Effect of Tetrazolium Salts on Selected Bacterial Species.* (26112)

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The reduction of tetrazolium salts by living cells has been utilized extensively as an indicator of active metabolic processes(1-13). However, proper evaluation of the histochemical technic involving tetrazolium depends on understanding the effects of these salts *per se* upon living systems. Attempts have been made to characterize the effects on bacteria by studying their influence on growth and on isolated enzyme systems(15). The following communication deals with a detailed study of the growth inhibitory effects of these compounds on a number of bacterial species.

Method. *Escherichia coli, Salmonella typhimurium, Shigella sonnei, Proteus vulgaris, Staphylococcus aureus, Streptococcus pyogenes* and *Bacillus subtilis* cultures were isolated in our laboratory and carried on Trypticase Soy Agar (B. B. L.). Heart Infusion Broth (Difco), dispensed in 4 ml amounts in 15 x 125 ml tubes fitted with metal closures, was used in the experiments. The tetrazolium compounds used included 2 monotetrazolium salts, triphenyl tetrazolium chloride (TTC) and iodo-nitrotetrazolium (INT) and three ditetrazolium salts, neotetrazolium (NT), blue tetrazolium (BT) and nitro-blue tetrazolium (NBT).† Stock solutions of 0.5% were prepared in distilled water, autoclaved and diluted in broth to final concentrations of 2.0 to 500.0 μg/ml (10^{-6} to 10^{-2} moles/liter). Controls contained sterile distilled water instead of tetrazolium solutions. All experiments were carried out in duplicate. Each tube, inoculated with 0.05 ml of an 18 hour broth culture of the organism to be tested, was incubated at 37°C for 24 hours, at which time subcultures in tetrazolium-free media were made. The lowest concentration of each tetrazolium salt which completely prevented growth on subculture was considered the minimal lethal dose (MLD).

Results. MLD values for gram negative and gram positive organisms are shown in Tables I and II. The ditetrazolium salts (NT, BT, NBT) were more effective as bacterial growth inhibitors than the monotetrazolium salts (TTC, INT). This difference is even more striking when the molarity of the solutions is considered. The order of decreasing inhibitory action for both gram negative and gram positive organisms was generally NBT, NT, BT, INT, and TTC. Gram negative organisms were found to be more resistant than the gram positive organisms.

TABLE I. Minimal Lethal Doses of 5 Tetrazolium Compounds on Gram Negative Organisms (24 Hr, 37°C).

Tetrazolium salts	Organisms	Minimal lethal dose μg/ml	Molarity
Monotetrazolium			
TTC	*E. coli*	>500	10^{-2} *
	Sal. typhimurium	>500	10^{-2} *
	P. vulgaris	>500	10^{-2} *
	Sh. sonnei	>500	10^{-2} *
INT	*E. coli*	>500	10^{-3} *
	Sal. typhimurium	>500	10^{-3} *
	P. vulgaris	>500	10^{-3} *
	Sh. sonnei	500	10^{-3}
Ditetrazolium			
NT	*E. coli*	125	10^{-4}
	Sal. typhimurium	250	10^{-4}
	P. vulgaris	500	10^{-3}
	Sh. sonnei	32	10^{-5}
BT	*E. coli*	250	10^{-4}
	Sal. typhimurium	500	10^{-3}
	P. vulgaris	>500	10^{-3} *
	Sh. sonnei	125	10^{-4}
NBT	*E. coli*	125	10^{-4}
	Sal. typhimurium	125	10^{-4}
	P. vulgaris	125	10^{-4}
	Sh. sonnei	8	10^{-5}

* Molarity of highest concentration tested; no inhibition observed.

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† TTC, triphenyl tetrazolium chloride; INT, 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride; NT, pp'-diphenylene-bis-2-(3,5 diphenyl tetrazolium chloride); NBT, 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'(3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride; BT, 2,2',5,5'-tetraphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride.

TABLE II. Minimal Lethal Doses of 5 Tetrazolium Compounds on Gram Positive Organisms (24 Hr, 37°C).

Tetrazolium salts	Organisms	Minimal lethal dose μg/ml	Molarity
Monotetrazolium			
TTC	*Staph. aureus*	>500	10^{-2} *
	Strep. pyogenes	32	10^{-3}
	B. subtilis	32	10^{-3}
INT	*Staph. aureus*	64	10^{-4}
	Strep. pyogenes	4	10^{-5}
	B. subtilis	16	10^{-5}
Ditetrazolium			
NT	*Staph. aureus*	8	10^{-5}
	Strep. pyogenes	<2	10^{-6}
	B. subtilis	<2	10^{-6}
BT	*Staph. aureus*	8	10^{-5}
	Strep. pyogenes	4	10^{-6}
	B. subtilis	4	10^{-6}
NBT	*Staph. aureus*	8	10^{-5}
	Strep. pyogenes	<2	10^{-6}
	B. subtilis	<2	10^{-6}

* Molarity of highest concentration tested; no inhibition observed.

With one exception all the salts tested were lethal to gram positive organisms in concentrations ranging from less than 2 up to 64 μg/ml. However, *Staph. aureus* was resistant to the action of TTC even at 500 μg/ml, the highest concentration used. In the case of the gram negative organisms tested, TTC was found ineffective, even in concentrations of 500 μg/ml, while the other salts showed varying degrees of antibacterial activity. *Sh. sonnei* was found to be most sensitive to the inhibitory effects of tetrazolium salts, and *P. vulgaris* the least sensitive.

Intense reduction of the tetrazolium salts to colored formazans was generally associated with a decreased lethal effect. This was more apparent in the case of the gram negative organisms, which were less affected by the tetrazolium salts, than in the case of the gram positive organisms.

Discussion. The previously reported(1) inverse relationship between inhibition of bacterial growth by the tetrazolium compounds and reduction of these compounds to their respective formazans was confirmed. It has also been shown that the tetrazolium compounds are more lethal to gram positive organisms than to gram negative organisms. These results confirm the previously reported effects of NT(1). In the living state the gram positive bacteria are generally more permeable to dyes. They are also more susceptible than gram negative organisms to the bacteriostatic effect of triphenylmethane dyes and to the action of surface tension reducing agents(14). These properties could account for the different reactivity to tetrazolium of the 2 bacterial groups, since tetrazolium salts are surface tension reducing agents and have certain structural resemblance to triphenylmethane dyes. Quantitative differences in the metabolism of the 2 groups of bacteria might also play a role.

The inverse relationship between tetrazolium reduction and growth inhibition reflects the necessity for active cellular metabolic processes in the reduction to formazan. Growth inhibition is probably due to interference with cellular enzyme systems, thus blocking pathways for continuous transference of electrons to the tetrazolium salt.

A similarity may be noted between the inhibitory effectiveness of the tetrazolium salts on bacterial growth reported here and their influence on pseudo - cholinesterase(15) While this enzyme is not commonly found in bacteria, tetrazolium salts may exert inhibitory effects on bacterial enzyme systems through a similar mechanism. The presence of the biologically reactive quaternary amine group in the tetrazolium molecule tends to support this contention. An earlier study (16) reporting higher toxicity for BT in mammals as compared with the monotetrazolium derivatives is in accord with findings in bacteria herein described.

Summary. The influence of 5 tetrazolium compounds on growth of various bacterial species is reported. Gram negative organisms are more resistant to the inhibitory effects of these compounds than are the gram positive organisms. The ditetrazolium salts are more lethal to the bacteria than are the monotetrazolium salts.

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Immunologically Competent Cells in Adult Mouse Liver: Studies with Parent-to-Hybrid Radiation Chimeras. (26113)

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Evidence has been accumulated that tissues other than spleen, lymph nodes, and bone marrow contain immunologically competent cells. Peripheral white blood cell fractions (1,2), thymus(3,4), and liver, kidney, and heart muscle(4) contain cells able to confer adoptive immunity to transplantation antigens and to cause rejection of heterologous or homologous bone marrow following implantation in X-irradiated mice.

To obtain further evidence of the immunologic potentialities of adult liver tissue, we grafted liver cells into sublethally irradiated F_1 hybrids, using inbred parental strain mice as donors. In this genetic situation, it is expected that only immunologically active cells of the grafted liver can react positively against the F_1 hybrid antigens. Therefore, a fatal reaction should occur in the F_1 recipients of certain strain combinations, similar to the wasting disease described after parental spleen(5,6), thymus(7), or leukocyte(8) implantation.

Methods. Sixteen- to 20-week-old male and female (C57BL $\times$ 101)F_1, (C3H $\times$ 101)F_1, and (101 $\times$ C3H)F_1 recipient mice were exposed to a single dose of total-body radiation of 600 or 900 r. Mice were irradiated in a revolving partitioned lucite cage under the following conditions: 300 kvp, 20 ma, 4.7 mm of Be inherent and 3 mm of Al added filtration, 100 cm target-to-mouse distance, hvl 0.45 mm of Cu, 76 r/min.

Within 1 hour after irradiation, each 600-r irradiated mouse received intraperitoneally a suspension of parental strain liver cells, equivalent to 0.25 of a whole liver in 0.5 to 1 ml of Tyrode's solution. Liver cell suspensions were prepared by chopping the organ with scissors and by repeated aspiration of small fragments through a syringe. The preparation was finally passed through a wire mesh screen, and penicillin was added.† Each 900-r irradiated mouse received intravenously within 1 hour, *via* the tail vein, 1 femur-equivalent (about 10×10^6 nucleated cells) of isologous bone marrow (IBM). Subsequently these mice were given an intraperitoneal inoculation of parental liver cells equivalent to 0.1 of a whole liver. Marrow

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† The final suspension of liver cells contained in all instances a mixture of parenchymal, littoral, and biliary cells; these are referred to throughout the text as simply "liver cells."