the low sodium diet.

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Received September 27, 1963. P.S.E.B.M., 1964, v115.

Antimicrobial Activity of Thiobenzoate and Its *O*-Fluoro- and *M*-Fluoro-Derivatives.* (28869)

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An earlier report from this laboratory showed that the antifungal peptide, Ro2-7758 (Hoffmann-LaRoche), was stimulatory to the yeast sulfate reductase system at low concentrations but inhibitory at higher concentrations(1). Even though some discrepancies were encountered in attempting to correlate this action with all aspects of the activity spectrum of Ro2-7758, it was concluded that the inhibitory effect on sulfate reduction may play a role in its antibiotic action. The suggestion was also offered that a search should be made for synthetic compounds which would inhibit this metabolic pathway which is unique in microorganisms, with an aim toward development of effective antifungal agents.

* This work was aided by grant from Nat. Inst. Health, U.S.P.H.S.

The high sulfur content of Ro2-7758(2) prompted an investigation of the effects of certain sulfur-containing compounds on microbial growth, and an attempt to correlate any observed action with effect on the sulfate reduction system. The following is the result of studies with thiobenzoate and its *o*-fluoro- and *m*-fluoro- derivatives.

Materials and methods. All microorganisms were stock laboratory strains which had been maintained in this laboratory for several years. Tryptic soy medium and Sabouraud dextrose medium were used for cultivation of bacteria and fungi, respectively.

Thiobenzoic acid (TBA) was obtained from Aldrich Chemical Co., Milwaukee, Wisc. The fluorinated derivatives were synthesized by Hynes Chemical Research Corp., Durham, N. C. All 3 thiobenzoates are liquids at room

temperature and relatively insoluble in water but soluble in ethyl alcohol. The *o*-fluoro- (O-FTBA) and *m*-fluoro- (M-FTBA) derivatives, previously undescribed, boiled at 59-61°C at 0.6 mm Hg and 61-62°C at 0.8 mm Hg, respectively. Elemental analysis showed (*ortho-*) : C, 53.45%; H, 2.90%; F, 12.92%; S, 21.3%; (*meta-*) : C, 54.86%; H, 3.59%; F, 12.84%; S, 19.54%; calculated for C_7H_5OFS : C, 53.8%; H, 3.2%; F, 12.15%; S, 20.5%. Solutions of each were prepared by suspending in water, saline, or buffer and neutralizing by the slow addition of NaOH or KOH. Some H_2S was evolved during the process of neutralization, but could not be detected from these solutions afterwards. Also upon neutralization, an insoluble residue formed which represented about 5% of the starting material. After filtration from the solution and exhaustive washing with water, elemental analysis and infrared spectrometry of the residue were entirely compatible with a disulfide structure, the expected oxidation product of the sulfhydryl compound. Neutralized, filtered solutions were discarded after 5 days. All other chemicals were reagent grade and were used without further purification.

The unfractionated cell-free sulfate reductase system was prepared and its activity assayed by the method of Wilson, Asahi, and Bandurski(3); protein content of each enzyme preparation was estimated by absorption of the solution at 260 λ and 280 λ. Radioactivity of $S^{35}O_3$ formed from $S^{35}O_4$ was determined in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co.). An unfractionated pyruvic carboxylase was prepared and its activity assessed as described in step 1 of reference (4) Activity of a crystalline urease (Sigma, Type V) was followed as CO_2 evolution from urea in a Warburg respirometer at 30°C in 0.2 M potassium citrate buffer, pH 5.85. Hydrogen sulfide was detected with the N,N'-dimethyl-*p*-phenylenediamine reagent(5). Anaerobic glycolysis and oxygen uptake of a non-proliferating *Candida albicans* suspension were measured as described earlier(6). Tests for reversal of growth inhibition by the thiobenzoates were done with the crossed filter paper strip method(1). Acute toxicity determinations were performed with albino Swiss male mice which averaged 20 g in weight. Chronic toxicity tests were done with adult albino male rabbits at a dosage of 50 mg/kg daily for 40 days. Upon sacrificing the rabbits at the end of the test period, tissues were fixed in buffered formalin and stained with hematoxylin-eosin stain for microscopic examination. Hippuric acid was estimated by the method of Umberger and Fiorese(7).

Results. Initial investigations of the antimicrobial spectra of the thiobenzoates showed that filamentous fungi (*Aspergillus niger, Microsporum gypseum, Penicillium* sp., *Rhizopus oryzae,* and *Trichophyton mentagrophytes*), yeast-like fungi (*Candida albicans* and *Cryptococcus neoformans*). and gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) were inhibited by concentrations of 10 to 100 μg/ml in broth culture, whereas gram-negative bacteria (*Aerobacter aerogenes, Pseudomonas aeruginosa,* and *Escherichia coli*) were inhibited only at concentrations of 1000 μg/ml or greater. Detailed data for 2 representative organisms are shown in Table I. The size of inoculum was found to influence the percent inhibition obtained with each compound at any given concentration, as would be expected with an inhibitor which combines with the cell at a finite number of binding sites.

Prolonged incubation of inhibited cultures caused resumption of growth at concentrations of 10 to 50 μg/ml of each inhibitor, a concentration range which gave complete inhibition when measured after 16 to 24 hours incubation. The thiobenzoates were not inactivated by incubation in sterile broth for 7 days, however, and 32 transfers in the presence of a sublethal concentration of TBA did not evoke the emergence of a resistant population. Initiation of growth after prolonged incubation may thus be due to enzymatic degradation of the inhibitor.

To determine if onset of inhibition of growth by thiobenzoates was immediate or delayed, a 16-hour broth culture of *C. albicans* was divided into 9.0 ml aliquots, 1.0

TABLE I. Inhibition of Growth of *B. subtilis* and *C. albicans* by Thiobenzoates.

Conc, μg/ml	*B. subtilis*						*C. albicans*					
	O-FTBA		M-FTBA		TBA		O-FTBA		M-FTBA		TBA	
	18 hr	24 hr	18 hr	24 hr	18 hr	24 hr	18 hr	24 hr	18 hr	24 hr	18 hr	24 hr
.1	9%	4%	59%	7%	13%	20%	0%	5%	0%	0%	0%	0%
1.0	69	27	62	20	26	25	3	4	5	0	5	0
10	59	18	59	12	33	24	21	17	88	71	92	86
25	86	46	97	91	92	86	84	72	100	100	95	96
50	100	100	100	100	100	100	100	100			100	100

Tubes of liquid media containing inhibitors at indicated concentrations were inoculated with one drop of a dilute cell suspension and incubated at 37°C. Growth of cultures at 18 hr and 24 hr was determined by optical density of each tube at 640 λ. Data expressed as % inhibition of growth as compared with control.

ml TBA, O-FTBA, or M-FTBA in water was added to give a final concentration of 1000 μg/ml, and the optical density of each culture was measured at intervals thereafter for 7 hours. Fig. 1 shows that the control culture maintained a linear growth rate for at least 6 hours; cultures containing the TBA and M-FTBA ceased growing immediately, the decreased optical density possibly resulting from a change in size of the cells. The variance obtained with O-FTBA probably reflects only a difference in inherent activity of this compound, since reversal experiments (see below) indicated a similar fundamental mechanism of action for all 3 inhibitors.

To determine if death of the cells occurred at the time growth ceased, each inhibitor was added to a log phase broth culture of *C. albicans* to give a final concentration of 1000 μg/ml and the cultures incubated at 37°C. Washed, non-proliferating cells of *C. albicans* in saline, adjusted to the same optical density as the proliferating broth cultures, were set up in parallel. At intervals aliquots were taken from each flask, the cells washed twice with distilled water, resuspended in water to the original volume, and a loopful streaked on an agar plate. Results obtained after 12 hours' incubation of the plates are shown in Table II. Irreversible

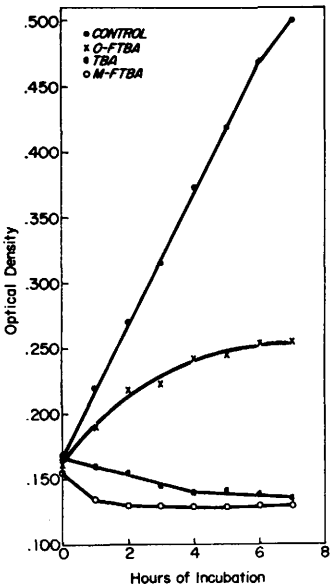

FIG. 1. Immediate fungistatic effect of thiobenzoates on *C. albicans*. See text for details.

TABLE II. Survival of *C. albicans* After Exposure to Thiobenzoates for Various Intervals.

Hr exposure	TBA		O-FTBA		M-FTBA	
	Proliferating cells	Non-proliferating cells	Proliferating cells	Non-proliferating cells	Proliferating cells	Non-proliferating cells
2	++	++	++	++	++	++
4	++	++	++	++	++	++
6	++	++	++	++	+	++
8	++	++	++	++	—	++
10	+	++	++	++	—	++
12	—	++	++	++	—	++

Final concentration of each thiobenzoate was 1000 μg/ml. ++, growth equivalent to that of control; +, markedly reduced growth; —, no growth.

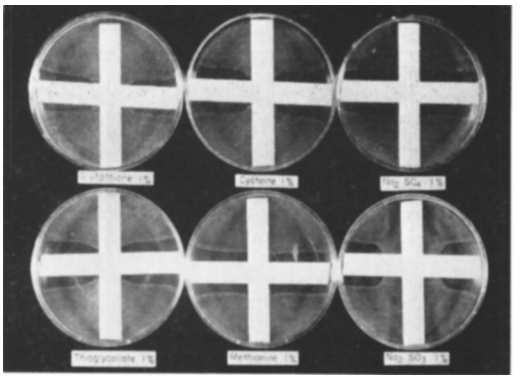

FIG. 2. Effect of certain sulfur-containing compounds on inhibition of growth of *B. subtilis* by M-FTBA.

The horizontally-viewed strips on each plate were impregnated with 1% M-FTBA. Each vertically-viewed strip was saturated with a neutralized 1% solution of the compound designated on label. Incubation was at 37°C for 18 hr.

damage to all proliferating cells occurred between 6 and 8 hours with M-FTBA, and between 10 and 12 hours with TBA. O-FTBA did not have a fungicidal action within the period of the experiment, even though growth in broth of *C. albicans* ceased after about 6 hours (Fig. 1). Non-proliferating cells were not affected by either compound within the 12-hour period.

A reversible bacteriostasis was also demonstrated with *B. subtilis* as test organism. Filter paper strips impregnated with a 1% solution of each thiobenzoate were placed on agar plates seeded with the bacterium. At intervals up to 8 hours, a plate was removed from the incubator, a filter paper strip impregnated with a 1% sulfite solution was applied (as in Fig. 2), and plates were returned to the incubator. After 24 hours' incubation, reversal of inhibition was as complete in each case as occurred with sulfite in the experiment shown in Fig. 2.

A possible relationship of inhibition of sulfate reduction to inhibition of cell growth was demonstrated in reversal experiments on agar plates and in broth. Fig. 2 shows results obtained with the crossed filter paper strip method of reversal. Sulfite, cysteine, glutathione, and thioglycollate reversed the inhibition to varying degrees, while sulfate, methionine, and 70 other compounds tested did not; reversal was consistently more marked with sulfite, the immediate product of sulfate reduction. Similar results were obtained with all 3 thiobenzoates, and with either *B. subtilis* or *C. albicans* as test organism. It was concluded that this reversal was a specific metabolic action and not a result of creation of a reducing environment in the agar, since nitrite caused no annulment of inhibition, and the inhibitory action of each thiobenzoate was the same when the plates were incubated at 0.2 atmosphere as when incubated at ambient pressure. Reversal experiments in broth, in which growth of cells was measured as an increase in optical density of each culture, gave results comparable to those obtained with agar plates (Table III).

Evidence against production of H_2S as the possible inhibitory mechanism of the

TABLE III. Reversal by Certain Sulfur-Containing Compounds of Inhibition of Growth of *C. albicans* by Thiobenzoate.

mg/ml	Sulfate	Sulfite	Thio-sulfate	Methio-nine	Cystine	Cysteine	Gluta-thione	Thiogly-collate
	% reversal							
.001	2	7	2	—	—	—	—	—
.01	1	68	2	0	26	66	30	20
.1	3	63	4	0	43	39	48	30
1.0	3	*	—	*	—	*	27	58
10.0	*		—		—		*	*

Tubes of Sabouraud broth containing thiobenzoate at a final concentration of 20 μg/ml, along with the compound being tested for reversal activity at concentration shown in left column, were inoculated with one drop of a dilute suspension of *C. albicans*; control tubes contained each sulfur-containing compound but no thiobenzoate. Growth was assessed by optical density measurements after 24 hr at 37°C. Percentage reversal was calculated by comparing readings of each tube containing thiobenzoate with appropriate control.

* Denotes concentration at which compound being tested for reversal activity inhibited growth; — indicates that a compound was not tested at that concentration.

TABLE IV. Effect of Thiobenzoates on an Unfractionated Cell-Free Sulfate Reduction System from Yeast.

	TBA		M-FTBA		O-FTBA	
mg/ml	CPM	% inhibition	CPM	% inhibition	CPM	% inhibition
0	3004	—	4798	—	3104	—
1.0	1735	42	2061	57	2486	20
2.5	730	76	750	84	1235	60
5.0	313	90	269	94	364	88
10.0	127	96	41	99	135	96

Assays were performed by method of Wilson et al.(3). Values in CPM columns are averages of 2 determinations and represent activity of $S^{35}O_3$ formed from $S^{35}O_4$. Total protein in each assay was 8.1, 8.5, and 6.5 mg/ml for TBA, M-FTBA, and O-FTBA, respectively.

thiobenzoates was obtained by incubating a concentrated solution of each compound, with and without a heavy suspension of *C. albicans*, in Warburg vessels containing dilute NaOH in the center wells. Spot tests of the NaOH solutions after 2 hours' incubation were negative for H_2S.

Effects of the thiobenzoates on a cell-free sulfate reduction system from yeast are shown in Table IV. Even though the amount of each compound required to effect virtually complete inhibition was much greater than that required to inhibit growth of cells from an inoculum in culture, the amount of sulfate reducing enzyme(s) present in the Warburg vessel in the *in vitro* cell-free assay system was undoubtedly several orders of magnitude larger than the amount present in the small inoculum. The relatively low order of activity against the cell-free system thus may be more apparent than real.

Assays against other enzyme systems with each thiobenzoate at a final concentration of 5.0 mg/ml showed that pyruvic carboxylase was inhibited 92%, 67%, and 100% by TBA, O-FTBA, and M-FTBA, respectively. Activity of crystalline urease was, however, inhibited only 19%, 50%, and 47% by TBA, O-FTBA, and M-FTBA, respectively. None of the thiobenzoates had any significant effect on oxidation of glucose by washed cells of *C. albicans;* endogenous oxygen uptake was stimulated slightly. Anaerobic glycolysis by *C. albicans* was completely inhibited by all 3 compounds at 5.0 mg/ml. Oxidation of glucose by *C. albicans* was still unaffected by all thiobenzoates after the cells had been exposed to a 30-min period of anaerobiosis (100% N_2) prior to addition of glucose from the side arms of the Warburg vessels and restoration of the aerobic atmosphere.

Results of acute toxicity tests of each thiobenzoate in mice are shown in Table V. Surviving mice showed no effects during 14 days' observation. Death of the non-survivors occurred from 10 to 30 minutes after intraperitoneal injection, and was usually preceded by coma and signs of respiratory distress. Convulsions preceded coma only occasionally, and opisthotonus was not noted.

M-FTBA was chosen for chronic toxicity tests in rabbits because its acute toxicity was intermediate between that of TBA and O-FTBA. Each rabbit received a total dose of 2.0 g/kg over a period of 40 days. Weight gain and general appearance of the test rabbits were similar in all respects to those of the control rabbits over the test

TABLE V. Acute Toxicity of Thiobenzoates in Mice.

	Dose, mg/kg								
	0	100	200	250	300	350	400	450	500
TBA	6	6	6	0					
O-FTBA	6	6	6		6		5		1
M-FTBA	6	6	6		6	3	3	1	

Numbers indicate survivors in each group of 6 mice 14 days after a single injection of designated dose.

period, and there was no necrosis of the injection sites. Post mortem examination after sacrificing all animals with intravenous Nembutal showed the rabbits which received M-FTBA had a congestive glomerulitis with hyperplasia of both endothelial and epithelial cells. Protein-like material occupied the Bowman's capsules. Tubules appeared normal. There was generalized congestion of the liver with parenchymal cell damage and bile duct proliferation. Multinucleate hepatic cells were numerous. The spleen showed sinusoidal eosinophilia, congestion, and increased mitoses in germinal centers. There was epithelial hyperplasia of colloid follicles in the thyroid, along with a fatty infiltrate and numerous microfollicles. Bone marrow displayed a diffuse hyperplasia with numerous and prominent megakaryocytes and myelocytes. Erythrocyte and leucocyte counts of the peripheral blood were within normal limits.

Excretion of hippuric acid by rabbits was demonstrated after a single intramuscular injection of TBA. Excretion was approximately 75% of that obtained after a similar intramuscular injection of sodium benzoate. An enhanced uptake of O_2 by a rat liver homogenate was also demonstrated upon addition of each thiobenzoate to the Warburg vessel. This uptake was proportional to initial concentration of each compound, and rates of oxidation of all 3 were similar.

To determine if TBA would produce methemoglobin *in vitro,* 1.0 ml of human blood was added to 19 ml of distilled water to induce hemolysis. One ml of the hemolyzed blood was then added to 10.0 ml of TBA in saline to give a final concentration of 1.0 mg/ml. No change in the characteristic absorption spectrum of oxyhemoglobin was noted. None of the thiobenzoates at a concentration of 1.0 mg/ml induced *in vitro* hemolysis of human erythrocytes.

Discussion. Experiments with compounds capable of antagonizing the antifungal and antibacterial actions of the thiobenzoates imply that inhibition of the sulfate reduction system is the principal antimicrobial mechanism of these compounds. Even though absolute specificity for this system is not revealed by enzymatic data, experiments with intact cells of *C. albicans* demonstrate an absence of effects on the aerobic hexose dissimilation pathway. Likewise, some degree of specificity may be inferred from the fact that proliferating cells were irreversibly damaged after 6 and 10 hours' incubation with TBA and M-FTBA, respectively, while washed, non-proliferating cells in a parallel experiment remained viable for at least 12 hours. Depletion of an essential metabolite(s) subsequent to inhibition of formation of that metabolite(s) thus appears to be one possible mode of action, and is compatible with an hypothesis of sulfate reduction inhibition. The significance of inhibition of pyruvic carboxylase is difficult to evaluate; inhibition of cell growth could not be reversed by pyruvate, and certain compounds which are highly inhibitory to a partially purified carboxylase(8) do not possess antimicrobial activity (Gale, unpublished data). A general effect on sulfhydryl groups of enzymes seems unlikely in view of the relatively low order of activity against crystalline urease.

Data obtained previously(1) with the antifungal sulfur-containing peptide, Ro2-7758, and the present data suggest that the sulfate reduction system is inhibited by certain sulfur-containing compounds; it remains to be determined whether or not enhanced specificity and lower mammalian toxicity may be achieved with other substituted sulfhydryl compounds.

Summary. Thiobenzoate and its *o*-fluoro- and *m*-fluoro-derivatives were shown to inhibit the growth of certain fungi and gram-positive bacteria at concentrations below 100 μg/ml. Gram-negative bacteria were not affected at concentrations below 1000 μg/ml. Inhibition of growth of susceptible cells was antagonized by sulfite, cysteine, cystine, thioglycollate, and glutathione; sulfate and methionine were not antagonistic. A cell-free sulfate reduction system from yeast was inhibited by each thiobenzoate. It was concluded that inhibition of the microbial sulfate reduction system may be the principal antimicrobial mechanism.

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Received September 27, 1963. P.S.E.B.M., 1964, v115.

Effect of N-Dichloroacetyl-dl-Serine on Regeneration in the Forelimb of *Diemictylus viridescens.** (28870)

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The urodeles among amphibians possess a high degree of regenerative capacity in adult life. Since the process or regeneration presents many of the phenomena of embryonic development, adult amphibians can be used for the study of developmental processes. It therefore seemed of interest to use regeneration blastemas as testing material for the study of the biological effect of the Na salt of N-dichloroacetyl-dl-serine, which was shown by Levi *et al*(1) to be carcinostatic in certain cases.

Material and methods. Adult salamanders of the species *Diemictylus viridescens* were obtained from commercial dealers in New York City and kept in finger bowls. They were fed tubifex worms for several days before the experiment.

Amputation of the forelimb was carried out at the distal end of the humerus. No narcotics were used for the amputation. The animals were placed in batches of 6 each in a finger bowl. An hour after amputation each of the animals was injected with 1 ml of the Na salt of N-dichloro-acetyl-dl-serine, made up in amphibian Ringer's solution. Two concentrations were used, 0.1 and 0.2 %, in 2 series of experiments. The injections were repeated daily throughout the experiment. A control series using 1 ml of amphibian Ringer's solution per day was also set up. In other controls no injections were used. Injections were given subcutaneously as near to the region of amputation as possible. When this was not possible (usually after about 15 to 20 days) intramuscular or intraperitoneal injections were given at any region of the body.

The animals were occasionally fed with tubifex worms during regeneration, but they did not eat regularly. The water in the bowls in which the animals were kept was changed every other day.

Camera lucida drawings were made of the regenerates at 35, 45, and 56 days after amputation. The experiment was then terminated.

The various phases of regeneration may be split into 4 distinct phases following the account of Hay and Fischman(2); (1) a wound-healing phase: within 24 hours after amputation, when the transected surface of the limb was covered with a well-defined wound epithelium; (2) a dedifferentiation phase: usually by the fifth day after amputation, when wound healing was complete and a disintegration of the damaged muscles was apparent; (3) a growth phase: following the establishment of a definitive blastema, when extensive dedifferentiation ceased and growth and mitosis became the dominant features of the regenerative process; and (4) a differen-

* The data in this paper are taken in part from a thesis, presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Fordham University.