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SHORT COMMUNICATION

Radiation-Induced Cell Killing Is Highly Dependent upon Buffer Treatment (Filtration Compared to Autoclaving) due to Metal-Catalyzed Formation of Hypochlorite: A Cautionary Note

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Buffer solutions used in experiments in radiation biology may be sterilized by either autoclaving or filtration. We show here that for phosphate-buffered saline such differences in buffer treatment may result in widely differing dose–effect curves for cell killing. The temperature-dependent transformation of monophosphate ions into di- or polyphosphate evidently proceeds to an appreciable extent upon autoclaving the buffers at 120°C for 10 to 20 min. This increases the capability of the buffer to chelate spurious metal contaminations and, as a consequence, to reduce the amount of cytotoxic hypochlorite being produced. Depending on conditions of buffer treatment we have observed dose modification factors for the colony-forming ability of yeast cells up to the order of 3. Thus effects due to buffer treatment might easily outweigh the effect which the experiment was originally designed to determine. We strongly advise, therefore, that results of parallel sets of experiments in which different methods of buffer sterilization have been used should not be compared directly.

INTRODUCTION

It has been shown for many different cell types, including bacterial cells (*1–4*), phage and mammalian cells (*5*), that phosphate-buffered saline (PBS)[1] is considerably toxic when it has previously been subjected to ionizing radiation. This toxicity is not due to short-lived radicals but rather to chemically stable species as it decreases only slowly when the interval between irradiation of the solution and cell incubation is extended to minutes or even hours (*2*, *5*). Hydrogen peroxide, as the most prominent molecular product of the irradiation of water, is not toxic to cells in the concentration range which can be accounted for by the radiation dose applied. In buffers containing chloride, however, cytotoxicity is observed to be proportional to the concentration of sodium chloride, and hypochlorite has in several instances been invoked as a candidate for toxicity (*2*, *3*, *5*). Based on the radiation chemistry of chlorine-derived radicals (*6–9*), several reaction schemes have been proposed to account for the concomitant production of H_2O_2 and HOCl (*3*, *5*) which, incidentally, are the same compounds that cooperate in the microbicidal mechanism of phagocytosis (*10*). Concerning the parallel existence of these substances in aqueous solution, however, a serious ambiguity exists. At physiological pH they are known to react with each other, forming the chemically inert products O_2 and Cl^- (*11*). Hence it is obvious that the species and the amount which "survives" at the end of the irradiation of buffer to finally convey cytotoxicity must depend on the *relative* concentrations of H_2O_2 and HOCl during irradiation and thus very stringently on the conditions of gas saturation, pH, chloride and phosphate concentration. Because it depends on so many parameters, the yield of the cytotoxic reactant in a biological environment is difficult to predict and, despite numerous attempts to establish an exact correlation between radiation dose, reactant concentration and the resulting cytotoxicity, the results have been contradictory and hampered by poor reproducibility. Minimization of the data scatter was sought by applying extreme experimental standardization, e.g. baking glassware, treating plastic petri dishes with nitric acid, treating solutions with Chelex prior to use and so forth (see e.g. ref. *4* for a discussion). When recently re-investigating all parameters for production of HOCl (which is optimal in slightly acidic solutions purged with a stream of N_2O/O_2 in a 7:1 v/v ratio), we realized that some consistent experimental error could be traced back to buffer handling: The autoclaved solutions behaved differently than those which were sterilized by fil-

[1]Unless otherwise indicated, PB is a 50 m*M* solution of NaH_2PO_4; PBS is PB + NaCl (added to a final concentration of 140 m*M*).

tration. Such observations have been noted previously in the literature (e.g. ref. *1*), where the authors state that their experiments were done in sterile-filtered solution since in autoclaved phosphate buffer some "radioprotective substance" was evidently produced. As current knowledge of radiochemistry does not allow for such a substance (phosphate is considered relatively inert under irradiation and no plausible radioprotector is known to derive from it), we decided to compare the cytotoxicity of irradiated autoclaved and sterile-filtered solutions systematically.

Crucial for the experiments was a reliable method to determine the amounts of the presumed toxicants H_2O_2 and HOCl. The standard method for detecting oxidizing species, i.e. the oxidation of potassium iodide, is applicable only to determining the combined presence of both compounds. As it does not differentiate between the two species, however, the contribution of HOCl to the overall reaction must be determined separately to calculate the contribution of H_2O_2 by subtraction. HOCl chlorinates fluorescein by a two-step mechanism in the 4′ and 5′ positions (*12*) and causes a red-shift of the absorption peak of fluorescein from 493 nm toward 502 nm. Hence difference spectroscopy around 500 nm can be used conveniently to measure the amount of HOCl present.

MATERIALS AND METHODS

All HOCl measurements were performed at pH 4.2 to 4.5, where the chlorination of fluorescein runs to completion within minutes and the absorption of 4′,5′-dichloro fluorescein remains stable for days. In each case 3.8 ml of test solution was added to 200 µl of 1.7 m*M* uranin (the water-soluble sodium salt of fluorescein, obtained from Merck).

Buffers were prepared from NaH_2PO_4 (Baker analyzed® or Fluka, p.a.) and NaCl (Fluka, purissimum, p.a.) and were used at the pH that was reached upon dissolution of the salts. (The resulting minor variations in pH, depending on salt concentration or presence of cells, never exceeded the narrow limits between 4.2 and 4.8.) Where it is indicated that buffers were made "metal-free" by treatment with Chelex 100 (Bio-Rad Laboratories), the solutions were incubated overnight with the resin and were tested for deficiency of essential metals according to Buettner (*13*). Irradiations were carried out in a commercial ^{60}Co-GammaCell (Atomic Energy of Canada, Ltd.) delivering a dose rate of 18 Gy/min.

Cytotoxicity was determined using a diploid repair-proficient strain of *Saccharomyces cerevisiae* (BK0); aliquots containing 10^7 cells/ml of freshly harvested, buffer-washed stationary cells were incubated with the irradiated buffers for 20 min, then diluted appropriately and plated in triplicate on yeast extract peptone dextrose (YEPD) medium. Colonies were counted after 4 days of incubation at 30°C. All other experimental conditions are given in the figure legends.

RESULTS AND DISCUSSION

Figure 1 compares two sets of experiments: (1) survival of yeast cells irradiated in suspension, either in sterile-filtered (**a**) or autoclaved PBS (**b**); and (2) irradiation of the respective buffers alone, followed by subsequent incubation of the cells before plating (curves **c** and **d**). In both cases a significantly steeper dose–response curve is obtained for sterile-filtered solution (**a** compared to **b** and **c** compared to **d**, respectively). Clearly, in the second case, the existence of long-lived cytotoxic chemicals must account for the cytotoxicity as none of the cells had been exposed directly to radiation.

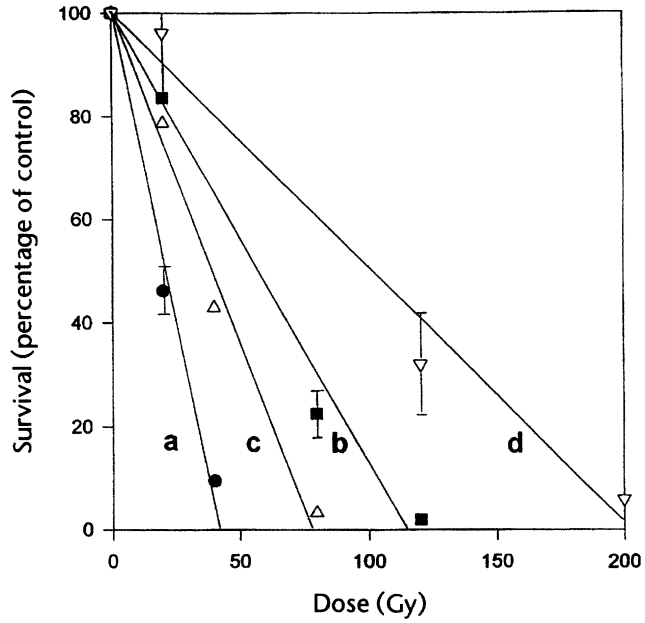

FIG. 1. The survival of yeast cells after irradiation depends on buffer treatment (autoclaving compared to sterile filtration). Curves **a** and **b** compare the effect of buffer treatment when the cells are irradiated together with the buffer, curves **c** and **d** the effect when the buffer is irradiated separately and the unirradiated cells are incubated thereafter. *Saccharomyces cerevisiae* (10^7 cells/ml); data points are means of triplicate colony counts. Irradiation conditions: Solutions purged with N_2O/O_2 = 7:1 (v/v); ^{60}Co γ rays, dose rate: 18 Gy/min. ●, **a**, Cells irradiated in sterile-filtered PBS; ■, **b**, cells irradiated in autoclaved PBS; △, **c**, sterile-filtered PBS irradiated, then cells incubated; ▽, **d**, autoclaved PBS irradiated, then cells incubated.

Figure 2, showing the changes in absorption at 500 nm which are indicative of 4′,5′-chlorination of fluorescein, allows three conclusions: (1) The presence of NaCl is absolutely required, which in a rather trivial way suggests a chloride-derived chemical to be the responsible species; (2) the buildup of HOCl is metal-dependent, as it is completely suppressed by rendering the buffer metal-free with Chelex; (3) it is strongly suppressed by autoclaving the buffer once (circles) and eliminated completely after the third round of autoclaving (inverted triangles). The inset shows that at different doses the ability of autoclaved buffer to prevent HOCl formation depends on phosphate concentration.

The results shown in Fig. 3 substantiate the effect of metal catalysis: Addition of increasing amounts of the metal chelator DTPA completely suppresses HOCl buildup above 5 µ*M* concentration. What remains to be established is the mechanism of the alleged "radioprotective" effect of autoclaving phosphate. The conversion of monophosphate to di- and higher polyphosphates is known to be a temperature-dependent process (*14*). As these compounds are efficient chelators for metal ions, such a conversion would increase the chelating properties of the buffer. Based on this assumption one would expect—at a given tempera-

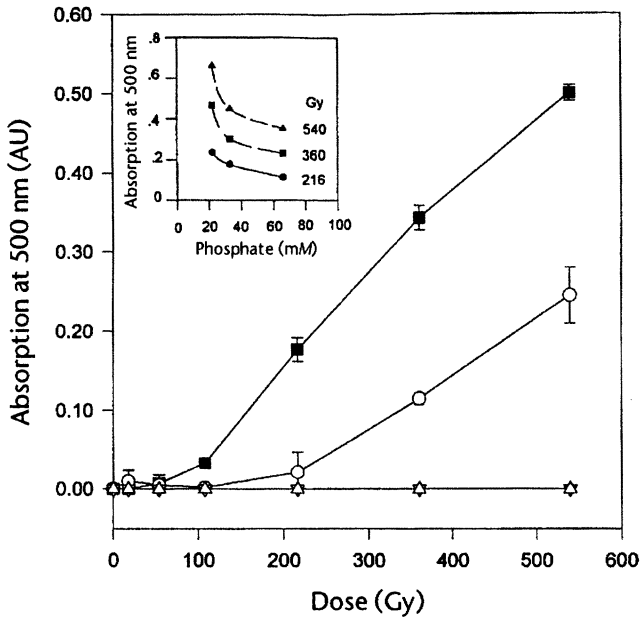

FIG. 2. The production of fluorescein-chlorinating product depends on spurious metals and autoclaving. After addition of irradiated buffer to fluorescein the absorption at 500 nm changes due to 4′,5′-chlorination (squares); if the buffer has been sterilized by autoclaving before irradiation (or rendered metal-free by treatment with Chelex) no chlorinating product is formed. (■) Irradiated PBS added to fluorescein; (○) PBS 1× autoclaved (121°C for 20 min) and then added to fluorescein; (▽) PBS 3× autoclaved and added to fluorescein; (△) PBS treated with Chelex overnight and added to fluorescein. The effect of irradiated PBS, when added to fluorescein, is not discernible. Irradiation conditions are the same as for Fig. 1. The inset shows that fluorescein chlorination inversely depends on phosphate concentration: (▲) 540 Gy, (■) 360 Gy, (●) 216 Gy.

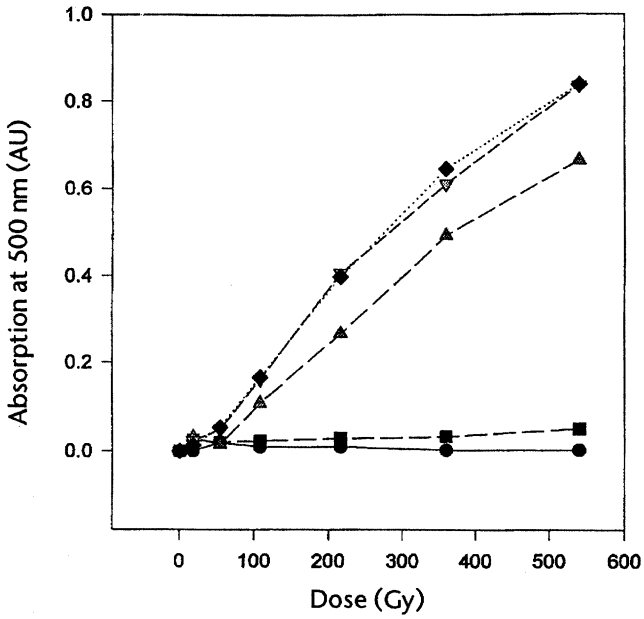

FIG. 3. The formation of fluorescein-chlorinating product can be suppressed by small amounts of the metal chelator diethylenetriamine pentaacetic acid (DTPA). Phosphate content of PBS was reduced to 11 m*M*; irradiation conditions were the same as for Fig. 1. (●) 8 μ*M*; (■) 5 μ*M*; (▲) 1 μ*M*; (▼) 200 n*M*; (◆) control: no DTPA.

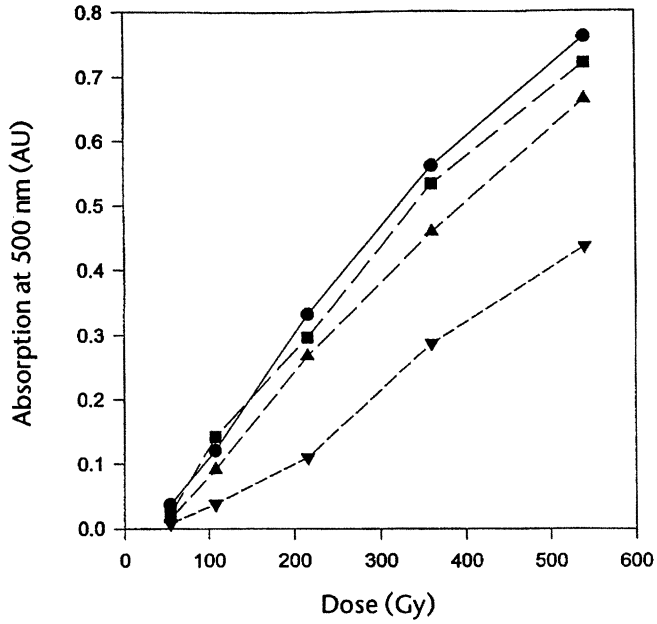

FIG. 4. The formation of fluorescein-chlorinating product is suppressed by repeated autoclaving of the buffer before irradiation. (●) 11 m*M* PBS, not autoclaved; (■) 11 m*M* PBS, 1× autoclaved; (▲) 11 m*M* PBS, 2× autoclaved; (▼) 11 m*M* PBS, 3× autoclaved.

ture—the level of chelator to be dependent upon the concentration of phosphate in the buffer. This prediction is verified by the results in the inset of Fig. 2 showing that by autoclaving the production of HOCl decreases with increasing phosphate content at any given dose.

The results shown in Fig. 4, which depicts the effects of repeated autoclaving, also support the hypothesis of phosphate-dependent chelator formation. Note that, in contrast to the high phosphate concentration in the experiments shown in Fig. 2, where one round of autoclaving was sufficient to suppress the fluorescein-chlorinating properties of the buffer substantially, it takes more than three rounds of autoclaving to produce a similar decrease if the phosphate content is reduced to 11 m*M*.

By a series of further tests we determined the persistence of the proposed phosphate-derived chelator: It proved to be rather long, with a reduction in activity of approximately 20% per day. This is not in contradiction to the behavior of polyphosphates as these are known to hydrolyze only slowly in aqueous solution (*14*).

Attempts to identify polyphosphates *directly* were unsuccessful. No differences in the spectra of irradiated autoclaved solution compared to irradiated sterile-filtered solution could be resolved by UV/vis spectroscopy. One means to differentiate between mono- and diphosphate would be the addition of $AgNO_3$, as diphosphoric acid is known to form white precipitates of $Ag_4P_2O_7$ with silver ions, in contrast to yellow precipitates with monophosphoric acid (*14*). Minute differences in the spectra of autoclaved and filtered buffers after addition of $AgNO_3$ can be observed, but they

are too small to allow for a quantitative spectrophotometric differentiation between mono-, di- and polyphosphates in the irradiated solutions.

The most straightforward control experiment, i.e. trying to suppress the production of fluorescein-chlorinating product by the addition of commercial diphosphate (Sigma) before irradiation of the buffer, leads to ambiguous results. We have to assume that commercial diphosphate introduces an amount of metal impurities that is sufficient to override the converse effect of metal chelation. From other experiments we know that the level of trace metal necessary for the catalysis of HOCl buildup is less than micromolar, a fact which is also evident from Fig. 3, where micromolar concentrations of the chelator DTPA suppressed HOCl formation completely. Any buffer for an *in vitro* experiment, e.g. a 50 millimolar phosphate buffer of physiological salt content, which is prepared from chemicals of the highest purity available commercially (typically metal impurities of less than 0.0001% are guaranteed by lot analysis), may contain metals in the range of several hundred nanomolar. If during the autoclaving process there was only partial transformation of the buffer to diphosphate, the latter might be able to chelate the metal traces essential for HOCl production and thus explain our results reasonably well.

We do not understand at the moment why the effects of buffer treatment and metal catalysis become evident only at slightly acidic pH and why the production of measurable HOCl falls off toward physiological pH to levels below the detection limit. Thus we cannot decide from the experiments presented here the extent to which HOCl might be responsible for damage to cells when they are irradiated in their "physiological" environment, where chloride and catalytic metal sites are available, but it follows that radiobiological *in vitro* experiments under the quoted conditions depend dramatically on buffer treatment.

SUMMARY AND CONCLUSIONS

If chloride-containing phosphate buffer is autoclaved, its metal-chelating properties increase with increasing phosphate concentration and with the duration of the autoclaving procedure. Effects due to successive autoclaving are additive and the chelating properties of the buffer remain rather stable, persisting over several days. The production of HOCl by irradiation of PBS is a complex metal-catalyzed process, depending strongly on pH, oxygen content and the production rate of OH radicals (viz. the N_2O effect). Even though the details of the mechanism of the formation of HOCl (or the concomitant production of another cytotoxic chlorine-derived species?) are not entirely understood at the moment, a cautionary note derives from the observations: The enormous differences in the radiation-induced production of the compound after different buffer treatments require that experiments be performed with carefully standardized buffer preparations. Besides the reported consequences for the relatively gross end point of cell death, other cellular effects which might conceivably derive from HOCl, e.g. signals due to its reaction with SH groups of membrane channels or transport systems, might respond even more sensitively and thus even more urgently require stringent precautions in buffer handling.

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