creosote is also relatively low (Table 5), and the release of creosote from wood samples into water is minimal (Becker et al., 2001). Therefore, it is not important to measure water-extractable phenols in commercially-available wood samples.

4. Conclusion

This study demonstrated that varying amounts of PAHs and water-extractable phenols are present in creosote and creosote-treated wood products such as railway sleepers and stakes that are sold for agricultural purposes. Among carcinogenic PAHs, benz(*a*)anthracene was detected in the highest concentration, varying between 228 and 6328 μg/g in creosotes. Benzo(*b*)fluoranthene, benzo(*k*)fluoranthene and BaP were found in the range of 67–3541 μg/g. Almost all creosotes contained more than 50 μg/g of BaP, which is the upper limit level that is permitted in the EU standard. Creosote-impregnated wood products, such as brand-new or secondhand railway sleepers and foundations, also contained significant amounts of BaP (58–749 μg/g) and benz(*a*)anthracene (250–1282 μg/g). The concentration of phenols was low in creosotes and creosote-treated wood, and was not related to PAHs content. The effects of the water-extractable phenols on health might to be negligible. In Japan, creosotes containing a high concentration of BaP have been sold, and consumers are free to use them for wood preservation. This situation may cause impermissible health damage to persons handling creosotes and creosote-treated wood products, and the government has scheduled a restriction of the use of creosotes containing elevated amounts of carcinogenic PAHs.

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一般報文

ラット頭蓋冠由来骨芽細胞のALPase活性を促進する硫酸化ヒアルロン酸の効果

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Enhancement action of sulfated hyaluronan on the ALPase activity of rat calvarial osteoblasts

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Abstract : The purpose of this study was to clarify the effect of hyaluronan (Hya) and sulfated hyaluronan (SHya) on rat calvarial osteoblast (rOB) cells proliferation and differentiation *in vitro*. rOB cells were cultured in the presence of Hya with different molecular weights (0.2, 2, 30, 90, 120 x 10^4) for 10days. Hya did not affect the proliferation of rOB cells. However, SHya suppressed the proliferation of rOB cells. The alkaline phosphatase (ALPase) activity of rOB cells cultured with SHya for 10 days was significantly enhanced in comparison with control (in the absence of polysaccharides) and with Hya. Hya suppressed the ALPase activity of rOB cells. As a result, SHya can control rOB cells proliferation and differentiation. SHya suppressed the rOB cells proliferation in a few culture days and promoted the differentiation. It was suggested that these effects were based on the sulfate groups of SHya. Therefore, it is considered that SHya is useful for the biomedical material, which promotes the differentiation of rOB cells.

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1. 緒　言

硫酸化多糖であるヘパリン（Heparin; Hep）やヘパラン硫酸（Heparan sulfate; HS）はheparin-binding growth factors（HBGFs）と複合体を形成し，組織が損傷を受けた場合，速やかにHBGFsを放出して，周辺の組織を活性化することが明らかとなっている[1-3]．HBGFsは，骨の修復にも重要な役割を果たしていることが知られ，骨芽細胞の増殖や分化の過程でオートクライン，パラクライン的に骨の形成や吸収を制御する[4-6]．病気やケガなどで骨組織が損傷した場合，修復するために人工骨や人工関節などの人工材料が用いられている．しかし，これらの人工材料は様々な問題があり，近年組織工学的手法を用いた骨再生が期待されている．この手法を用いて骨組織の再生に利用される細胞は，骨組織を形成する骨芽細胞である．骨芽細胞は間葉系由来の細胞で，未分化な間葉系の細胞から骨原性細胞を経て次第に成熟した骨芽細胞へと分化する．骨が大きく欠損した場合，細胞が増殖，分化するための足場が失われるため，仮の足場が必要となる．しかし，足場が優れていても細胞の数が少ないと十分な組織の再生は望めない．そこで、生体材料と増殖因子の組み合わせによる組織再生の方法が，近年多く報告されている[7, 8]．しかし，これらの増殖因子はたんぱく質であり，生体内での寿命が短く，不安定であるため，増殖因子を保持する担体が必要である．グリコサミノグリカンの構成成分であるヒアルロン酸（Hya）は，眼球、関節をはじめとする多くの結合組織に存在し，細胞外マトリックスの構成成分として，組織の創傷治癒や形態発生に重要な働きをしていることが報告されている[9-11]．近

年，Hyaのレセプターとして CD44 が発見されて以来，Hyaを介した生物学的機能の研究が盛んに行われている[12-14]．Pilloni らは，骨芽細胞の前駆細胞である間葉系細胞を用いて，分子量の異なるHyaの影響を検討しており[15]，Hya は骨芽細胞の石灰化を促進すると報告している．しかし，細胞の増殖性，分化マーカーについての詳細な検討は行っていない．そこで本研究では，骨再生用材料の開発を目的として，生体適合性の高い多糖類を用いて骨組織の再生を試みた．本研究では、ヒアルロン酸と硫酸化多糖の機能を併せ持つ高分子量の硫酸化多糖を作製し，ラット頭蓋冠由来骨芽細胞[rat calvarial osteoblast（rOB cells）]の初期骨分化マーカーであるAlkaline phosphatase（ALPase）に対する影響について検討を行った．

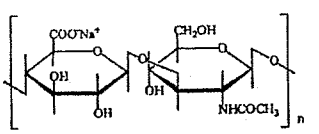
2. 実験方法

2.1 材料

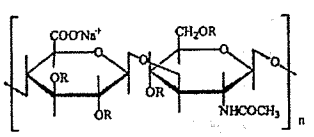
SHya は以前に報告した方法にて合成した[16]．使用した硫酸化多糖の硫酸化度（D.S.；2 糖残基当たり硫酸基の量）を Table 1 に示した．HyaX(X は分子量を示す)の分子量は 0.2, 2, 30, 90, 120 x 10^4 のもの使用した．Hya, SHya, コンドロイチン硫酸 typeC（Chs-C），Hep は 0.5 mg/l の濃度になるように培地に溶解し，0.22 μm の孔径を有する

Table 1 Characteristics of polysaccharides

Polysaccharides	Number of sulfate groups per two saccharide rings	MW (x10^4)
Hya	0	0.2-120
1.2SHya	1.2	55
2.1SHya	2.1	20
3.4SHya	3.4	5
Chs-C	1	0.5
Hep	2.5	1



Hyaluronan (Hya)



Sulfated hyaluronan (SHya)

R = $SO_3^-Na^+$ or H^+

Fig.1 Structure of hyaluronan and sulfated hyaluronan

filter で滅菌をおこなった．Hya および SHya の構造式を Fig. 1 に示した．

2.2 細胞培養

生後 48 時間以内のウィスター系ラット（Charles River）の頭蓋冠から、酵素消化法により rOB cells を分離した[17]．その後，10% fetal bovine serum（FBS, GIBCO）を含む Dulbecco' s modified Eagle' s medium（DMEM, Nissui-seiyaku）を用いて，初代培養を行った．3 日毎に培地を交換しながら通常の継代培養を行い，継代数 4-6 の rOB cells を実験に使用した．

2.3 細胞増殖

多糖類と 10% FBS を含む DMEM を用いて調製した rOB cells（1x10^4 cells/well, 24 multiwell plate）を播種し，5% CO_2 下，37℃で培養した．所定時間培養後の細胞数を，下記のタンパク質量測定によって計測した．上澄みを除去し，well を phosphate-buffered salines（PBS; pH7.6）で 3 回洗浄した。0.04% nonidet P-40（NP-40, Nacalai tesque）を含む 1ml PBS を各 well に添加し，37℃で 10 分間インキュベートした．懸濁液を超音波破砕機を用いてホモジナイズした後，1000rpm, 4℃, 5 分間遠心を行った．この上澄液を細胞溶液として，Bio-Rad protein assay（protein assay, Bio-Rad Lab.）法により，595nm の吸光度を EIA READER を使って総タンパク質量を測定した．細胞数とタンパク質量の検量線を作成し，検量線により総タンパク質量から細胞数を算出した．検量線の作成法を以下に示す．0, 1, 5, 10, 30x10^5 cells/ml に調製した細胞懸濁液を各試験管に入れ，1000rpm, 4℃, 5 分間遠心を行った．上澄みを除去し，0.04% NP-40 を含む 1ml PBS を各試験管に入れ，総タンパク質量を求め，細胞数と総タンパク質量の検量線を作成した．

2.4 Alkaline phosphatase（ALPase）活性

ALPase 活性の測定は以下のようにして行った．細胞増殖の測定時に得られた細胞溶解液 0.1ml と基質水溶液 0.4ml（16mM p-nitrophenylphosphate disodium salt hexahydrate）を混合して，30 分間，37℃でインキュベートした．その後，反応を停止するため，混合液に 0.2N NaOH 水溶液を 0.5ml 添加し，410nm の吸光度を EIA READER を用いて測定した．総タンパク量は Bio-Rad protein assay によって測定し，Albumin(Bovine Albumin Fraction V）の検量線から算出した．

全ての実験において，実験数 n=6 として測定を行い，その平均値を求めた。

3. 結果

分子量の異なる Hya を添加した rOB cells の増殖曲線を，Fig. 2 に示した．培養 7 日目までは，Hya の分子量に関係なく rOB cells は増殖し，コンフルエントに達した．しかし，培養 10 日目になると，高分子量の Hya を添加した rOB cells において，わずかに細胞数の増加が示され

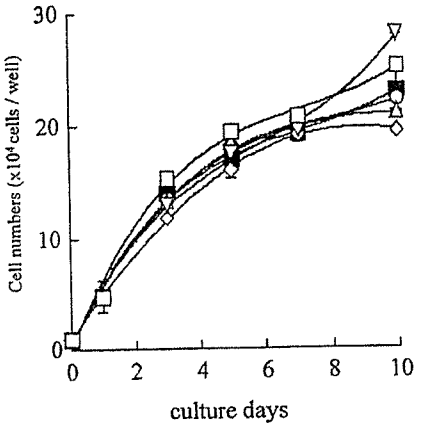


Fig.2 Effect of 0.5mg/ml hyaluronan on the proliferation of rOB cells

■ none ○ Hya0.2 △ Hya2 ◇ Hya30 ▽ Hya90 □ Hya120

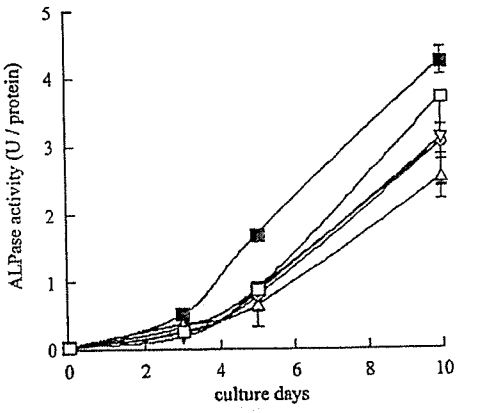


Fig.4 Effect of 0.5mg/ml hyaluronan on the ALPase activity of rOB cells

■ none ○ Hya0.2 △ Hya2 ◇ Hya30 ▽ Hya90 □ Hya120

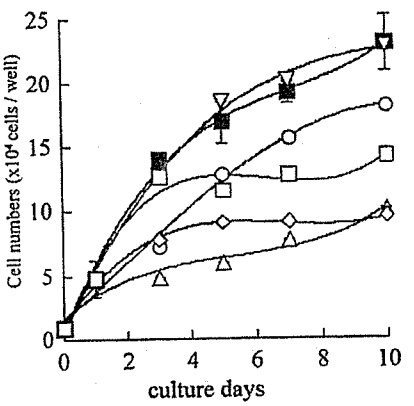


Fig.3 Effect of 0.5mg/ml sulfated polysaccharides on the proliferation of rOB cells

■ none ○ 1.2SHya △ 2.1SHya ◇ 3.4SHya ▽ Chs-C □ Hep

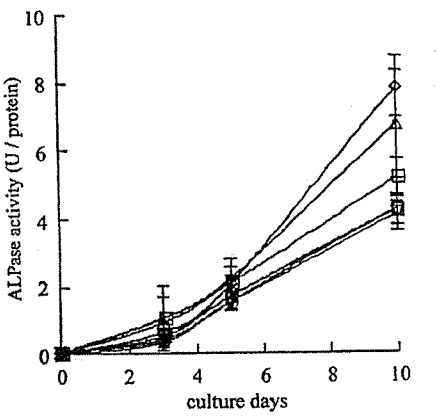


Fig.5 Effect of 0.5mg/ml sulfated polysaccharides on the ALPase activity of rOB cells

■ none ○ 1.2SHya △ 2.1SHya ◇ 3.4SHya ▽ Chs-C □ Hep

た.

Fig.3に，硫酸化度の異なるSHyaを添加したrOB cellsの増殖曲線を示した．Hyaを添加した場合と異なり，SHyaを添加したrOB cellsは，培養3日目から非添加系に比べて増殖が抑制された．さらに，SHyaの硫酸基の導入率が高くなるほど，rOB cellsの増殖は抑制された．これに対し，同じ硫酸基を有する多糖類であってもChs-Cではほとんど影響は見られず，Hepでも抑制効果は小さかった．

Fig.4に，Hyaを添加したrOB cellsのアルカリフォスファターゼ(ALPase)活性の経時変化を示した．Hyaは分子量に関係無く，骨芽細胞の初期分化マーカーであるALPaseの活性は非添加系に比べて低い値を示した．Fig.5に、硫酸化度の異なるSHyaを添加したrOB cellsのALPase活性を示した．Hyaとは異なり，SHyaを添加したrOB cellsのALPase活性は非添加系に比べて上昇が認められた．特に、高硫酸化度になるほど，ALPase活性の

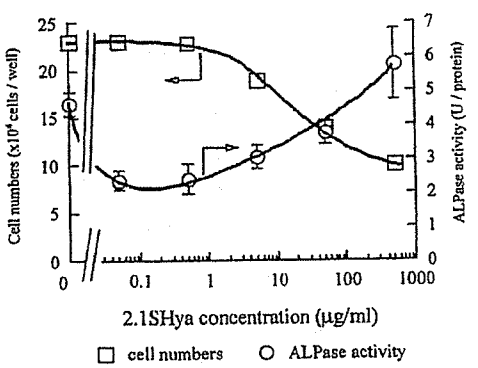


Fig.6 Dose-dependence of 2.1SHya on the proliferation and ALPase activity of rOB cells after 10days

上昇率は高かった．そこで，rOB cells の増殖と ALPase 活性に対する 2.1SHya の添加濃度の影響を検討した (Fig.6)．高濃度の SHya は rOB cells の増殖を抑制し，ALPase 活性を促進させたのに対し，低濃度の SHya は増殖を促進し，ALPase 活性を抑制させることが認められた．

4. 考 察

本実験で我々は，分子量の異なる Hya を骨芽細胞に添加し，骨芽細胞の増殖と ALPase 活性について検討を行った．分子量 2000 から 120 万の Hya を rOB cells に添加したところ 非添加系とほとんど変わりなく増殖し，Hya 添加やその分子量の違いによる影響は見られなかった (Fig.2)．しかし，ALPase の活性は分子量に関係なく非添加系に比べてすべて低いため (Fig.4)，Hya は rOB cells の分化を抑制することが示唆された．Hep, HS は細胞外あるいは細胞表面に広く存在し，多くの種類のタンパク質と特異的な相互作用を示すことが知られている[18]．特に，ヘパラン硫酸プロテオグリカン (HSPG) は，細胞と ECM の相互作用や細胞同士の相互作用を介して，接着，凝集，シグナル伝達などに関与している．このように，多岐にわたる HSPG の機能の中で，増殖因子との相互作用については多くの報告があり，注目されている[1-3]．FGF, transforming growth factor-β(TGF-β), bone morphogenetic protein(BMP)などの細胞増殖因子は，Hep や HS などの硫酸化多糖と相互作用し，細胞の増殖を制御することが報告されている[1,4]．Hep, HS, Chs の分子量は，Hya に比べて非常に小さい．そこで本研究では，高分子量である SHya の骨再生用材料への応用を目的として，SHya 単独での rOB cells に対する影響を検討した．SHya の硫酸化度が高くなるにつれ，細胞の増殖は抑制され，Hep もある程度の抑制効果を示した(Fig.3)．ALPase 活性に対しては，硫酸化度が高くなるにつれて活性が上昇した(Fig.5)．これより，硫酸化多糖は細胞の増殖を抑制し，分化を促進させることが示された．次に，影響が最も大きく現れた 2.1SHya を用いて，濃度依存性について検討を行った．Fig.6 より，2.1SHya は低濃度では細胞の増殖を促進し，高濃度になるにつれ増殖を抑制した．これに対して ALPase 活性は低濃度では活性が低く，高濃度になるにつれ上昇した．これより，2.1SHya は濃度を変化させることで，rOB cells の機能を制御することが可能であることが示された．Hep, HS と増殖因子との協同的な作用の細胞の増殖に対する影響も，濃度によって大きく異なることが報告されている．Blanquaert らは，Hep 及び硫酸化多糖の RGTA(Heparin-like polymers derived from dextran)と増殖因子との協同作用による，マウス頭蓋冠由来骨芽細胞 MC3T3-E1 への影響を報告している[1]．RGTA は増殖因子と共に用いることで，増殖に対しては抑制的に働き，ALPase の活性が上昇することを明らかにした．この作用は RGTA のみでも影響が現れるが，増殖因子が存在することにより，さらに顕著に影響が現れた．今回，我々は SHya 単独の影響を検討したが，彼らの結果と一致する結果が得られた．以上の結果から，Hya に硫酸基を導入することにより，SHya は骨芽細胞の増殖や分化機能を制御することが可能であると示された．

5. 結 論

rOB cells に Hya を添加すると，rOB cells の増殖は促進され，分化は抑制された．しかし，SHya を添加すると，rOB cells の増殖は抑制され，分化の促進が示された．SHya の効果は SHya の硫酸化度，濃度に大きく依存した．従って，SHya は骨芽細胞の機能を制御することが明かとなった．骨形成促進作用を持っている BMP, FGF2, TGF-β などの増殖因子を臨床応用に用いる場合，これらの増殖因子に適した担体の開発が必要である．SHya は分子量が高く，粘性があるため，増殖因子を保持する能力は Hep、HS などの他の硫酸化多糖に比べて高いことが考えられる．今後，SHya と増殖因子との相互作用について検討を行うことにより，SHya の分化促進作用の機序を明らかにできると同時に，SHya の骨再生用材料への応用が期待される．

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Review

Importance of Considering Injured Microorganisms in Sterilization Validation

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Disinfection or sterilization treatment by heating, irradiation, or chemicals can cause injury to microorganisms at sublethal levels. Microbial injury is the inability to grow under conditions suitable for the uninjured microorganisms. This inability of injured microorganisms to grow is explained in terms of more complex or different nutritional requirements or in terms of increased sensitivity to environmental conditions such as incubation conditions (time or temperature) or to chemical agents such as halogen compounds. Injured microorganisms can be distinguished from those that are dead or mutated by their ability to regain normal physiological activity when placed in appropriate conditions for cultivation. The return to normal physiological function has been termed repair. The extent and severity of sublethal injury, the mechanisms of injury, and the mechanisms and degree of recovery vary with the sterilization procedures, the species, the strains, the condition of the microorganism, and the methods of repair. Injury to spore formers has been detected at different stages of the spore cycle. The sites of injury include damage to enzymes, membrane disruption, and/or damage to DNA or RNA. Information on the sublethal injury and recovery of microorganisms is very important in evaluating sterilization/disinfection procedures. This paper supplies academic as well as practical information dealing with the repair, and detection of injured microorganisms for performing reproducible sterilization validation.

Key words : Injured microorganisms/Injury/Repair/Damage/Sterilization validation

INTRODUCTION

The injury to microorganisms by various sterilization procedures is a common occurrence. The detection of injured microorganisms is of significance for evaluating the sterilization process in health care products, and attainment of sterility assurance and reproducible sterilization validation (Busta, 1978; Hurst, 1977; Hurst, 1984a). Inadequate detection of injured microorganisms in health care products can lead to potential spoilage and hazards if the injured microorganisms undergo repair and proliferate during product storage. Therefore it is important to employ techniques that will optimize the detection and enumeration of injured microorganisms. Ignorance or poor recovery of injured microorganisms may lead to an overestimation of lethal effects, which can result in faulty sterilization validation. This must be avoided. An understanding of injury can supply valuable information to optimize the proliferation and maintenance of microbial cultures.

It is important to understand the resistance, injury, repair, and detection of the bioburden to attain satisfactory and reproducible sterilization validation.

1. Importance of inactivating spore formers

The spore cycle is described in Figure 1. Spores are formed in the vegetative cell during sporulation and are released into the environment during cell lysis. Spores can withstand high doses of heat, irradiation, and chemicals.

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The typical structure of bacterial endospore consists, from the inside to the outside, of a protoplasmic core, a spore wall, a cortex, the spore coats, and exosporium (Figure 2). The spore coats protect the spores and respond to germinants. The cortex and the cell wall are comprised of peptidoglycan (Figure 3). The spore cell wall forms the cell wall of the germinated cell. The dehydrated core consists of DNA, ribosomes, enzymes, and other cellular components including calcium (Barach et al., 1976), magnesium, manganese, and dipicolinic acid (Denyer et al., 2004). The chemical structure of dipicolinic acid is shown in Figure 4. Bacterial spores in the injured state are not considered as threats unless they regain metabolic activity.

The objective of health care product sterilization is to destroy bacterial spores, rendering the product safe while retaining acceptable quality for long periods of time. The repair of injured spores may lead to product quality loss and/or safety problems to people. Thus, it is quite important to detect undamaged as well as injured spores in products to avoid any risks concerned. The recovery and enumeration of normal or injured spores which are susceptible to injury and repair involve the completion of the various stages in the spore cycle in Fig. 1 (Gould, 1984).

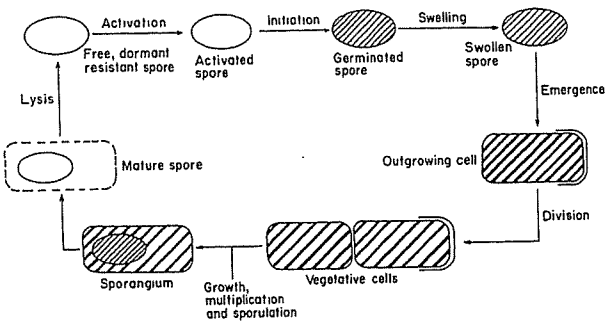

FIG. 1. Diagrammatic representation of the cycle of bacterial endospore formation, germination and outgrowth

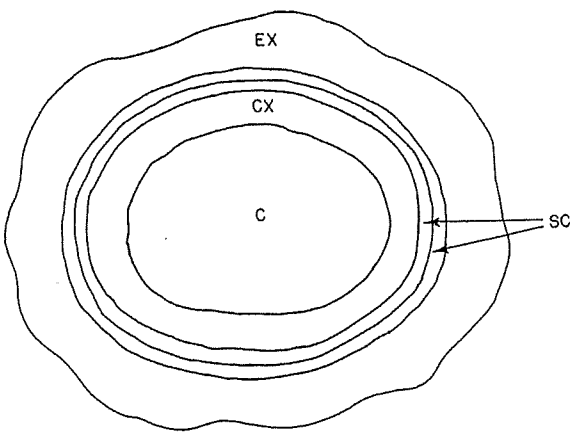

FIG. 2. A typical bacterial spore
EX, exosporium; SC, spore coats; CX, cortex; C, core

2. Definition

The representative cycle of bacterial endospore activation, germination, outgrowth and growth is presented in Fig. 1.

2-1. Activation

Activation is generally accomplished by sublethal levels or other treatments (heating, irradiation, chemical agents exposure and so on) which do not cause significant changes in the properties of the dormant spore, but accelerates the germination process. This treatment also inactivates the vegetative cells in the medium.

2-2. Germination

The sequence of major changes accompanying endospore formation and germination is presented in Table 1. During the stage of germination, the dormant state of the spore is irreversibly terminated. When spores germinate they lose their characteristic resistance to heat, radiation, chemicals, and other stresses. Germination is initiated by several agents. They include nutrients (e.g., amino acids and sug-

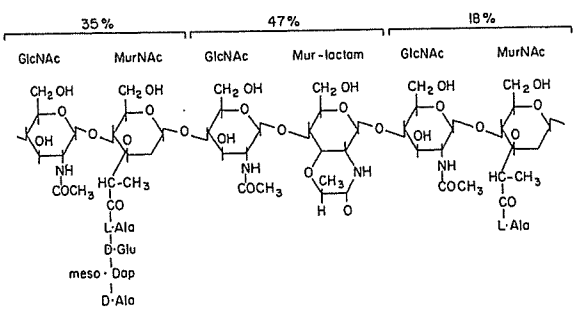

FIG. 3. Chemical structure of peptidoglycan

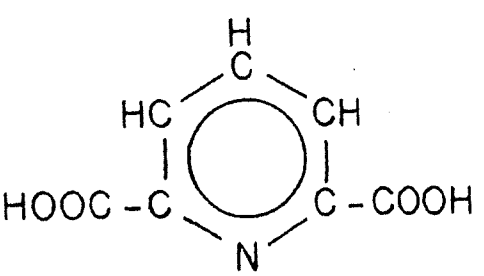

FIG. 4. Chemical structure of dipicolinic acid

TABLE 1. Sequence of major changes accompanying endospore formation and germination

Spore formation (timescale commonly 8 h)	Spore germination (timescale commonly 5 min)
End of vegetative growth	Addition of germinants
Chromatin filament formed	Heat resistance lost
Spore protease excreted	
Forespore septum formed	Calcium and dipicolinic acid excreted
Forespore protoplast engulfed	Temporary rise in resistance to ultraviolet
Heat resistant catalase formed	irradiation
Peptidoglycan cortex synthesized	Refractility loss observable by phase
Spore becomes refractile	contrast microscopy
Dipicolinic acid synthesized	
Calcium taken up	Resistance to stains lost
Proteinaceous spore coats assembled	Release of fragments of hydrolysed
Resistance to organic solvents acquired	peptidoglycan
Resistance to heat acquired	Fall in extinction of spore suspensions
Mother cell lyses to release mature spore	Onset of metabolism

ars), nonnutrient germinants (e.g., metal ions, bicarbonate, and calcium dipicolinate), enzymes (e.g., lysozyme), and physical treatments (Adams, 1973; Adams, 1974; Alderton et al., 1974). According to the present author's experiment, Ca, vitamin mixtures, L-alanine, puruvate and glucose are appropriate agents to promote germination (Shintani, 2006). Mg ion does not successfully promote germination contrary to reported results (Cazemier et al., 2001; Busta et al., 1976).

2-3. Outgrowth

In this stage, the germinated spore is transformed into the first vegetative cell. It is a metabolic step involving the synthesis of RNA, proteins, DNA, and the cell wall and membrane, which differentiate the spore into a vegetative cell (Adams, 1978).

2-4. Growth

The stage involves metabolic growth which can be observed as an increase in the number of vegetative cells. The inhibition of the growth or the doubling process will prevent detection of the organism.

3. Spore injury

Injury is defined as damage incurred during exposure to sublethal environmental stresses. Injury may cause a longer lag period, increased sensitivity or decreased tolerance to a variety of chemical agents and the inability to multiply until repair of the injury has been attained. Repair is characterized as a return to normality and is defined as the correction of the injury-mediated damage and the process involved therein.

One major difference between bacterial spores and vegetative cells is the high resistance of the spore to environmental stresses. Several types of injury have been reported for bacterial spores (Adams, 1978). Spore injury is more complex than injury to vegetative cells (Hurst, 1984b) because any of the several steps in the spore cycle can be independently affected (Fig. 1). The injury and repair mechanisms of bacterial spores are different from those of vegetative cells due to the dormant and resistant state of spores. Several stages are involved in the transformation of a spore into a vegetative cell, and there is the possibility that a spore is defective in nature during sporulation (Gould, 1984). Some treatments may injure spores by mechanisms different from those involved in the injury to the vegetative cells. Treatments such as freezing and drying, which injure vegetative cells, are not usually implicated in the injury of spores. The physical treatments involved in damage to bacterial spores include heating, ionizing and UV radiation, and hydrostatic pressure. Chemical injury to spores occurs due to changes in the pH, ion exchange treatments, and disinfectants such as hypochlorite, phenol, hydrogen peroxide, peracetic acid, and ethylene oxide. Enzymatic treatments can cause spore characteristics and spore coat permeability is altered with lysozyme and hydrolytic enzymes. Metabolic factors may be active during sporulation and include the lack of important nutrients and altered levels of divalent cations of Ca, Mg, Mn and so on. Injury towards bacterial spores is expressed in various forms. There is a need for nonnutrient germination stimulants by the injured spores, modified optimum incubation temperatures for the enumeration of survivors, an increased sensitivity of the survivors to inhibitors and selective agents, and altered nutritional requirements by the survivors (Adams, 1978). Other factors required for recovery includes changes in pH, the oxidation-reduction potential, the recovery medium, the

incubation period and so on.

3-1. Injury by heating

Elevated temperatures may cause stress and injure bacterial spores (Foegeding and Busta, 1981; Gould, 1984). Bacterial spore injury by heating has been observed in the temperature range of 50 to 170℃ and on spores of both aerobic and anaerobic bacteria (Adamas, 1978). Heat-injured spores are unable to grow under conditions that are optimal for unheated spores. They exhibit sensitivities to antibiotics and curing agents, such as sodium chloride (Briggs and Yazdany, 1970), fastidious growth requirements, the need for nonessential agents, such as starch, activated charcoal, lysozyme, and other lytic enzymes, altered incubation temperature and an extended lag phase (Gould, 1984).

3-2. Radiation injury by ionizing and UV irradiation

Spore injury caused by irradiation has been reported (Farkas et al., 1995; Foegeding and Busta, 1981). It is an interesting phenomenon that spores injured by irradiation can generally initiate germination at rates even faster than those of unirradiated controls. This appears different from the injury caused by heat, which is expressed as damage to germination (Gould, 1984). An explanation for this is that irradiation damages spore DNA that is not needed for the initiation of germination. From this, irradiation injury is usually expressed during outgrowth (Rowley et al., 1983).

The significant difference between UV and gamma-ray irradiation is that the former involves base dimer formation (e.g., thymine-thymine, thymine-cytosine, cytosine-cytosine mostly pyrimidine dimers) leading to messenger disorder while the latter consists mainly disruption of single strand or double strand of DNA. In both cases, disorder of DNA messenger to produce proteins has occurred and dinfection can be evaluated from the death or survival of targeted microorganisms.

3-3. Injury by several sorts of chemicals

Several chemicals used in disinfection and/or sterilization can injure bacterial spores, especially through their action on spore coats (Gould, 1984). Spores with damaged spore coats are more sensitive than intact organisms to chemicals. Bacterial spore injury through the action of chemicals has been reported with adverse pH conditions (Gould, 1984), ethylene oxide (Futter and Richardson, 1970a, b, Davis et al., 1978), hydrogen peroxide (Wallen and Walker, 1979), hypochlorite (Foegeding and Busta, 1983a,b), alcohols (Craven and Blankenship, 1985) and/or nitrite (Gould, 1984).

Hypochlorite injures bacterial spores such as *Clostridium botulinum* (*C. botulinum*) (Foegeding and Busta, 1983a, b). Hydrogen peroxide has injured spores of *Bacillus atroaeus* (*B. atrophaeus*) (Wallen and Walker, 1979), *B. cereus* and *C. sporogenes* (Neal and Walker, 1977). Ethylene oxide injures bacterial spores (Roberts, 1970). They are, for example, *C. perfringens* (Futter and Richardson, 1970a,b), *B. subtilis*, and *Geobacillus stearothermophilus* (*G. stearothermophilus*) (Davis et al., 1978). Alcohol treatment can reduce the activation of *C. perfringens* spore, and higher levels injure them. Ozone has been reported as an effective sporicide, especially at low pH and higher humidity at more than 90% (Sakurai et al., 2003). The removal of spore coat proteins in *Bacillus* and *Clostridium* spores enhanced their inactivation by ozone (Foegeding, 1985). Spores lacking intact coats were significantly more sensitive to inactivation by chlorine dioxide than strains with intact spore coats (Foegeding et al., 1986). Copper increases spore sensitivity to hydrogen peroxide (Bayliss and Waites, 1976; Waites et al., 1979).

3-4. Injury by combination

The phenomenon of combined chemical and/or physical treatments causing bacterial spore injury is reported by Waites and Bayliss (1984). Combination effects have also been observed between gamma-irradiation and heating. Irradiation reduced heat resistance (Gomez et al., 1980) and preheating reduced radiation resistance (Ma and Maxcy, 1981). The presence of free radical producing compounds (e.g., iodide, iodate, or iodoacetate) during radiation treatment results in strong synergistic effects expressed as the inhibition of spore germination. This is thought to be due to inactivation by the free radicals of spore proteins or enzymes involved in germination (Gould, 1984; Waites et al., 1979). Irradiated spores are more sensitive to subsequent heat treatment than unirradiated spores. The increased heat sensitivity was not observed when the spores were heated in the presence of sucrose or glycerol (Foegeding and Busta, 1981; Gomez et al., 1980). One reason is that sucrose or glycerol served as a radical scavenger with their OH functional groups and another is the damage of an osmoregulatory mechanism of rehydration involved in the development of heat sensitivity in irradiated spores. Spore damage is greater in the presence of both heat and chemical treatments and either one of these treatments can be used to sensitize the spores to the other (Waites and Bayliss, 1984). The pretreatment of spores with hydrogen

peroxide and/or peracetic acid makes them more susceptible to damage by heat. Heated spores are more sensitive to inhibition by chemicals such as hydrogen peroxide or glutaraldehyde.

4. Injury during outgrowth

The outgrowth stage of spore formers may also involve injury by several sorts of sterilization treatments. Shifts in optimal temperatures to lower levels for the recovery of heated spores have been suggested as favoring outgrowth because germination can occur in a wider temperature range (Prentice and Clegg, 1974; Busta, 1967). Injury during outgrowth may involve any of several structures and metabolic pathways (Adams, 1978). Injury may involve damage to the spore membrane, DNA or other vital components and structures of the cell. After injury, the spores were germinated with lysozyme but they retained their injury because they continued to be sensitive to chemicals. The results suggested that injury had occurred in the outgrowth stage of the life cycle of spore formers.

5. Vegetative cell injury

The injury of spores is practically more important than that of vegetative cells, because spores are more tolerant to sterilization procedures. Research is needed on the sporulation of injured cells to determine any inherent changes in the resulting spores that may influence subsequent destruction, injury, and repair processes (Hurst, 1984b). The injury of vegetative cells may be expressed as sensitivity to several sorts of chemicals, modified metabolic activity, and leakage of intracellular material (Tsuchido, 2003).

Electron microscopic observations have indicated holes and fractures in the membranes of heated B. cereus cell (Hurst, 1977). The disappearance of ribosome and the coagulation of cytoplasmic protein were also observed. Studies with *B. subtilis* and *B. cereus* have indicated reversible damage to cell membranes, DNA, and RNA.

6. Factors causing injury

The factors affecting bacterial spore destruction can also affect injury (Foegeding and Busta, 1981). The conditions influencing resistance to stress, injury, mechanisms of injury, and repair vary with the genera, species, and strains of spore formers. Spore of *B. subtilis* is less resistant to irradiation injury than that of *C. botulinum*. It is interesting that the mechanism of DNA damage by heat was different for the spore and vegetative cell of *B. subtilis* (Uchida and Kadota, 1979). Cells in the log phase are more susceptible to injury than spores or cells in the stationary phase (Mackey, 1984; Mossel and van Betten, 1984; Manas and Mackey, 2004). Important properties of the substrate include the water content, the concentration and type of ions and pH, the oxidation-reduction potential, the gas atmosphere, the presence of protective agents, and contamination with other microorganisms. Handling, storage, and treatment of the injured spore suspension after exposure to stress will also affect recovery. Important considerations include the recovery enriched media, the germinants, the nutrients, the selective agents, the antimetabolites, the oxidation-reduction potential, the gas atmosphere, the pH, the water activity, the incubation temperature, and the prolonged cultivation period (Foegeding and Busta, 1981).

Thermally injured spores have demonstrated sensitivities to sodium chloride (Briggs and Yazdany, 1970) and other curing salts (i.e., sodium nitrate or sodium nitrite), antibiotics, other chemical inhibitors, certain culture media, varying lots of culture media, the pH of the recovery medium, the oxidation-reduction potential, and the gas atmosphere during recovery. In addition, thermally injured spores have demonstrated delayed germination, a sensitivity of the germination process to media components, the need for a modified incubation temperature, and the need for a longer incubation period for optimum colony formation (Hurst, 1984a; Adams, 1978; Foegeding and Busta, 1981). The spore injury by irradiation has led to a sensitivity to sodium chloride and other components, pH, certain gas atmospheres, dilution and incubation temperature, and a requirement for a longer incubation period for recovery (Foegeding and Busta, 1981). Spores injured by chemicals are sensitive to pH, gas atmosphere, sorts of media, and incubation temperature. In that meaning, to attain successful and reproducible sterilization validation, these factors must be seriously studied and a scientific rationale attained to avoid falsified validation results.

Recovery has been improved with lysozyme, high L-alanine levels, lactate, and malate, which improved germination with ferrous sulfate ($FeSO_4$), manganous sulfate ($MnSO_4$), yeast extract, glucose, and vitamin-free casamino acids, and with a longer incubation period (Foegeding and Busta, 1981, Shintani, 2006).

7. Sites and mechanisms of spore injury and repair

Sites and mechanisms of spore injury are presented in Table 2 (Waites and Baylis, 1984). Sites and mechanisms of spore injury by heat include as follows: damage to germination systems, the loss of

TABLE 2. Site of bacterial spore injury by combined treatment

Site of injury	Treatments
Coat	Heat + chlorine
	Heat + hydrogen peroxide
	Glutaraldehyde + formaldehyde
Membrane	Heat + neomycin and polymyxin
	Glutaraldehyde + formaldehyde
Cortex	Heat + hydrostatic pressure
	Heat + irradiation (γ)
	Heat + chlorine
	Heat + hydrogen peroxide
	Heat + Cu^{2+}
	Irradiation (γ) + hydrostatic pressure
	Ultrasonic waves + glutaraldehyde
Exosporium	Ultrasonic waves + hydrogen peroxide
Proteases	Heat + Cu^{2+}
Calcium removal	Glutaraldehyde + ionizing cation
DNA	Heat + irradiation (γ)
	Heat + hydrogen peroxide
	Heat + ethidium bromide
	Irradiation (γ) + hydrostatic pressure
	Irradiation (UV) + hydrogen peroxide

Cited from Waites and Baylis (1984), pp.223

cortex lytic activity through enzyme inactivation or inactivation of the mechanism involved in enzyme release, damage to the spore membrane (plasma or cortical) structures related to the need for suitable osmolarity or water activity during recovery, and damage to DNA.

Thermal injury does not cause single-strand DNA breaks. Chemical injury has been associated with the inactivation or alteration of spore-germination systems, while injury by irradiation is almost associated with single-strand or double-strand DNA breaks. This means depending on the sort of sterilization procedure, sites of injury differ, which means recovery mechanisms also differ.

7-1. Damage to germination enzymes

The germination of bacterial spores may be inactivated or altered by heat and chemical treatments (Hurst, 1984a; Gould, 1984). Damage to germination systems by heat has often been characterized by a need for specific germinants or enzymes in the medium (Foegeding and Busta, 1981; Gould, 1984). For example, Ca, glucose or alanine are considered as germinants and thus inclusion of them into the culture medium is indispensable to attain reproducible and successful sterilization validation (Shintani, 2006; Shintani et al., 2000; Sasaki et al., 2000). Damage to the germination system has been demonstrated with the use of calcium dipicolinate (Edwards et al., 1965a,b), lactate (Foegeding and Busta, 1983a), lysozyme (Busta and Adams, 1972; Adams and Busta, 1972; Barach et al., 1974; Adams, 1974; Duncan et al., 1972; Alderton et al., 1974), and mixtures of amino acids (Uchida and Kadota, 1979; Gurney and Quesnel, 1981; Gurney and Quesnel, 1980) and so on. As most chemicals that promote improved recovery (e.g., lysozyme, other lytic enzymes, calcium dipicolinate, and amino acids) are involved in promoting spore germination (Gould, 1984), it is speculated that they act by helping the spores bypass the injured L-alanine germination system (Gould, 1984).

The mechanism of injury to germination involves the inactivation of lytic enzyme systems, or the mechanism that releases these lytic enzymes, which are involved in degradation during germination (Adams, 1978). This is supported by the effect of lysozyme, other lytic enzymes, and dipicolinate in allowing the injured spores to bypass the damage and germinate. The inactivation of germination enzymes has been supported by large losses of cortex lytic enzyme activity in thermally injured spores, and by thermodynamic values for the inactivation of the L-alanine germination system that were consistent with protein denaturation (Adams and Busta, 1972). Increased recovery by the use of complex amino acid mixtures may be effective through the generation of NADH and ammonium, since these could be unavailable in the L-alanine germination system through the inactivation of alanine dehydrogenase involved in the alanine

cycle (Gurney and Quesnel, 1981; Gurney and Quesnel, 1980). Treatment with chemical agents may render the germination process sensitive to lysozyme, which enhances the germination of spores (Hurst, 1977). One example is that the treatment with alkali removes spore coat proteins and renders the germination process sensitive to lysozyme. The other example is that chlorine treatment also removes coat proteins from spores (Wyatt and Waites, 1975).

In the mechanism of the injury to germination systems, lytic enzymes, e.g., lysozyme, are speculated to enhance germination by hydrolyzing the β,1-4 linkage of peptidoglycan in the spore cortex (Fig. 3), which then permits the hydration of the core of the spore (Gould, 1984). It may involve interference with membrane function as indicated by the increased sensitivity of heated spores (Flowers and Adams, 1976) to surface active agents including antibiotics (Gould, 1984).

7-2. Damage to the spore membrane

The spore membrane and structure have been proposed as the site of injury (Hurst, 1984a; Adams, 1978; Foegeding and Busta, 1981; Gould, 1984). Even when lysozyme is needed to germinate injured spores, the actual repair may be taking place during the outgrowth stage. This could indicate that injury might be associated not with a germination system, but with the membrane (Foegeding and Busta, 1981; Barach et al., 1974; Barach et al., 1975). Evidence to support the membrane damage theory includes the sensitivity of heated C. perfringens spore to several antibiotics and chemicals with surface-active properties (Barach et al., 1974; Flowers and Adams, 1976). Damaged membrane is also sensitive to sodium chloride, nitrate, nitrite, and fatty acids (Flowers and Adams, 1976; Chumney and Adams, 1980). The lack of repair during germination occurred when transferred in a medium supporting outgrowth (Barach et al., 1974; Flowers and Adams, 1976) and at the completion of repair during outgrowth. It occurred even in the presence of inhibitors of RNA, proteins, DNA, and cell wall synthesis (Flowers and Adams, 1976; Chumney and Adams, 1980). The injured spores were osmotically fragile (Gomez et al., 1980). It was observed by electron microscopy that in heat-treated spores, the plasma membrane was separated from the core of *C. botulinum*. From this it can be speculated that heat causes damage to the plasma and cortical membrane of the spore, which become the vegetative cell membrane and cell wall, respectively (Hurst, 1984a; Adams, 1978; Foegeding and Busta, 1981).

Significant changes in pH may also cause damage associated with spore membranes (Gould, 1984). Alkali treatment dissolves protein components in the spore coat, which increases permeability and allows lysozyme to act on the cortex peptidoglycan. This also happens after treatments that rupture disulfide bonds (Gould, 1984). Milder alkali treatments increase the sensitivity of spores to various germinants (Vary, 1973) by improving access of the germinants to receptor sites (Gould, 1984). The effect of alkali treatment on spore coat permeability may also be responsible for the increased susceptibility of the spores to disinfectants and other chemicals, some of which (e.g., hypochlorite) also remove coat protein (Gould, 1984). Reduced pH may activate spores and enhance germination, while drastically low pH values may induce dormancy (Gould, 1984; Vary, 1973). High acidity is linked with the removal of cations, especially calcium, from the spores (Blocher and Busta, 1985; Rode and Foster, 1966), which may cause a reversible injury of the germination mechanism (Gould, 1984) because calcium is an indispensable agent for spore germination (Shintani, 2006). The exchange of cations caused by acid treatment also reduces the heat resistance of the spores (Alderton, 1964), which is also reduced by irradiation resistance (Gomez, 1980). Reduced heat resistance catalyzed by acid is reversed when the spores are reloaded with cations (Gould, 1984). The mechanism for the acid-catalyzed loss of heat resistance may be related to changes in spore hydration caused by changes in the ionic state of the spore cortex, which affects its osmotic and contraction state (Gould, 1984; Alderton, 1963).

7-3. Injury to DNA and RNA

Injury by irradiation or heat may also be due to changes in the genetic materials of the cell, especially DNA (Hurst, 1984a; Adams, 1978; Foegeding and Busta, 1981; Gould, 1984). Breaks in the single-strand of the spore DNA have been identified in irradiated spores (Grecz and Grice, 1978). The spore DNA is more resistant to single- and double-strand breaks than the DNA of vegetative cells. The ionizing and UV-radiation resistances of DNA isolated from cells and spores are similar. The increased resistance of spore DNA appears to be due to either increased structural integrity and/or its ability to repair single-strand DNA breaks after the initiation of spore germination (Grecz and Grice, 1978).

The recovery of DNA appears to take place in dormancy. The repair of DNA during dormancy is apparently catalyzed by an enzyme which appears to be a magnesium-dependent DNA ligase (Gould, 1984; Durban et al., 1974). Spore injury by UV irradiation

also involves DNA but it appears to be different from the DNA breaks caused by ionizing radiation as mentioned in 3.2.

Damage of spore DNA by UV irradiation appears to be different from that of vegetative cells. Spore damage is thought to be due to changes in the configuration of DNA (Gould, 1984). Injury and repair by thermorestoration is speculated to occur at the site of DNA (Tanooka, 1978). The synergistic effect of irradiation and heat treatments, which is expressed as an increased heat sensitivity of pre-irradiated spores (Gombas and Gomez, 1978), is probably due to the increased fragmentation of spore DNA, and the inactivation of DNA repair enzymes by heat (Gould, 1984). This indicated that spores treated only with irradiation were active (Gould, 1984). The increased heat sensitivity of preirradiated spores may be due to damaged peptidoglycan in the spore cortex, which can allow the hydration of the core and result in reduced heat resistance (Hurst, 1984a; Gould, 1984). In addition to germination enzymes and membrane damage, DNA has also been suggested as a target of spore injury by heat (Hurst, 1984a; Adams, 1978; Foegeding and Busta, 1981; Gould, 1984; Gombas, 1983).

Some of the more complex nutritional requirements of heat-injured spores have been attributed to heat-induced mutations (Adams, 1981). The need for amino acid supplementation to induce the recovery of heat-injured spores has suggested that heat may damage spore DNA (Uchida and Kadota, 1979). The alteration of amino acid metabolism or amino acid-stimulated germination may be another effect of thermal injury (Gould, 1984). Dry heat also caused mutations which were expressed as reduced sporulation (Gould, 1984). Chemical damage may also occur on DNA and result in lower heat resistance (Hanlin et al., 1981).

8. Repair of injured spores

Several papers have been published on this subject so far (Hurst, 1984a; Gould, 1984; Ray and Adams, 1984; Waites and Baylis, 1984; Mossel and van Netten, 1984; Mackey, 1984, Gilbert, 1984a,b; Johnson and Busta, 1984). The role of medium constituents in the recovery of injured spores has been discussed (Blocher and Busta, 1982; Shintani, 2006; Shintani et al., 2000; Sasaki et al., 2000). They described the effects of inhibitors on recovery and the modified metabolic requirements of injured spores. Their conclusion is the constituents to enrich the culture medium are required by injured microorganisms. This means an enriched culture medium is more desirable than the selective medium to avoid falsified results in a sterilization validation study.

Injury was initially recognized in terms of cultural inadequacies observed in microorganisms exposed to various stresses. Most of these inadequacies need to be addressed before the organisms present any evidence of vitality through cell division. Restoration to the original undamaged condition is accomplished through the process known as repair or recovery (Busta, 1978). There is a need for the development of standardized methods for the recovery and enumeration of both injured and uninjured spores (Johnson and Busta, 1984). There are several complex steps (i.e., activation, germination, outgrowth, and growth) to be completed before the dormant spores can grow and be enumerated (Fig. 1). The same set of events should be completed for the detection and enumeration of injured spores.

The culture media and environmental conditions employed should support these events for appropriate spore recovery. Injured spores may require modified conditions for their recovery and enumeration compared with uninjured entities. The sublethal damage of spores results in various sensitivities and additional requirements for the injured spores to repair their injury and grow. Factors to be considered include sensitivity to activation treatments, germinants, selective agents, chemical additives, other inhibitors, oxidation-reduction potential, pH, water activity, osmolarity, nutritional requirements, and incubation requirements including gas atmosphere, temperature, and incubation period.

The repair and recovery of injured cells usually requires richer culture media, the removal of inhibitors, and optimum incubation conditions, including pH, relatively lower temperatures for cultivation, and an extended incubation period. The influence of these factors in specific situations appears to be dependent on the type of microorganism, the nature of the stress, and the type and extent of injury (Johnson and Busta, 1984). An activation treatment usually consists of heating at 80℃ for 10 min, but specific requirements may vary with the strain, the injury, the suspending medium, and the recovery medium (Johnson and Busta, 1984). Some specific factors to be monitored during recovery are the condition of the population before its exposure to stress, handling and storage of the sample before enumeration, the composition of the recovery medium (e.g., the type of medium, nutrients, and inhibitors), the dilution procedures and diluents, the mode of inoculation, the enumeration procedure (e.g., plating or MPN), and the conditions of incubation (Mossel and van Netten, 1984). The recovery requirements may be different with the type and extent of injury, the species, strains,

and individual spore suspensions (e.g., the conditions of sporulation and handling before and after exposure to stress), storage and handling after exposure to stress and before recovery, and the conditions of recovery. Research on specific recovery media and constituents for injured spores is limited and not systematic (Table 3). The responses may be different with varying strains and the types of injury (Blocher and Busta, 1982; Farkas and Roberts, 1982) Thus, specific media may be appropriate for recovery under certain conditions, and different individual components may be needed for enumeration under different conditions of injury. Variations in media, their constituents, purity, and storage period may be influential in the repair of injured spores. Media components that may enhance recovery under certain conditions include potential nutrients such as sugars, yeast extract, glucose, and amino acids, absorbants such as starch, reducing agents such as thioglycollate and cysteine, lysozyme and egg yolk, bicarbonate, cultural filtrates and divalent cations (Blocher and Busta, 1982). In addition to the media and their constituents, the appropriate pH, gas atmosphere, oxidation-reduction potential, incubation temperature, and incubation period should be selected for the individual strains to recover from specific types of injury.

Injured spore formers have significant sensitivity to sodium chloride (Feehery et al., 1987), nitrate and nitrite (Chumney and Adams, 1980), surface-active agents including antibiotics (Chumney and Adams, 1980), pH (Feehery et al., 1987), water activity and osmolarity (Mattick et al., 2001), gas atmosphere (Feehery et al., 1987), culture media, media brand and their lots (Pflug et al., 1979; Pflug et al., 1981; Sasaki et al., 2000; Shintani and Akers, 2000; Shintani et al., 2000), and incubation temperature and period (Feehery et al., 1987; Shintani et al., 2000). The type and extent of these sensitivities vary with the species and the type of injury.

Irradiation-injured spores in general were less affected by pH than by heat-damaged spores (Futter and Richardson, 1970). It was indicated that only two modifications have been recommended widely and adopted in the specific detection of injured and uninjured spores (Ray and Adams, 1984). They include the use of starch as a binder of potential inhibitors in the recovery medium and extension of the incubation period for the detection of survivors. Other potential requirements and modifications for injured spore

TABLE 3. Effect of added agents to culture media on the recovery of injured bacterial spores

Ingredients	*Clostridium*	*Bacillus*
Nutrients		
Glucose	0,+	0,+
Fructose		+
Sucrose	0	+
Galactose		+
Maltose		0,+
Egg yolk	+	+
Yeast extract	+	0,+
Casamino acids		+
Lactate	+	
Absorbants		
Starch	0,+	+
Charcoal	+	+
Serum albumin	+	
Reducing agents		
Thioglycollate	−,+	0
Cysteine	+	0
Cations		
Magnesium		+
Calcium	+	+
Iron		+
Others		
Bicarbonate	+	
Lysozyme	+	0
Cultural filtrates	+	+

0: No effect, −: Reduced recovery, +: Increased recovery
Cited from Blocker, J.C. and Busta, F. F. (1982).

formers to recover are discussed in the following.

8-1. Culture medium

The culture medium used to detect injured spore formers will influence their recovery (Johnson and Busta, 1984; Pflug et al., 1979; Wallen and Walker, 1979; Shintani et al., 2000; Sasaki et al., 2000; Shintani and Akers, 2000; Shintani, 2006). Several media have been used for the recovery of injured spores (Table 4). The recovery of *B. pumilus* spores injured by UV irradiation was greater in double-strength soybean casein digest broth (SCDB) than in soybean casein digest agar (SCDA) (Abshire et al., 1980). Heat-injured *C. sporogenes* P.A. 3679 spore recovered better in Anderson's pork-pea infusion than in trypticase peptone agar, yeast extract agar, pork infusion agar, and T-Best agar (Polvino and Bernard, 1982). This suggested that recovery differed when medium differed and additionally suggested that the pour-plate procedure was not adequate for the recovery of heat-stressed spores. The repair rate of *C. perfringens* was much higher in trypticase yeast extract broth than in 0.1% peptone (Traci and Duncan, 1974). The recovery of heat-stressed *C. perfringens* was also better on tryptone-sulfite-cycloserine medium without egg yolk than on sulfite-polymyxin-sulfadiazine medium (Orth, 1977). More heat-stressed *C. sporogenes* recovered on modified P.A. 3679 agar than on yeast extract agar and peptone trypticase agar (Grischy et al., 1983). Treatment of *B. megaterium* with chlorhexidine resulted in a sensitivity to potassium chloride during recovery in SCDA, but recovered in SCDB (Nadir and Gilbert, 1982). Recovery is influenced not only by the kind and constituents of the culture medium, but also by the condition of the medium (Pflug et al., 1981; Pflug et al., 1979), the quality of the constituents (Johnson and Busta, 1984; Shintani et al., 2000; Sasaki et al., 2000), and the brand and the lot of the medium (Pflug et al., 1981; Pflug et al., 1979; Shintani et al., 2000; Sasaki et al., 2000). Different lots of soybean casein digest, even from the same manufacturer, resulted in different rates of recovery of heated *G. stearothermophilus spores* (Pflug et al., 1981; Shintani et al., 2000; Sasaki et al., 2000; Shintani and Akers, 2000; Shintani, 2006). This lot to lot variation

TABLE 4. Culture media used for the recovery of injured spore formers

Treatment	Organism	Medium
Heat	*Clostridium sporogenes*	Yeast extract starch bicarbonate agar
		Yesair's pork infusion+thioglycollate
		Pork-pea agar
		Pork-pea or beef infusion extract
		Thioglycollate milk+bicarbonate
		Beef infusion or yeast extract agar
		Anderson's pork-pea infusion
		Modified P.A. 3679 agar
	C. botulinum	Yesair's pork infusion+starch
		Yeast extract starch bicarbonate agar
		Yeast extract agar
	C. perfringens	Tryptone-sulfite-cycloserine
		Shahidi-Ferguson perfringens agar
		Trypticase yeast extract broth
		Sulfite-polymyxin-sulfadiazine medium
	Bacillus cereus	Mannitol egg yolk polymyxin
	B. stearothermophilus	Antibiotic assay medium A
		Eugon broth
	B. subtilis	Beef infusion
		Columbia broth
	Desulfotomaculum niginficans	Soytone-sulfite agar
Irradiation	*C. sporogenes*	Eugon agar cystine
		Pork-pea infusion
	C, botulinum	Pork-pea infusion
	B. pumilus (UV)	Double-strength trypticase soy broth
Ethylene oxide	*B. subtilis*	Columbia broth
Hydrogen peroxide	*B. subtilis*	Yeast extract, glucose, casamino acid agar + $FeSO_4$+$MnSO_4$

Cited from Blocher and Busta (1982).

and brand to brand variation may be due to the differences in the nutrient composition among the same culture medium. New lots or brand of media and components should be validated to avoid falsified sterilization validation because consecutive success in validation studies is indispensable (Johnson and Busta, 1984; Shintani et al., 2000; Sasaki et al., 2000; Shintani and Akers, 2000). In that meaning equivalency between the old culture medium and the new culture medium must be confirmed in sterilization validation studies (Shintani et al., 2000; Sasaki et al., 2000; Shintani, 2006).

8-2. Nutritional requirements

Injured spores have fastidious requirements and may need nutrient supplementation in the recovery medium (Mackey, 1984; Shintani et al., 2000; Sasaki et al). Nutritional requirements vary with species and strains of microorganisms and are variable with respect to the injured state (Johnson and Busta, 1984). The recovery of heated *Bacillus* spores was improved when glucose or blood was added to the nutrient agar (Johnson and Busta, 1984). Several compounds (yeast extract, liver extract, glucose, fructose, mannose, galactose, sucrose, maltose, soluble starch, pyruvate, glycerol phosphate, amines, amino acids, vitamins and divalent cations) increased the recovery and apparent heat resistance of *B. subtilis* spore (Johnson and Busta, 1984). Other compounds (xylose, arabinose, trehalose, lactose, glycerol, mannitol, glycogen, lactate, acetate, and succinate) did not significantly improve the recovery of injured *B. subtilis* spore (Johnson and Busta, 1984).

Several individual and some combinations of amino acids (glycine, alanine, homoserine, threonine, valine, glutamine, arginine, isoleucine, aspartic acid, and methionine) also improved the recovery of injured *B subtilis* spore (Johnson and Busta, 1984). The recovery of hydrogen peroxide-damaged *B. atrophaeus* spore was improved with yeast extract, glucose, and casamino acids (Johnson and Busta, 1984; Wallen and Walker, 1979). In every case of injury, addition of glucose, pyruvate, alanine and divalent cations (Ca and Mg) will cause significant recovery (Shintani, 2006).

8-3. Germinants

Useful agents to initiate germination are lysozyme, egg yolk emulsion, initiation protein, and calcium dipicolinate. Among them, lysozyme is the most common additive that has improved the recovery of heat-injured *C. perfringens* (Barach et al., 1974) and *C. botulinum* spores (Alderton et al., 1974). An initiation protein produced by *C. perfringens* has also increased the heat resistance (Duncan et al., 1972). An initiation factor was also detected during the growth of *G. stearothermophilus* in certain media, and promoted the recovery of thermally injured *G. stearothermophilus* spore (Labbe, 1979). This factor appeared to be influential on outgrowth.

Calcium dipicolinate has also improved recovery, and increased the heat resistance of *B. subtilis* spores (Busta and Adams, 1972).

8-4. Detoxifying agents

The stressed microorganisms indicate the increased sensitivity to several inhibitors. Traces of inhibitory agents (e.g., unsaturated fatty acids such as linoleic acid and linolenic acid) which may be present in the culture media are speculated to be absorbed and neutralized through the addition of starch, activated charcoal, and serum albumin to the medium. Increased recovery with the inclusion of such compounds in the medium has been recognized with various spore formers (Labbe, 1979; Labbe and Change, 1995). Another study has reported no improved recovery with starch in the culture medium (Blocher and Busta, 1982). The speculated discrepancy between the results is due to the presence of inhibitors not absorbed by starch, or the presence of inhibitor concentrations higher than those absorbed by starch (Blocher and Busta, 1982).

8-5. Inhibitors

Damaged spores are sensitive to several agents, such as antibiotics and surface-active agents (e.g., sodium lauryl sulfate, sodium deoxycholate, and quaternary ammonium compounds), chloride, nitrite, nitrate, acids, alkali, unsaturated fatty acids, and so on (Barach et al., 1974; Flowers and Adams, 1976; Chumney and Adams, 1980; Tsuchido et al., 1983; Tsuchido et al., 1987). Injured vegetative cells presented a reduced recovery in media with selective agents.

However undamaged cells were perfectly tolerated and were not influenced the recovery rate. The recovery substrate should be applied to support the recovery of injured organisms (Blocher and Busta, 1982).

Modifications may include adjustments in pH, the removal of inhibitory agents, the use of different selective agents, or the use of chemicals that will support recovery (Blocher and Busta, 1982). These modifications will depend on the microbial strains and the type of injury (Gould, 1984). Injured spores are often sensitive to pH variations in the recovery medium (Futter and Richardson, 1970a). Heat-injured spores in general recovered optimally at a neutral pH (Cook and Brown, 1965; Yokoya and York, 1965). In

addition to heat-injured spores, spores damaged by irradiation are more sensitive to the pH of the recovery medium than undamaged spores (Farkas and Andrassy, 1985). Other chemicals that improve spore recovery include sucrose and glycerol (Busta, 1978), bicarbonate (Odlaug and Pflug, 1977), and thioglycollate (Odlaug and Pflug, 1977). Several cations, such as iron, magnesium, copper, calcium, and manganese can improve recovery by enhancing germination or cell growth (Busta, 1967). Sucrose and glycerol are considered to be radical scavengers.

8-6. Oxidation-reduction potential (Eh)

The oxidation-reduction potential of the medium and the gaseous conditions can be influential on the recovery of normal as well as damaged anaerobic spore formers (Shoesmith and Worsley, 1984).

Anaerobe spore of *C. perfringens* damaged by heat recovered best in a pure nitrogen or in hydrogen atmosphere than in one in which they are mixed. Irradiated spores, however, recovered better under nitrogen than hydrogen or their mixture. Spores damaged by ethylene oxide gas also preferred nitrogen in their recovery environment (Futter and Richardson, 1970b). These phenomena indicate that the recovery of the damaged spores differs depending on the sort of sterilization procedures.

The environments of carbon dioxide are known to enhance the germination and growth of *Clostridium* spp (Foegeding and Busta, 1983c). Hydrogen, in combination with carbon dioxide or nitrogen, however, did not alter germination, compared with a carbon dioxide or nitrogen atmosphere (Foegeding and Busta, 1983). Carbon dioxide levels at atmospheric pressure inhibited the germination of the aerobe *B. cereus*, but enhanced the germination of clostridia. Increased carbon dioxide levels under high pressure caused the complete inhibition of clostridial spore germination (Enfors and Molin, 1978). Although the type of gas atmosphere is important in the recovery of anaerobes, the oxidation-reduction potential of the substrate has been more important than the gas composition of the environment (Johnson and Busta, 1984). Improved recovery with sodium thioglycollate has been attributed to its reducing properties.

Reducing agents including thioglycollate may be inhibitory, depending on the type, the concentration, the strain, and the exposure time (Shoesmith and Worsley, 1984). Thus, the use of cysteine as a superior reducing agent, which is noninhibitory to clostridia, has been recommended (Shoesmith and Worsley, 1984).

8-7. Incubation temperature

The most appropriate and optimum incubation temperature varies for the enumeration of various species and types of spore formers. The optimum temperature for injured spores is in general different than for uninjured spores. This has been observed with heat-injured spores, which often require lower or more restrictive temperature ranges for optimal recovery (Adams, 1978; Futter and Richardson, 1970a; Shintani, 2006). The lower or more restrictive temperature range for better recovery of injured spores may be influential either on germination (Adams, 1978) or on outgrowth (Prentice, 1974). This phenomenon has been observed with a variety of heat treatments, heating substrates, and recovery media.

The species of spore formers that have required a lower or more restricted incubation temperature range include *B. subtilis* (Prentice and Clegg, 1974; Shintani, 2006), *G. stearothermophilus* (Cook and Gilbert, 1968; Shintani, 2006), *C. perfringens* (Futter and Richardson, 1970a), and *C. botulinum* (Odlaug and Pflug, 1977). Spore of *B. subtilis* type heat-injured at ultra-high temperature recovered more effectively at relatively lower temperature than at an optimum temperature of 45℃ for uninjured spores (Edwards et al., 1965b; Shintani, 2006). Heated spore of *B. subtilis* also recovered better at 30℃, while uninjured spores were enumerated equally well at temperatures in the range 15 to 50℃ (Prentice and Clegg, 1974). Maximum counts for heated *G. stearothermophilus* spore were obtained at 45 to 50 ℃, compared with at 50 to 65℃ for unheated spores (Cook and Gilbert, 1968; Shintani, 2006). The maximum enumeration of heated *C. perfringens* spore was achieved at 26℃ (Futter and Richardson, 1970a) compared with 34℃ for the control. Injured spore of *C. botulinum* recovered best at 25℃ compared with at 31 to 37℃ for uninjured control (Adams, 1978).

Changes in the optimal incubation temperature have also been observed with irradiation-treated spores. Unirradiated spore of *C. botulinum* 62A was well enumerated at 45℃, while the detection of irradiated spore showed a maximum enumeration at 35℃ (Chowdhury et al., 1976).

8-8. Incubation period

Injured spore formers exhibit a longer lag phase during recovery than uninjured spore formers. The period of lag phase can be as short as a few hours or as long as several months. Thus, the incubation period should be adjusted for optimum recovery (Blocher and Busta, 1982; Mossel and van Netten, 1984).

Delayed germination and growth of injured spores may result in the underestimation of heat resistance if

the incubation period has not been prolonged (Lynt et al., 1983). Longer incubation period has been required for heat, irradiation, and chemically injured spores (Futter and Richardson, 1970a; Chowdhury et al., 1976; Wallen and Walker, 1979; Neal and Walker, 1979). In some instances, repair may be accomplished during storage at refrigeration temperature (Edwards et al., 1965b). The incubation period may also be shortened by the addition of certain ingredients (e.g., yeast extract, glucose, pyruvate, alanine and so on) in the recovery medium (Wallen and walker, 1979).

9. Conclusion

The presence of injured microorganisms in health care products and foods may cause any hazardous problems. It is necessary to diminish totally the injured and uninjured microorganisms to attain successful sterilization validation. Otherwise, faulty results may be attained in terms of sterilization validation. The phenomenon of superdormant spores (Gould et al., 1968) and their relationship to injury needs additional validation research.

The relationship of tailing in survivor curves relative to spore injury also needs evaluation (Ababouch et al., 1987; Ababouch et al., 1987, 1995). Preservation with combination of physical (e.g., heat) and chemical (e.g., sodium chloride, nitrite or sorbate) treatments may lead to spoiled or toxic products after a longer period of time than that for control treatment. It must be clarified whether this delay could involve some type of injury and repair process. Although heat injury has been studied more extensively than injury by other treatments, there is a need for additional work on injury caused not only by heating, but also by other sterilization processes, less well-investigated injuries, such as those caused by radiation, chemicals, drying, or cold temperature.

Future research should clarify the following matters. Why is injury expressed in various ways among species, strains, and processing treatments? Why does the same treatment sometimes result in injury that is expressed differently in various species or even within the same strain? Can knowledge on the repair requirements for some species be applicable to other species under similar conditions of injury? What is the extent and importance of injury in real healthcare product fabrication systems? Studies should be designed to clarify and to answer these and other pertinent questions. If such questions can be successfully addressed in the future, then guidelines and standardized procedures could be formulated for the recovery and enumeration of both normal and injured spore formers in culture media, as well as in complicated healthcare product fabrication systems.

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