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Experimental data and theoretical considerations concerning the validity of the SAL concept to characterize non-thermal antimicrobial treatments

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Dedicated to Professor Dr. Dr. h.c. Peter Pflegel, Greifswald, on the occasion of his 65th birthday

For sterilization processes the pharmacopoeias demand a sterility assurance level (SAL) of 10^{-6}, i.e. a probability of not more than one viable microorganism among one million sterilized products. This SAL concept is based on the assumption that the inactivation of microorganisms by physical or chemical means generally follows first-order kinetics. In this paper it is demonstrated that this is not absolutely true for non-thermal antimicrobial processes. Using *Bacillus subtilis* spore test preparations the sporicidal efficacy of gamma and ultraviolet irradiation on the one hand as well as the treatment by glutaraldehyde and hydrogen peroxide containing solutions on the other hand was investigated. A range of mean spore contamination between 10^8 and 10^{-2} spores per test item could be supported by experimental data. It was demonstrated that the antimicrobial treatment parameters which are sufficient to reduce a high spore burden were not valid for an adequate reduction of the remaining lower spore burden. It is concluded that any extrapolation of such experimental data to the SAL range as usual in the validation of sterilization process parameters may be not permitted. Possible theoretical explanations of the non-homogeneity of the spore inactivation by non-thermal methods as well as consequences for the safety evaluation of sterilization processes are discussed.

1. Introduction

The definition of sterility in its absolute sense means a total absence of all viable microorganisms. Because that cannot be proved experimentally the pharmacopoeias demand a sterility assurance level (SAL) of 10^{-6} for sterilization processes. However, the practical demonstration of such an extremely low probability of one non-sterile item among a population of one million equally treated objects is likewise impossible. Starting from thermal sterilization processes the SAL concept is based on the assumption that the inactivation of microorganisms by physical or chemical means follows an exponential law. That means that with a given microorganism species the same intensity (i.e. time, temperature, dose, concentration, etc.) of a special antimicrobial treatment will result in the same degree of germ inactivation independent of the absolute number of microorganisms present. Consequently, to find sufficient sterilization process conditions which will meet the SAL requirements usually test objects with a known bioburden (i.e. microbial contamination) are exposed to fractions of the sterilization process in a way that a decreasing number of germs will survive which can be quantified experimentally. These experimental results are then extrapolated to the SAL range to find the final parameters for a sterilization treatment. If the antimicrobial effect is based on several components whose coaction is not known exactly or may change during the process, the so-called half-cycle method is used. A limited number of bioindicators which are usually contaminated by about 10^6 bacterial spores each will be exposed to a routine process in a way that no test objects show growth. This exposure time then is doubled to get the final sterilization conditions [1–3].
However, it is generally assumed that the inactivation kinetics are the same both in the experimentally controllable range of relatively high germ contamination and in the range of decreasing probability of microbial survivors up to the SAL range of 10^{-6}. Up to now there is very little knowledge about the dependence of microbial death kinetics on the number of germs which are present in the sterilization goods particularly in the case of a very low bioburden. To examine these problems more closely, several experiments on the sporicidal efficacy of non-thermal biocidal processes as irradiation and chemical treatment, were evaluated regarding their suitability to predict sterilization process parameters.

2. Investigations and results

Testing the sporicidal efficacy of gamma irradiation, the first sterile *Bacillus subtilis* spore tests strips were found after a dose of 5.5 kGy. Using doses higher than 9.0 kGy all spore strips tested were found sterile. This corresponds to an experimentally proved spore reduction from a declared starting contamination of about 10^6 spores per strip to a mean number of surviving spores of 0.1 per strip, i.e. a $\log_{10}$ reduction factor ($\log_{10}$RF) of 7.0 at 9.0 kGy gamma irradiation (Table).
To test the sporicidal activity of a so-called universal homogeneous ultraviolet (UHUV) irradiation a specially designed equipment [4] was used with a constant irradiation energy of $0.3\ \mathrm{mW \cdot cm^{-2}}$ whereas the dose was va-

Table: Inactivation of *Bacillus subtilis* spores immobilized on paper strips (BAG BioStrip) by gamma irradiation

Gamma irradiation dose (kGy)	Number of spore strips tested (n)	Number of sterile spore strips (n_0)	Mean number of surviving spores ($m = -\ln n_0/n$)
2.3	20	0	> 3.0
5.5	10	1	2.3
5.5	20	0	> 3.0
6.0	10	10	< 0.1
6.6	20	0	> 3.0
6.9	10	5	0.7
8.0	10	10	< 0.1
9.0	10	9	0.1
11.1	20	20	< 0.05
16.7	20	20	< 0.05
24.4	20	20	< 0.05

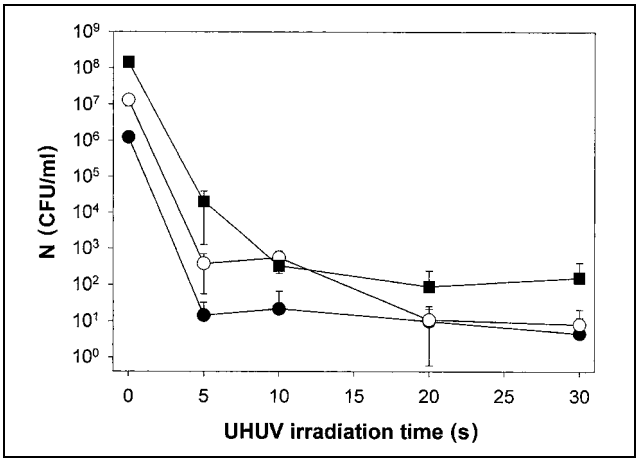

Fig. 1: Inactivation of suspended *Bacillus subtilis* spores by 0.3 mW · cm^{-2} UHUV irradiation (mean ± SD, n = 10 spore suspensions per data point)

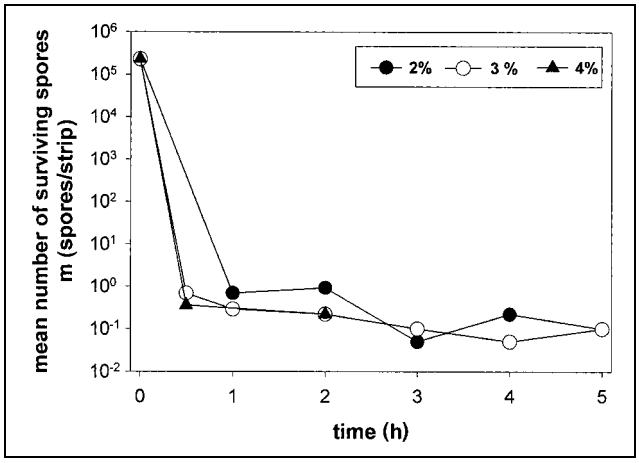

Fig. 3: Inactivation of *Bacillus subtilis* spores immobilized on paper strips (BAG BioStrip) by alkalinized glutaraldehyde solutions (mean number of surviving spores calculated on the basis of 20 spore strips per data point)

ried by the time. A 5 s lasting irradiation of suspensions containing a mean *Bacillus subtilis* spore concentration of $1.23 \cdot 10^6$ CFU · ml^{-1} resulted in a $\log_{10}$RF of 4.9. However, a six-fold prolongation of the irradiation time did not lead to a substantial additional spore reduction. The mean spore number remained more or less stable in the region of about 10^1 CFU · ml^{-1}. This was nearly the same using $1.32 \cdot 10^7$ CFU · ml^{-1} spore suspensions. Also in this test series the main part of spore inactivation ($\log_{10}$RF 4.6) was realized within the first 5 s of irradiation whereas after 20 as well as 30 s irradiation time the spore concentration was not further reduced below a mean value of about 10^1 CFU · ml^{-1}. Nevertheless, the same tendency of a steep spore decrease within the initial part of the time-dependent inactivation curve was found using spore suspensions with a mean content as high as $1.42 \cdot 10^8$ CFU · ml^{-1}. However, in this case the spore contamination within the total irradiation time of 30 s stagnated on a level between 10^2 and 10^3 CFU · ml^{-1} (Fig. 1).

Using paper-based *Bacillus subtilis* spore test strips the sporicidal efficacy of UHUV irradiation was much lower compared to spore suspensions. But, also in these tests for the initial decrease of a high bioburden of about 10^6 spores per strip a lower UHUV irradiation time was needed than for the further spore inactivation (Fig. 2).

Using solutions of alkalinized glutaraldehyde (2–3%), the spore count on the test strips was reduced by a $\log_{10}$RF of 5.5 after an exposure time of one or half an hour. A further prolongation of the glutaraldehyde treatment time up to 5 h resulted in additional spore reduction by less than one $\log_{10}$ cycle. In the glutaraldehyde concentration range which was tested here the spore inactivation kinetics was nearly independent on the glutaraldehyde concentration which was confirmed by two tests using a 4% alkalinized glutaraldehyde solution over 0.5 as well as 2 h treatment time (Fig. 3).

The corresponding phenomenon of non-homogeneous inactivation kinetics over the whole range of the experimentally detectable spore count was found with hydrogen peroxide solutions. A concentration of 0.6% resulted in a mean spore reduction from about 10^6 to about 1 per test strip within a 3 days exposure time. However, with a prolongation of the exposure time no substantial increase in sporicidal activity of 0.6% H_2O_2 was seen. Also after a 14 days treatment period a mean spore contamination of about 0.1 spores per strip was found, i.e. every tenth of the spore strips tested remained non-sterile (Fig. 4).

Almost the same was found if inclusion compounds of hydrogen peroxide and tensides in urea were tested which are proved to have an increased antimicrobial activity in relation to pure hydrogen peroxide solution [5]. Over an

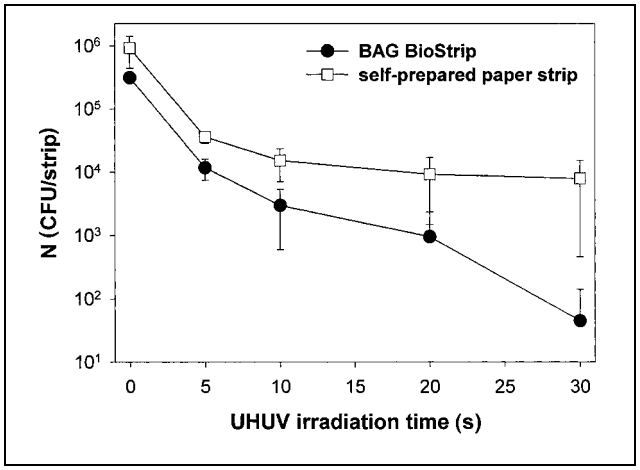

Fig. 2: Inactivation of *Bacillus subtilis* spores immobilized on paper strips by 0.3 mW · cm^{-2} UHUV irradiation (mean ± SD, n = 10 spore strips per data point)

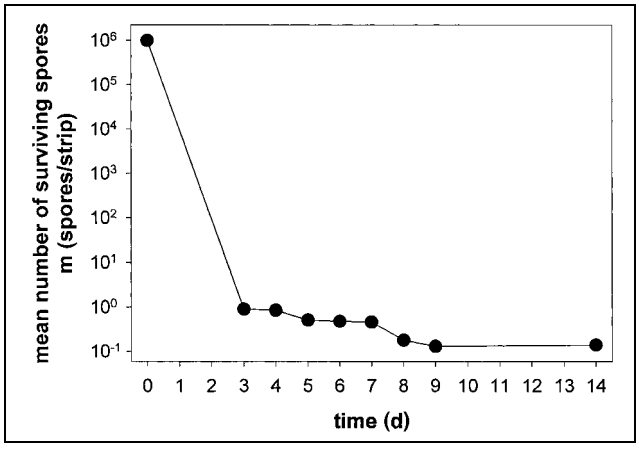

Fig. 4: Inactivation of *Bacillus subtilis* spores immobilized on paper strips (BAG BioStrip) by 0.6% hydrogen peroxide solution (mean number of surviving spores calculated on the basis of 105 to 159 spore strips per data point)

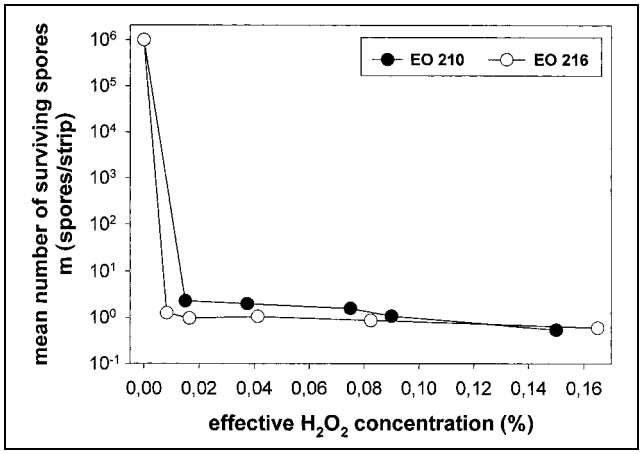

Fig. 5: Inactivation of *Bacillus subtilis* spores immobilized on paper strips (BAG BioStrip) by different concentrations of solutions of inclusion compounds of hydrogen peroxide and tensides in urea (mean number of surviving spores calculated on the basis of 20 spore strips per data point)

effective H_2O_2 concentration range from 0.008 to 0.16% during an exposure time of 3 days the mean spore contamination of the test strips could not be reduced substantially below a mean of 1 spore per strip (Fig. 5).

3. Discussion

Generally, it is not so very complicated to get a single product sterile. But, after any proof of its sterility by microbiological means it remains no longer in this state. Consequently, the main challenge of sterilization is to validate a process in a way that sterility may be guaranteed with a sufficient degree of probability. In other words, during a sterilization validation the antimicrobial efficacy has to be proved using a limited number of test objects and the results must be transferable to a large number of products which are to be sterilized.

During a microbial inactivation process no point of absolute sterility of all goods or test objects, will be reached. Rather the inactivation kinetics is characterized by a decrease in probability of microbial survivors, e.g. a decrease of the mean number of surviving germs on the objects treated. Therefore, in an initial part of a sterilization procedure all products will remain non-sterile although the absolute number of microorganisms is reduced to some extent, because the non-sterility of one item may be based on one surviving germ in an extreme exampe. Not until the mean number of surviving microorganisms is very low the first products will become sterile. Further treatment will then result in an increasing number of sterile products or in an increasing probability of sterility of a single product, respectively. The relation between sterile and non-sterile products as well as the distribution of the remaining microbial survivors on the products can be described statistically by a Poisson distribution [6, 7]. Accordingly there is a correlation between the number of sterile products n_0 and the whole number of equally treated products n on one hand and the mean number of surviving germs per test object m on the other hand:

$$n_0/n = e^{-m} \quad (1a)$$

or

$$m = -\ln n_0/n \quad (1b)$$

Obviously, with a decreasing mean number of surviving germs an increasing number of objects has to be investigated to have a chance to find the remaining non-sterile items. Thus, a sterilization testing using process parameters which result in complete sterility of a limited number of test objects will be of very small significance because the level of sterility assurance tested in this manner strongly depends on the number of test objects [7]. A verification of the efficacy of an antimicrobial procedure will only be reliable if an experimentally quantifiable number of test germs will survive the procedure. Based on such kinds of experimental data it has to be decided subsequently if or on which scale, respectively, it is permitted to extrapolate the results to the SAL range which is experimentally not accessible.

An important precondition to find microbial survivors is the use of test microorganisms which are highly resistant against the antimicrobial procedure under investigation. Generally, bacterial spores combine this characteristic with an easy and non-dangerous manageability in laboratory scale. For our studies we used *Bacillus subtilis* spores because they are known to be highly resistant against gamma and UV irradiation as well as against chemical treatment by glutaraldehyde and hydrogen peroxide [2, 4, 8–12]. The high spore count of the test preparations was used because commercially available bioindicators for sterilization monitoring are usually contaminated with at least 10^6 spores per test strip or per millilitre, respectively.

The problems which may arise if a highly effective antimicrobial procedure is tested using a limited number of test objects are illustrated by the results of the sporicidal activity of gamma irradiation as presented here. After gamma irradiation in doses lower than 5.5 kGy all spore strips remained non-sterile. On the other hand, irradiation doses higher than 9.0 kGy resulted in a complete sterility of all spore strips. Sterile as well as non-sterile test strips were found simultaneously between 5.5 and 9.0 kGy whereas the number of sterile items did not increase continuously with an increasing dose but both complete sterile (after 6.0 and 8.0 kGy) and complete non-sterile (after 5.5 and 6.6 kGy) test charges occurred in this range.

As described above the mean number of surving spores can be estimated based on a Poisson distribution of the survivors only if sterile and non-sterile spore strips occur simultaneously (see eq. 1b). Testing a limited number of 20 spore strips per irradiation dose as it is usual for sterilization monitoring the range of surviving spores per strip which may be estimated quantitatively is between 3.0 (1 sterile among 20 tested strips) and 0.05 (19 sterile among 20 tested strips) in the mean. Consequently, using this statistical method a real spore inactivation curve based on quantitative data can only be got over a range of 1.8 $\log_{10}$ cycles but a complete sterility of all test strips allows not more than the conclusion that the mean degree of contamination was reduced below a level of 0.05 spores per strip. Nevertheless, the data presented here seem to indicate a non-homogeneous trend of spore inactivation because a gamma irradiation dose of 5.5 kGy was sufficient to reduce the mean spore contamination by 5.5 $\log_{10}$ cycles from about 10^6 to 2.3 spores per strip. A further dose increase by 3.5 kGy up to 9.0 kGy resulted in an additional $\log_{10}$ spore reduction factor of about 1.4 only. In the dose range between 5.5 and 9.0 kGy homogeneity of the data is not given. Consequently, it seems to be very problematic to get valid spore inactivation kinetics for highly effective antimicrobial procedures as gamma irradiation where sterility of the first single test objects will be reached at relatively low doses.

UV irradiation which is in contrary to gamma irradiation not accepted by the pharmacopoeias as a sterilization procedure, is an electromagnetic irradiation, too, but it possesses much lower energy. Therefore, in our study UV tests were used to get some more general information concerning irradiation-induced spore inactivation kinetics. The limitations of the antimicrobial application of UV are not caused by a failure of biocidal activity but by a lack of penetration power. We used spore suspensions which were irradiated in quartz glass vessels of 10 mm in diameter. The UV transmission of 0.9% sodium chloride is quite high, only 14% of the 254 nm UV rays are absorbed over a length of 10 mm in relation to air. Because of the special design of the irradiation source used in our study the mutual shadowing of the suspended spores was minimized or fully avoided [4]. Consequently, a lack of penetration power as well as shadowing effects could be widely excluded to be limiting factors for the sporicidal action of the universal homogeneous UV (UHUV) irradiation in our experiments. Because the real number of surviving spores was estimated using a direct germ counting method, additional data could be received in the initial range of non-sterility of all test objects.

In all UV irradiation experiments presented here non-homogeneous spore inactivation kinetics were found. On the one hand relatively low doses were required to reduce a high starting-bioburden to a substantially lower level. On the other hand a second part of the inactivation kinetics was unisonously characterized by a more flat run of the curve indicating that a higher irradiation intensity was needed for the reduction of a lower level of microbial contamination. It was not possible to reduce the spore count substantially below 10^1 CFU $\cdot$ ml^{-1} within 30 s, whereas the main part of inactivation already took place within the first 5 s. Only in the test series using 10^8 CFU $\cdot$ ml^{-1} spore suspensions the lower sporicidal efficacy of UHUV may be related to the considerable turbidness of the test suspension caused by the high spore concentration. Similar non-homogeneous spore inactivation curves were found with spore test strips. As expected the total sporicidal effect of the UHUV irradiation was substantially lower if the spores were immobilized on paper surfaces because the universal spore irradiation was partially prevented by the non-transparent paper.

However, already within the experimentally controllable range of spore contamination it became obvious that the irradiation parameters which were necessary to reduce a high spore number were not valid to an adequate reduction of the remaining lower spore burden. Consequently, from our point of view it is very problematic to make any prediction about the inactivation kinetics caused by electromagnetic irradiation in the contamination range near the SAL level.

Almost the same applies for chemical sporicidal agents. Solutions of alkalinized glutaraldehyde are used for so-called "chemical wet sterilization" of medical instruments like endoscopes. This is not an official pharmacopoeial sterilization method yet. A minimum soak for 6 to 10 h in a solution of at least 2% alkalinized glutaraldehyde is recommended for sterilization purposes [2, 9]. As demonstrated in our study a reduction of *Bacillus subtilis* spores by nearly 6 $\log_{10}$ cycles was possible within 0.5 h (3 and 4% glutaraldehyde) or 1 h (2% glutaraldehyde). However, a prolongation of the glutaraldehyde treatment time did not result in a better sporicidal effect. Quite contrary to that, contamination remained nearly stable at a mean level of around 10^{-1} spores per test strip even if the treatment time was prolonged up to 5 h. Consequently, a real sterilization effect of glutaraldehyde in terms of the SAL could not be achieved neither by experimental tests nor by data extrapolation.

In these experiments a statistical estimation of the mean number of surviving spores per test object based on the Poisson distribution (see eq. 1b) was much more suitable than in the gamma irradiation experiments. Under all glutaraldehyde concentrations tested as well as treatment times (up to 5 h) sterile as well as non-sterile test objects occurred simultaneously.

The same phenomenon of a drastically decreased antimicrobial efficacy of a chemical agent in the case of a low microbial contamination was found with pure H_2O_2 solutions as well as with H_2O_2 containing preparations. Thus, also for chemical treatments it must be concluded that data extrapolation to the SAL range to define treatment conditions beeing sufficient to the definition of sterility will be not realistic.

The assumption of homogeneous first-order kinetics of microbial inactivation is based on long lasting experiences with thermal sterilization procedures, especially with the sterilization by moist heat in an autoclave. Thermal inactivation of microorganisms is mediated by heat-catalyzed hydrolytic as well as oxidative processes which are strongly dependent on the presence of water. There are several targets in microorganism cells including the cytoplasmic membrane, enzymes, nucleic acids, and proteins, that are susceptible to heat-induced damage [12]. However, in thermal sterilization procedures the lethal "agent" is a global heat-excited molecular energy state capable of producing lethal changes in the cell [13]. In difference to that, the energy of electromagnetic irradiation is produced and delivered in discrete amounts called quanta. Its germicidal effects result from the absorption of a respective number of single energy quanta by susceptible cell structures [14]. The antimicrobially most effective UV wavelength of about 254 nm is absorbed most strongly by nucleic acids. Therefore the major target cell component for UV is DNA where mainly occur dimerisations of thymin bases [12]. It is presumed that single or, more probably, multiple "hits" of UV irradiation energy quanta on the target sites are responsible for the inactivation of a microorganism cell. Based on this "target theory" it may be concluded that a reduction of the number of microorganisms, i.e. a reduced "target density", must result in a relative decrease in antimicrobial efficacy of a given irradiation dose. Consequently, from this theoretical point of view and besides the additional aspect of possible repair processes of UV-induced damages it seems to be very improbable that a SAL of 10^{-6} may be reached by UV irradiation levels which are within the limits of practicability [12, 14]. Despite a substantial optimization of the irradiation conditions these theoretical considerations were supported by our experimental data.

Considering the germicidal activity of gamma irradiation the situation is much more complex. Gamma irradiation is a sterilization procedure accepted by the pharmacopoeias. As indicated by the usual declaration of microorganism specific decimal reduction doses [10, 15] homogeneous first-order inactivation kinetics is assumed in general. However, the main target site of microorganisms beeing susceptible to ionizing irradiation is DNA, too. But, its antimicrobial activity is explained not only by the "target theory" as discussed above but also by the "theory of indirect action". Different to UV light ionizing radiation strips off electrons from the atoms of the material through which it passes. Therefore, besides direct effects of gam-

ma irradiation on microbial DNA as strand breaks, additional ionizations occur in the surrounding medium and principally in water. The produced highly reactive, short-lived radicals cause additional damaging reactions in the DNA [12]. The higher germicidal efficacy of gamma irradiation compared to UV light may be explained by these both mechanisms as well as by the general higher energy input in comparison to UV. But, both the direct effects of irradiation energy quanta and the indirect effects of reactive radicals may be attributed to single or multiple "hits" of active components on susceptible targets. Consequently, also in gamma irradiation a reduction of the "target density" should result in a decrease of the relative antimicrobial efficacy. This may occur at lower germ numbers as with UV irradiation and, consequently, will be much more difficult to prove experimentally. But, a general homogeneous first-order inactivation kinetics up to the SAL range should be considered to be rather improbable, too.

Chemical agents are mostly used within gas or low temperature plasma sterilization procedures [3]. The only liquid compound which is sometimes characterized as a "chemosterilizing" agent is alkalinized glutaraldehyde [9]. Although for ethylene oxide and other sterilant gases as propylene oxide, beta propiolactone, chlorine dioxide, and ozone, decimal reduction times related to constant process conditions are given by several authors, there is very few data concerning antimicrobial inactivation kinetics of chemical agents. The data acquired in our study using liquid chemicals is in contrast to the assumption that the inactivation kinetics for a test germ reduction from 10^6 to about zero which can be the proved experimentally is the same for the following cycle to reach a probability of surviving germs of 10^{-6}. It was possible to reduce the spore contamination in a way that several test objects became sterile but the slopes of the inactivation curves in the range of low contamination were flat to such an extent that an extrapolation far beyond the experimentally covered range seems to be very problematic. Thus, sterility will only be achieved by chemical agents with extravagant expenses beyond the limits of practicability.

Surprisingly, a kind of "target theory" as described for irradiation-induced germicidal processes was never applied to chemical antimicrobial processes. However, its mechanisms are also based on direct reactions of active molecules with special susceptible target structures. Consequently, similiar to irradiation-induced germ inactivation a reduction of the number of microorganims, i.e. a relative decrease of target structures must result in a lower efficacy of a given concentration of an active chemical agent. From this point of view a real germ reduction to a degree as low as demanded for the SAL must be considered as very doubtful. Even if such non-homogeneous kinetics still must be verified for gaseous chemicals it can be assumed that the process parameters estimated by half-cycle treatments are possibly not useful to guarantee sterility required by pharmacopoeias.

In summary all, the experimental data for sporicidal efficacy of irradiation as well as chemical treatment indicate that the germ inactivation kinetics are more dependent on the available germ number than regarded previously. Any extrapolation beyond an experimentally accessible range of germ contamination should be considered very problematic. A real homogeneous first-order kinetics of germ inactivation should be presumed for thermal sterilization processes only. This is supported by the theoretical consideration that thermal germ inactivation is based on a global excited molecular energy state capable of producing lethal changes in the microorganism cell whereas non-thermal antimicrobial principles are based on specific "hits" of active units as irradiation energy quanta or reactive molecules, respectively, at specific susceptible target structures. From this point of view a reduction of the targets must result in a decrease of antimicrobial efficacy in a higher extent than with thermal processes. This is stated to be the main and general difference between thermal and non-thermal antimicrobial processes.

Even if experimental data and statistical evaluations are combined it is not possible to guarantee an assurance level of germ inactivation better than 10^{-2}. This corresponds to a high-level disinfection at most but is not a sterilization procedure. Any extrapolation of experimental results up to the SAL range is only permitted if first order inactivation kinetics is proved for the respective treatment which is also true for highly resistant germs including viruses as well as prions. In all cases where homogeneous first-order inactivation kinetics cannot be proved the antimicrobial efficacy of a respective procedure must be validated over a germ inactivation range of $2\ \log_{10}$ cycles at most using test objects with a low bioburden. Any extrapolations beyond an experimentally proved range of $2\ \log_{10}$ cycles seem to be highly speculative. Consequently, a SAL of 10^{-6} as recommended by the pharmacopoeias cannot be guaranteed in these cases.

The demands of microbiological safety have recently increased particularly with regard to viruses and prions. Because of this situation it must be strictly avoided to lower one's sights with respect to the sterility assurance. Consequently, it is mandatory to look for alternative approaches to guaranee the safety of antimicrobial processes on a sufficient level. As expatiated more detailed elsewhere [16] this must include from our point of view

- the validation of cleaning procedures which are the main precondition of effective sterilization
- the check of the effectiveness of the sterilization process using "worst case" simulating test objects, i.e. under conditions were the susceptibility of the test germs to the antimicrobial active agent is hampered
- the check of virus safety by biochemical analytics
- the combination of different antimicrobial treatments.

4. Experimental

4.1. Microbiological tests

Bacillus subtilis spore suspensions in 0.9% sodium chloride were prepared by an established procedure [17] using *Bacillus subtilis* SBUG 14 (University of Greifswald, Germany). The spore concentration was varied by graduated dilution with 0.9% sodium chloride.

To estimate the spore count of the suspensions the spatulation method was used. 100 µl each of graduated dilutions of a spore suspension in 0.9% sodium chloride were spread onto tryptic soy agar plates (BAG Biologische Analysensystem GmbH, Lich, Germany). After incubation over about 16 h at 37 °C the number of colonies per plate was counted. Taking into account the dilution factor the spore concentration of the spore suspension was calculated as the number of colony forming units per millilitre N [CFU · ml^{-1}].

To prepare paper-based test strips pieces from wood-free paper (32×8 mm, 190 g · m^{-2}) were inoculated over the whole area of the strip with 100 µl each of a 10^7 CFU · ml^{-1} spore suspension and dried at room temperature for approx. 4 h in the laminar flow of a safety cabin.

Additionally, commercially available paper-based bio-indicators for sterilization monitoring (BAG BioStrips, Biologische Analysensysteme GmbH, Lich, Germany; *Bacillus subtilis* var. *niger* ATCC 9372) were used.

To recover the spores from a test strip it was transferred in a sterilized test tube containing 10 ml tryptic soy broth (BAG Biologische Analysensystem GmbH, Lich, Germany) as well as 10 sterile glass spheres (5 mm diameter) and agitated for at least 15 min on a shaking machine THYS 2 (MLW Labortechnik Ilmenau, Germany) with the maximum frequency of 6 s^{-1} [18, 19]. The spore count in the resulting tryptic soy broth based spore suspension was estimated by the spatulation method as described above.

As an alternative to estimate the number of surviving spores on the test strips an end-point method was used. The spore strips were incubated in tryptic soy broth (BAG Biologische Analysensystem GmbH, Lich, Germany) at 37 °C for 7 days. Following this incubation, the culture media were visually inspected for evidence of microbial growth which was indicated by turbidness. According the rules of a Poisson distribution the mean number of surviving spores per test object m was calculated from the number of sterile test objects n_0 related to the total number of identically treated test objects n [7, 20]:

$$m = -\ln n_0/n \quad (1b)$$

This end-point method can be used only if sterile and non-sterile test objects occur simultaneously.
Log_{10} reduction factors (log_{10} RF) were calculated as the differences between the log_{10} of the numbers of spores before and after the respective antimicrobial treatment.

4.2. Irradiation

For gamma irradiation Cesium-137 (B. Braun Melsungen AG, Melsungen, Germany) or Cobalt-60 radioisotope sources (Hahn Meitner Institute Berlin GmbH, Berlin, Germany), respectivly, were used. Based on dosimetric measurements the absorbed radiation doses were controlled by the exposure time.
For UV irradiation a specially designed low-pressure mercury-vapour discharge tube was constructed (Institute of Nonthermal Plasma-Physics at the Ernst Moritz Arndt University of Greifswald, Germany) which generates high intensity UV resonance radiation at 254 nm. Because of the particular structure of the discharge tube the object to be treated is surrounded by an UV generating low-temperature plasma realizing an universal and spatially homogeneous irradiation called UHUV (universal homogeneous ultraviolet) irradiation [4].The value of the attainable radiant intensity immediately at the object to be irradiated was in the order of 0.3 $mW \cdot cm^{-2}$.

4.3. Chemical agents

A glutaraldehyde stock solution (50% in water, Fluka AG, Buchs, Switzerland) was diluted with distilled water to get the test concentrations. Immediately before use it was activated by alkalinization (pH 7.5–8.5) using sodium hydrogencarbonate 0.3%.
Hydrogen peroxide 30% (Ph. Eur., stabilized, Roth GmbH, Karlsruhe, Germany) was diluted by distilled water to get the test concentrations.
The inclusion compounds of hydrogen peroxide and tensides in urea EO 210 (containing 5% lauryl sorbitan ester and 15% H_2O_2) and EO 216 (containing 5% dodecyl thiouronium chloride and 16.5% H_2O_2) [5] were tested as solutions in distilled water.

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