PARA-HYDROXYBENZOIC ACID, A HYPOXIC RADIOSENSITIZER IN BACTERIAL CELLS

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The relative radioresistance of hypoxic cells present in some tumours is a serious limitation in attempts to increase the therapeutic ratio between tumour control and damage to normal tissue during radiotherapy. Hyperbaric oxygen, fast neutrons and π^- mesons are suggested methods of overcoming this problem. The use of chemical radiosensitizers which effectively act upon hypoxic cells is another approach (1). A major group of such hypoxic sensitizers resemble oxygen in their mode of action, and it has now been fairly well established that their efficiency as radiosensitizers, on a molar basis, is directly related to their electron-affinity (2-4). Sensitization in bacterial systems by p-nitroacetophenone (PNAP) (4-6) and its more soluble derivative NDPP (7,8) led to the testing of other nitro-aromatic compounds such as the nitroimidazoles.Two promising drugs to have emerged recently, metronidazole and misonidazole, have been shown to be effective both in vitro and in vivo, and, more recently, the results of tests with these drugs in patients have been encouraging (9,10). Both these compounds are of particular interest because of their clinical use as trichomonicides, with considerable pharmacological, toxicological and pharmacokinetic information available. It seems quite possible that they may prove to be of value in clinical radiotherapy.

The aim of the present investigation is to look for other compounds, whose medicinal use is established and which, by virtue of their electron affinity, might be anticipated to act as radiosensitizers. One such group of compounds are the esters of p-hydroxybenzoic acid which are used in many pharmaceutical formulations as antimicrobial preservatives. In the first instance, the effect of different concentrations of p-hydroxybenzoic acid on the radiation sensitivity of oxic and anoxic buffered suspensions of the bacterium Staphylococcus aureus has been examined.

MATERIALS AND METHODS

p-Hydroxybenzoic acid (PHBA) was supplied by Sigma Chemical Co. The test organism was Staphylococcus aureus Oxford (NCTC 8236) maintained on slopes of nutrient agar at 4°C. Suspensions were grown to log phase in nutrient broth, washed and resuspended in fresh 1/15M phosphate buffer saline (pH 7.0) at a concentration of approximately 10^8 cells per ml prior to irradiation. PHBA was incorporated into the buffer saline at suitable concentrations. Routinely the bacteria were in contact with the additive for at least 45 min prior to irradiation.

γ-irradiation was carried out at ambient temperature using either a M38-3 Gammator 2400Ci ^{137}Cs source or a Gammacell 220 24000 Ci ^{60}Co source. Irradiation vessels were glass vials of od 19.7mm sealed with gas-tight rubber closures. Using the ^{137}Cs source, arran-

gement of these vessels within the irradiation chamber permitted the simultaneous irradiation of 18 suspensions at an average dose rate of 1.75 krads per min. With the ^{60}Co source, 36 suspensions could be simultaneously irradiated at predetermined dose rates ranging from 5.1 to 7.4 krads per min. Deoxygenation was by bubbling oxygen-free N_2 (less than 3 ppm O_2) for 20 min through the suspensions immediately prior to irradiation. For maintenance of suspensions under aerated conditions O_2 was bubbled through the suspensions for 5 min.

Suspensions were irradiated for fixed time intervals such that at the maximum dose level tested at least two decades of inactivation were generally achieved. Following irradiation bacterial suspensions were appropriately diluted and four 0.05ml aliquots of each diluted suspension pipetted onto plates of nutrient agar. After overnight incubation at 37°C, the micro-colonies formed were scored. Each colony was taken as indicative of a single bacterium in the original suspension. Counts performed on unirradiated samples of test suspensions treated identically to those exposed to irradiation were used as control estimates of the number of viable cells in calculations of surviving fractions. Dose-ln survival curves were constructed from five experimental points. Variation in values of slopes of these curves (inactivation constants) with changes in test conditions have been used to demonstrate quantitatively changes in radiation sensitivity.

RESULTS

Typical dose survival curves for Staph. aureus suspensions irradiated in the presence of different concentrations of PHBA in anoxia are depicted in Figure 1. Included in this figure are the responses for suspensions irradiated in air and anoxia in the absence of PHBA. These curves are linear over the dose range tested. All our computed values of inactivation constants together with standard deviations have been plotted in Figure 2 as a function of PHBA concentration. The horizontal lines are the levels of sensitivity observed when cells are irradiated in the presence and absence of O_2 in buffer alone.The corresponding parallel dashed lines represent standard deviation about these values. Increasing concentrations of PHBA from 10^{-6} to 5 x 10^{-5}M in anoxic bacterial suspensions produced no change in the response characteristic of bacteria irradiated in anoxic buffer alone. However further increases in PHBA concentration to 6 x 10^{-3}M caused a marked increase in radiation sensitivity, with the maximal response very close to that for suspensions irradiated in the presence of oxygen. The testing of higher PHBA concentrations was confounded by problems of toxicity.

PHBA was also tested for radiosensitizing activity in the presence of O_2. These tests were carried out at concentrations of PHBA that cause significant sensitizing effect in anoxic suspensions. The responses obtained are very close to that characteristic of bacteria suspended in oxygenated buffer alone, clearly demonstrating that the enhancing effect of PHBA and oxygen are not additive and that the sensitizing action of PHBA operates within the oxygen effect.

Preliminary studies showed that PHBA was somewhat toxic to the bacterium at concentrations of and above 6 x 10^{-3}M. Experiments were done to ensure that PHBA at lower concentrations showed no toxicity

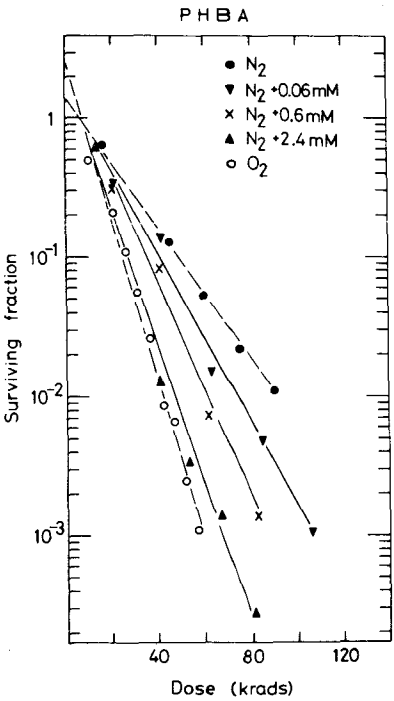

Figure 1. Survival data for hypoxic suspensions of Staph. aureus irradiated in the presence of different concentrations of PHBA.

towards the biological system. These control experiments demonstrated that the unirradiated PHBA had no effect on the viability of the unirradiated cells, that radiolysis products of PHBA at test concentrations had no effect on the viability of irradiated cells, and that these products carried over to the plating medium were without effect on the irradiated bacterium undergoing colony formation.

DISCUSSION

Our major finding is that PHBA acts as an efficient hypoxic radiosensitizer when tested against Staphylococcus aureus. The fact that the degree of sensitization is close to that observed in the presence of oxygen (DMF 2.5), suggests that PHBA may be simulating several of the previously proposed sensitizing actions of oxygen (11, 12). Only one such action may be as a result of its electron-affinity. Furthermore, the lack of sensitizing action in the presence of oxygen is evidence that this agent operates within the 'O_2 effect'.

Although the actual mechanism by which PHBA exerts its radiosensitizing action is unknown, several models have been proposed for the mechanism of sensitization by electron-affinic agents in general (for example, 3,13,14). The testing of PHBA in the presence of specific radical scavengers may help elucidate its mode of operation as a radiosensitizer. Whether the esters of PHBA, which are known to be less toxic than the parent compound, will likewise possess radiosensitizing properties remains to be investigated.

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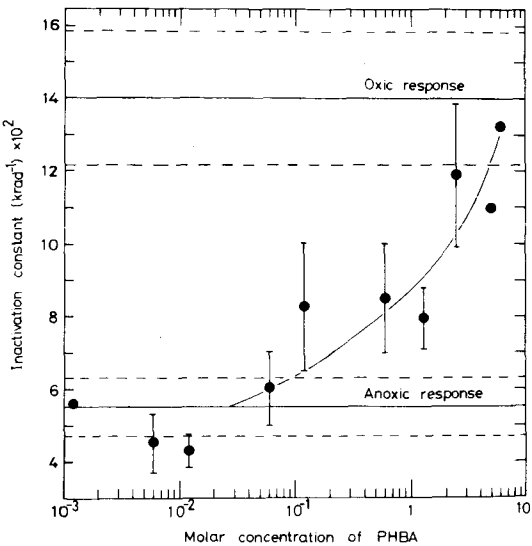

Figure 2. The effect of PHBA concentrations on the anoxic radiation response of Staph. aureus suspensions.

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ERRATUM:

The legend on the abscissa of Figure 2 should read:

"Molar concentration of PHBA (x 10^3)".