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Microbiological Studies on Drugs and Their Raw Materials. I. Experiments on the Reduction of Microbial Contaminants in Tablets during Processing

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Two forms of powdery sample for tabletting were prepared; one was the mixture of Avicell (crystalline cellulose) and freeze-dried skim milk containing *Escherichia coli* cells and the other was a freeze-dried powder of skim milk solution containing *E. coli* cells together with Avicell. *E. coli* cells were killed by tabletting to some extent depending on the pressure applied. Different patterns of surviving fraction/pressure relation were obtained for the above two forms of samples. Effects of temperature and moisture content of samples were also examined. Large microbial cells, such as *Rhodotorula glutinis*, were much more sensitive to tabletting than smaller *E. coli* cells. Spores of *Bacillus subtilis* were highly sensitive to tabletting. From these observations the major cause of killing action in the process of tabletting was considered to be the shearing force.

Keywords——tabletting; microbial death; *Escherichia coli*; *Bacillus subtilis* spore; *Rhodotorula glutinis*; crystalline cellulose; shearing force; freeze-drying

In the good manufacturing practice in drug processing, the reduction of contaminating microbial cells is of primary importance. Although various conventional methods of sterilization may possibly be applied to drugs or their raw materials, the application of these methods seems to be largely limited at an industrial scale.

According to Morii, *et al.*,[2] alkaline protease was inactivated partially under pressure up to 8900 kg/cm^2 in tabletting process. Horikoshi, *et al.*[3] suggested that this was caused by a shearing force applied on the enzyme protein. These observation imply that the shearing force generating in the course of tabletting may be applied more effectively on the contaminated microbial cells in powdery raw materials.

The present paper describes experiments on the reduction of microbial contaminants in tablets during their processing without any special sterilization treatment.

Materials and Methods

Organisms and Cultivation——*Escherichia coli* W3110 and *Bacillus subtilis* IAM 1144 were obtained from Prof. A. Nishi and *Rhodotorula glutinis* No. 753 was obtained from Prof. H. Kozuka of this University. *E. coli* was shaken cultured overnight in nutrient medium at 25° and cells were collected by centrifugations. *B. subtilis* was cultured on nutrient agar (Eiken Chemical Co.) at 30° and cells were harvested after 8 days, when about 80% of cells had formed endospores.[4] A clean spore sample was obtained from this cell suspension using Urographin (Schering AG) by the method of Irie and Morichi.[5] Clean spores were subjected to freeze-drying and stored in a refrigerator. *Rh. glutinis* was shaken cultured for 2 days at 25° in MY medium.

Preparation of Sample Powders Containing Test Organisms——The raw material used for tabletting was a crystalline cellulose powder, Avicell (Merck Inc.), which had been sterilized with ethylene oxide gas employing a Mini-Pack MPT-700 sterilizer (Mini-Pack Co.). Harvested *E. coli* cells were suspended in skim milk

1) Location: *Gofuku 3190, Toyama.*
2) M. Morii, A. Sano, N. Takeguchi, and I. Horikoshi, *Yakugaku Zasshi*, **93**, 300 (1973).
3) I. Horikoshi, N. Takeguchi, and M. Morii, *Chem. Pharm. Bull.* (Tokyo), **21**, 2136 (1973).
4) R. Irie and H. Uchiyama, *J. Gen. Appl. Microbiol.*, **10**, 237 (1964).
5) R. Irie and T. Morichi, *J. Gen. Appl. Microbiol.*, **19**, 421 (1973).

sterilized by autoclaving. The cell suspension was divided into two parts: One was freeze-dried after the addition of Avicell powder at the rate of one g per two ml suspension, and the other was freeze-dried without addition of any other material and the dried sample was mixed thoroughly with Avicell using a mortar and a pestle. The resulting dry sample of the former *E. coli*/skim milk/Avicell system was termed ESA and that of the latter *E. coli*/skim milk plus Avicell system was termed ESPA. All these samples were stored in a dessicator at 4° unless otherwise noted. In preparing *Rh. glutinis*-containing Avicell, the freeze-dried cells in skim milk was mixed thoroughly with Avicell (ESPA-type) and in preparing *B. subtilis* spore-containing Avicell, dried clean spores were mixed directly with Avicell.

Tabletting——When various data on tabletting were necessary, a single punch eccentric tabletting machine (Kimura, KT-2) with a variable speeder and flat type punches (16 mm in diameter) was used. This machine is equipped with a strain gauge and a differential transformer and the latter two are connected to a synchroscope, from which the pressure, speed and packing height can be determined as reported earlier.[6] When the application of higher pressures was required, a die assembly used for the KBr tabletting in the infrared analysis, whose flat punch diameter being 10 mm, and an oil press machine (Rikenseiki, R-18) were employed. The amounts of samples applied were *ca.* 530 and 400 mg for the tabletting machine and the die assembly, respectively.

Humidity Control——Samples for tabletting, when necessary, were kept at 20° under the atmospheres of different relative humidities controled by the presence of diferent concentrations of sulfuric acid.[7]

Viable Cell Counting——Viable cell numbers were counted by the conventional pour plate method using nutrient agar for *E. coli* and *B. subtilis* and MY agar for *Rh. glutinis*. Prior to the cell counting, three tablets were placed in 10 ml saline solution containing 0.2% Tween 80 and left standing or forced to crush, when necessary, with a glass rod until they crumbled to fine pieces. Then the resulting saline suspension of Avicell was agitated thoroughly using a mechanical agitator, and an aliquot was subjected to viable counting using triplicate Petri dishes for each dilution.

Results

Effects of Pressure and Speed of Tabletting on Surviving Fraction

Employing a tabletting machine, the effect of pressure on viable cell number in either ESPA or ESA was examined. As shown in Fig. 1, the pressure could simply be changed by using different amounts of samples under a constant tabletting speed and packing height.

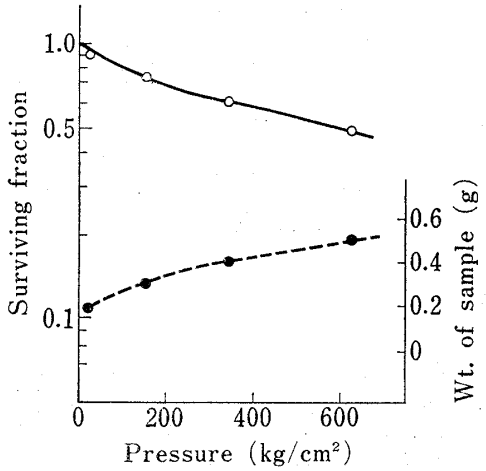

Fig. 1. Relationships between Weight of Sample and Pressure and between Surviving Fraction and Pressure

Different amounts of ESPA samples containing 2.1×10^5 *E. coli* cells/g were compressed in a tabletting machine.
--●--: weight of sample,
—○—: surviving fraction.

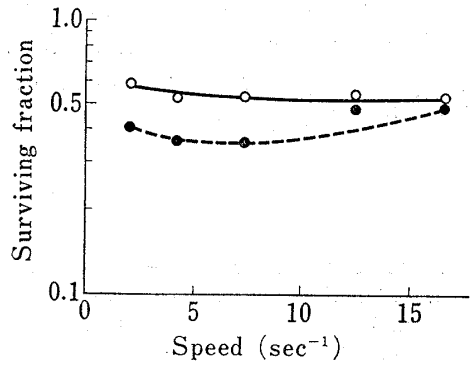

Fig. 2. Effect of Tabletting Speed on Surviving Fraction

Samples of ESPA containing 3.7×10^5 cells/g and ESA containing 2.1×10^8 cells/g were compressed at different speeds in a tabletting machine under pressures of 430 and 620 kg/cm², respectively.
—●—: ESPA, —○—: ESA.

6) I. Horikoshi, N. Takeguchi, and M. Morii, *Chem. Pharm. Bull.* (Tokyo), **22**, 327 (1974).
7) C.D. Hodgman, R.C. Weast, and C.W. Wallace (ed.), "Handbook of Chemistry and Physics," 35th Ed., Chemical Rubber Pub, 1953, p. 2310.

In either form of the sample, the surviving fraction, *i.e.*, the rate of surviving cell number in a processed tablet to that in an untreated sample, decreased as the pressure increased. A decrease in surviving fraction seemed to be much more marked in ESPA than in ESA (data not shown). As seen in Fig. 2, the effect of tabletting speed under a constant pressure on the surviving fraction was found to be exhibited even negligibly in either form of the sample in the range of speed so far examined.

Since it is mechanically difficult to yield higher pressure employing a tabletting machine and since we found the independency of tabletting speed on the surviving fraction, the effect of pressure on the surviving fraction was tested using a die assembly compressed with an oil press machine in the following experiments.

Using ESPA containing different number of *E. coli* cells, the effect of viable cell number on the surviving fraction/pressure relation was examined. Fig. 3A clearly shows that patterns of the dependency of surviving fraction on pressure remained almost unchanged irrespective of the wide difference in the starting cell numbers in samples. It should be noticed that in ESPA sharp breaks in these curves were found at the pressure around 500—700 kg/cm^2; the application of higher pressures over the point did not affect the rate of reduction in surviving fraction in ESPA. By contrast, in ESA no appreciable break in the dependency curve was observed (Fig. 3B); the rate of reduction in surviving fraction decreased almost steadily as pressure increased.

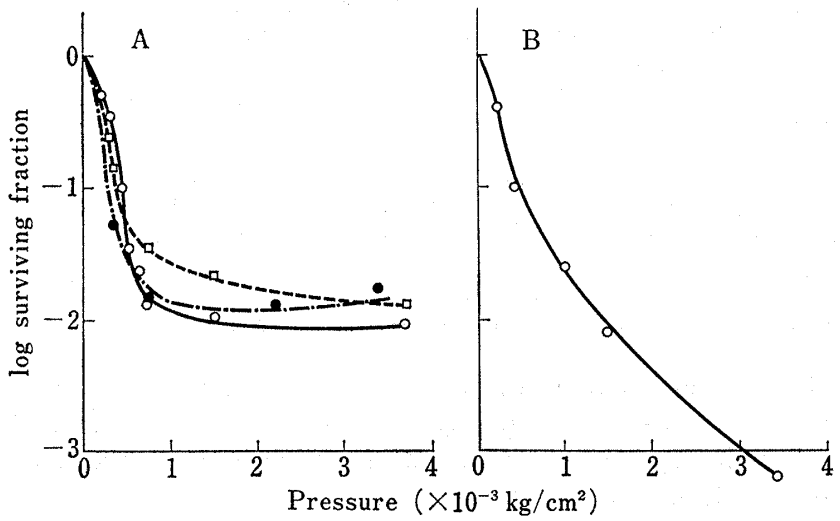

Fig. 3. Comparison of Patterns of Surviving Fraction/Pressure Relation using Two Different Forms of Samples

Samples were compressed in a die assembly under different pressures. A. ESPA containing varied numbers of cells. —○—: 6.1×10^4, —□—: 6.2×10^6, -·-●-·-: 6.3×10^7 cells/g. B. ESA containing 5.7×10^8 cells/g.

Effect of Moisture in Samples on Surviving Fraction

ESPA samples were stored overnight at 20° under atmospheres of different relative humidities and viable cell numbers before and after tabletting were counted. Fig. 4 shows that the starting cell numbers in samples kept under higher relative humidities were lower than those kept under lower ones, being caused by the death of dried *E. coli* cells under high humidity. The surviving fraction after tabletting was higher in samples kept under relative humidities lower than 40% than those kept under higher ones.

Effect of Temperature of Samples on Surviving Fraction

Each ESPA sample filled in a die assembly was kept at different desired temperatures (duration of temperature equilibrium had been measured preliminarily for each temperature)

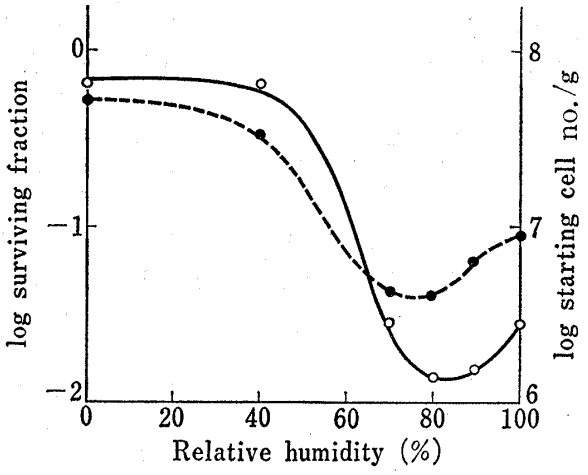

Fig. 4. Effect on Starting Cell Number and on Surviving Fraction of Atmospheric Relative Humidities under which Samples were stored

ESPA sample containing 7.9×10^7 cells/g were stored overnight under different relative humidities and those were compressed in a die assembly at the pressure of 744 kg/cm^2.
—●—: starting cell number, —○—: surviving fraction.

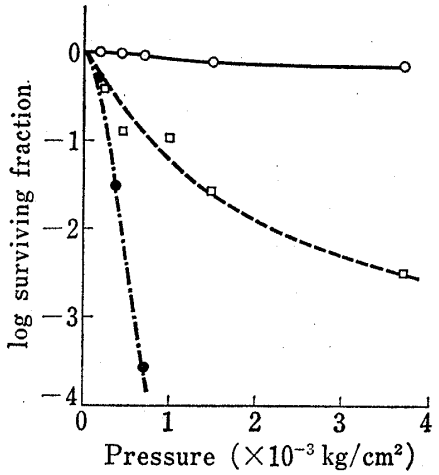

Fig. 5. Comparison of Susceptibilities of Various Organisms to Tabletting as Expressed by Surviving Fraction/Pressure Relation

—○—: *B. subtilis* spore, --□--: *E. coli* cells, -·●·-: *Rh. glutinis* cells.

Table I. Effect on Starting Cell Number and on Surviving Fraction of Temperature under which Samples were Compressed

Remarks	Cell number per g		
	30°	4°	−20°
Starting cell no. in sample	2.6×10^7	6.0×10^7	7.0×10^7
Cell no. in processed tablets	3.1×10^6	2.8×10^7	3.9×10^7
Surviving fraction	0.12	0.47	0.56

ESPA samples were compressed in a die assembly under the pressure of 744 kg/cm^2.

and the viable cell numbers before and after tabletting were counted. As seen in Table I, the starting cell number of a sample kept overnight at 30° was lower than those kept at 4° and −20°. The surviving fraction after tabletting was also low in the sample kept at 30°, whereas no appreciable difference in the surviving fraction was noted in samples kept at 4° and −20°.

Sensitivities of Different Organisms to Tabletting

Sensitivities to tabletting of microorganisms of different cell sizes and of different cell forms were examined employing samples containing vegetative cells of *E. coli* (ESPA) and *Rh. glutinis* (ESPA-type) and spores of *B. subtilis* (without skim milk). The surviving fraction/pressure relations obtained for these samples are shown in Fig. 5. *B. subtilis* spores were found to be highly resistant to tabletting in the wide range of applied pressure, whereas vegetative cells of *Rh. glutinis*, whose mean cell size was 7×2^2 μm^3, were much more sensitive to tabletting than those of *E. coli*, whose mean cell size was 2×0.4^2 μm^3.

Discussion

From the results of the present experiments, microorganisms contaminated in powdery samples were found to be killed by tabletting to some extent, depending on the applied pressure. The dependency on pressure of the surviving fraction seemed to be varied according to the state of subsistence of cells in samples as revealed by the use of ESPA and ESA. Figure

6 depicts schematically the state of subsistence of *E. coli* cells in both forms of samples. In the ESPA form of sample, cells were filled in skim milk particles and there should be interstices among Avicell and skim milk particles, since this sample was prepared by the simple mechanical mixing of Avicell particles and freeze-dried skim milk containing cells. By contrast, in the ESA form of sample, most of the interstices among Avicell particles are filled with dried skim milk containing cells, since this sample was prepared by the freeze-drying of skim milk solution containing cells together with Avicell particles. Assuming that the death of cells are caused by the disruption of dried cells by a shearing force generating in samples by compression, the degree of disruption of cells as a function of pressure may differ in both forms of samples.

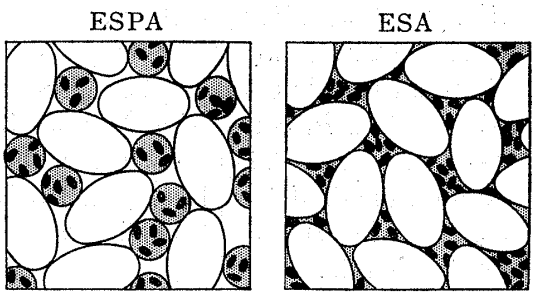

Fig. 6. Schematic Representation of the State of Subsistence of *E. coli* Cells in Two Forms of Sample

: Avicell particle, : dried skim milk, : dried *E. coli* cells.

In ESPA the shearing may occur in skim milk particles even under low pressure and a stronger shearing may also occur among skim milk and Avicell particles under higher pressures, thus resulting in the marked disruption of cells. The fact that under the pressure more than 500—700 kg/cm^2 a decrease in surviving fraction leveled off in ESPA suggests that skim milk particles still containing viable cells are compressed only in limited portions of interstices among Avicell particles and cells escape from the shearing action of the latter particles. In contrast to ESPA, in ESA shearing force in the range of lower pressures may be applied to cell likewise in ESPA, though less markedly, and it seems to be applied to cells even under higher pressures. This may probably be resulted from the situation that even fine portions of interstices among Avicell particles are totally filled with skim milk containing cells, thus even a minor displacement of Avicell particles at higher pressures may exhibit shearing action on cells.

The finding that in ESPA a decrease in surviving fraction leveled off under higher pressures tells us that the cause of death may not be only a pressure applied on samples. One of the possible candidate for the cause of death may be the heat of friction. In the present temperature experiment, at 30° the surviving fraction was found to be lower than at 4° and −20°. This suggests that at higher temperature the heat of friction may also contribute to some extent to the killing action of tabletting. By and large, a major cause of death by tabletting may be assigned to the shearing force. This was clearly demonstrated in the present experiment using microbial cells of different cell sizes. The larger the cell size was, the more sensitive to killing were the microbial cells. It should, however, be noted that bacterial spores were highly resistant to tabletting as they were so against various chemical and physical agents.

Effect of moisture content of samples seemed to be somewhat confusing. In the case of tabletting of alkaline protease, the higher the moisture content was, the more stable was the enzyme.[2] In the case of tabletting *E. coli* cells, reverse was true. In the explanation of this phenomenon, there may be one possibility that a high moisture content causes the swelling of cells and results in an increase in sensitivity to tabletting. Another adverse possibility is that a high moisture content causes an increase in the elasticity of cells and results in an increased resistance to shearing force. The fact that the decrease in surviving fraction in a higher humidity range leveled off or even restored (see Fig. 4) suggests that under humidities of 50—80% the effect of moisture content on the surviving fraction may depend largely on the former possibility and under humidities more than 80% it may depend, at least

partially, on the latter possibility.

In the raw powders employed for conventional tabletting, the state of subsistence of contaminating microbes may be represented simply by the model of ESPA rather than by that of ESA. And in conventional tabletting, the pressure applied is empirically controled at 300—500 kg/cm^2. Therefore, the reduction of bacterial contaminants may be in the order of around one-half the starting cell number. It may also be probable that a temperature rise expected during the operation of a tabletting machine may increase the rate of reduction of contaminants.

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