THE DISTRIBUTION AND SOME PROPERTIES OF ACCUMULATED SILICATE IN CELL-FREE BACTERIAL EXTRACTS

W. HEINEN,

Botanisch Laboratorium, Universiteit, Nijmegen

SUMMARY

On incubation with silicate and a suitable substrate, cellfree extracts of *Proteus mirabilis* incorporate the anion Si, which is successively destributed and linked to various compounds. The distribution pattern changes with increasing incubation time: On fractionation with alcohol-ether (AES), most of the Si appears first in the alcohol-ether-insoluble (AEI) fraction reaching a maximum after 2 hours, whereafter a steady decline of the Si-concentration is observed. This fraction was found to contain water-extractable Si-compounds. The amount of Si that can be extracted from the precipitated material increases with time and temperature; maximum yield is obtained extracting the residue at 50 °C for 3 hours. The amount of water-extractable Si-compounds present in the AEI-fraction also varies with increasing incubation time: The high concentration found after 30 min rapidly declines to a minimum after 4 hours, followed by a slow increase during further incubation.

Both the AEI- and AES-fraction contain acid-labile Si-compounds, liberating free silicic acid on acid hydrolysis. The percentage of these acid-labile compounds in both fractions was also found to vary with the time of incubation.

1. INTRODUCTION

Silicon has been denoted as a mere trace element for years, though a variety of biological objects contain relatively high amounts of silicate and other Si-compounds. In most cases, as in plants (YOSHIDA *c.s.* 1962; OKUDA and TAKAHASHI 1964) and diatoms (LEWIN *c.s.* 1966), it is mainly present in its polymeric form. Deposition however occurs at well-defined areas of the objects, leading to the assumption that the final polymerization is preceded by a number of more or less specific processes, including the uptake, concentration and transport of silicate. Similar reactions have been found to occur in animal tissue (SAUER *c.s.* 1959, BALLY 1960, POLICARD *c.s.* 1960), and Si-accumulation has also been observed in microorganisms (HOLZAPFEL & ENGEL 1954).

Various soil bacteria, for which inorganic as well as organic Si-compounds of the soil (DEUEL 1957, 1959) form a major part of their natural environment, have been shown to utilize Si-compounds after depolymerization of polysilicates (WEBLEY *c.s.* 1960). Previous work in this laboratory has shown that bacteria are capable to accumulate silicate; the final fixation depends on the inorganic and organic compounds supplied along with the silicate during aerobic incubation (HEINEN 1960, 1963 a, b). Further examination of these problems revealed that metabolic intermediates such as NADH and ATP are involved in the process of silicon accumulation by cell-free extracts (HEINEN 1965).

This paper deals with the distribution and some properties of Si-containing compounds formed by cell-free extracts of *Proteus mirabilis*.

2. MATERIALS AND METHODS

Cell-free extracts of *Proteus mirabilis* were used throughout these experiments. They are prepared by sonic treatment, according to the scheme described previously (HEINEN 1965a). For most of the experiments, cells grown in a MERCK Standard I medium were used for extraction, and only for a few experiments the harvested cells were incubated in a silicate-medium (A), or a silicate-medium with glucose (B) before extraction, as in previous experiments (HEINEN 1965a).

For aerated incubation (up to 48 hours) the same medium with 40 μg Si/ml as before was used (HEINEN 1965b).

Fractionation of the cell-free extracts after incubation (for time intervals see text) was done by following the method applied previously (HEINEN 1965a): An ethanol-ether (1:1) mixture was added to the extract (2 v/v); the precipitate formed was removed by centrifugation (15 min, 15000 x g), and taken up in bidestilled water. This suspension is referred to as the "main fraction" or alcohol-ether-insoluble (AEI) fraction. The supernatant of this fraction was collected and evaporated under vacuum at 35–37 °C to a small volume. A new precipitate appeared during this process, which was separated by centrifugation, yielding a yellow-orange insoluble material, which was suspended in water ("second fraction"). The supernatant, referred to as the alcohol-ether-soluble (AES) fraction was recovered from the last spin and filled up to a distinct volume with destilled water. Since the second fraction was found to have a very low Si-content, this precipitate was mostly combined with the AEI-fraction and treated together. Further treatments of these fractions (hydrolysis, water extraction etc.) and the methods applied for determination have either been described before (HEINEN 1965a, b), or are outlined briefly in the text.

Silicon was either determined as "total Si" using the fusion method (HEINEN 1960), or as "molybdate-active Si" (BAUMANN 1960) using the direct method (HEINEN 1965a). In this latter case only the Si present as free silicic acid with all ligands available reacts, while polymer or bound Si (Si-O-Si, Si-Si-, Si-C-, Si-O-C-compounds) does not react.

3. RESULTS

Since foregoing experiments had shown that the distribution of the silicate taken up by intact cells of *Pr. mirabilis* depends on time, and that the Si-concentrations of the fractions shift with the time of incubation (HEINEN 1965b), we decided to check whether similar processes would occur with cell-free extracts. According to previous results, Si-incorporation by cell-free extracts depends on a suitable substrate (HEINEN 1965a, b) and occurs with Si-adapted and non-adapted cells as well (HEINEN 1965b, 1967).

Table 1. Concentrations of total (fused) and free (molybdate-active) silicate in not-Si-adapted intact cells of Pr. mirabilis. The bacteria were incubated for 48 hours in (A) Si-medium, (B) Si-medium with glucose and (C) in a medium containing phosphate, silicate and glucose.

	incubation medium		
	A	B	C
µg total Si (fused)	28.7	99.5	59.3
µg molybdate-active Si	8.1	11.0	10.5
µg "bound Si"	20.6	88.5	48.8
% "mobile Si"	28.2	11.5	17.7

3.1. Si-incorporation in non-adapted cells

In order to prove, whether non-adapted cells would be suitable for the following experiments, the total Si and "molybdate-active" (BAUMANN 1960) and mobile Si (HEINEN 1963b) was determined in cells from the standard growth medium (not adapted to silicate) after a 48 hours incubation in Si-medium (A), Si + glucose-medium (B), or a corresponding medium containing 100 µg P/ml, 40 µg Si/ml and 1.2 mg glucose/ml (C). The data given in *table 1* confirm earlier results that more silicate is incorporated in presence of glucose, and that phosphate acts as a competitor for silicate uptake. The amount of "bound Si" calculated from the difference between the total and molybdate-active Si, which must be linked in such a way that it does not react without previous fusion, gives the highest value in the B-medium with silicate and glucose, the lowest value in absence of glucose (A), but is not very much affected by the presence of phosphate (C), though the total uptake is smaller. The mobile "molybdate-active" Si on the other hand is nearly equal in both glucose-containing media (B and C), but higher (about 30%) in the A-medium. Since these data were in accordance with previous results obtained with Si-adapted cells, extracts from non-adapted bacteria were used for all further experiments.

3.2. Si-distribution in cell-free extracts

When the cell-free extract was fractionated with ethanol-ether after 4 hours of incubation, the total Si determination revealed a typical distribution of the Si,

Table 2. Typical distribution of total (fused) silicate in the alcohol-ether-insoluble (AEI) main fraction, the second precipitate appearing on evaporation of the supernatant, and the alcohol-ether-soluble (AES) fraction, obtained from a cell-free extract of *Pr. mirabilis*.

	µg Si per fraction	% Si per fraction
main fraction (AEI)	655.2	92.0
"second fraction"	7.6	0.9
fraction AES	48.9	6.9

with the highest amount present in the precipitated AEI-fraction (92%), much less in the AES-fraction (about 7%) and a trace in the second fraction *(table 2)*.

As in the foregoing experiments with intact cells, the main fraction (AEI) again was found to contain Si-compounds which can be extracted with water. When the release of molybdate-active Si from the AEI-fraction was followed at different temperatures (21°, 37° and 50°C) by stirring an aliquot of suspended material for a certain time, the subsequent determination of silicate in the supernatant after centrifugation gave the following results *(fig. 1)*: During the extraction at room temperature (21°C) the Si-concentration of the supernatant increased throughout an extraction period of 3 hours. Rising the temperature to 37°C was slightly effective, whereas extraction at 50°C readily enhanced the transfer of the water-soluble Si-containing substances from the precipitate. This extraction was nearly completed after 2 hours, yielding about 24% of the total Si of the fraction after 3 hours.

3.3. The release of bound silicate

By determining the Si-concentration of the AES-fraction, the precipitated AEI-fraction and the water-soluble silicate released from the latter after 30 min extraction at 50°C in the course of time during incubation, the data given in *fig. 2* were obtained. They reveal that most of the silicate incorporated is first loosely bound to the material that is precipitated with alcohol-ether since it is readily released from the AEI-fraction as soluble Si by water extraction. The amount of water-soluble Si-compounds decreases during the first four hours,

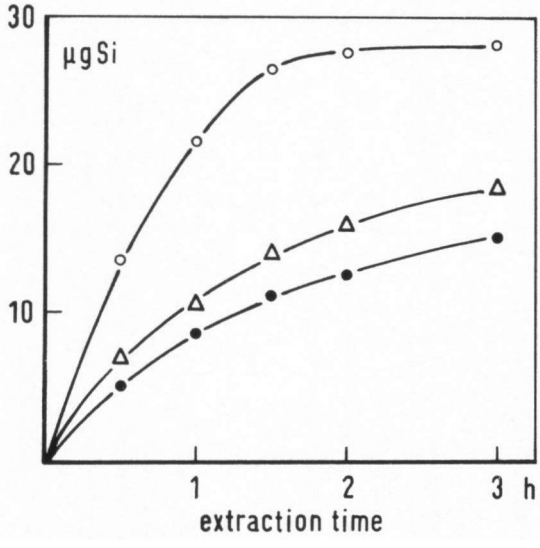

Fig. 1.
Solubilization of molybdate-active silica-compounds from the alcohol-ether-insoluble (AEI) fraction, depending on temperature and extraction time.
•—• = 21°C, △—△ = 37°C, o—o = 50°C.

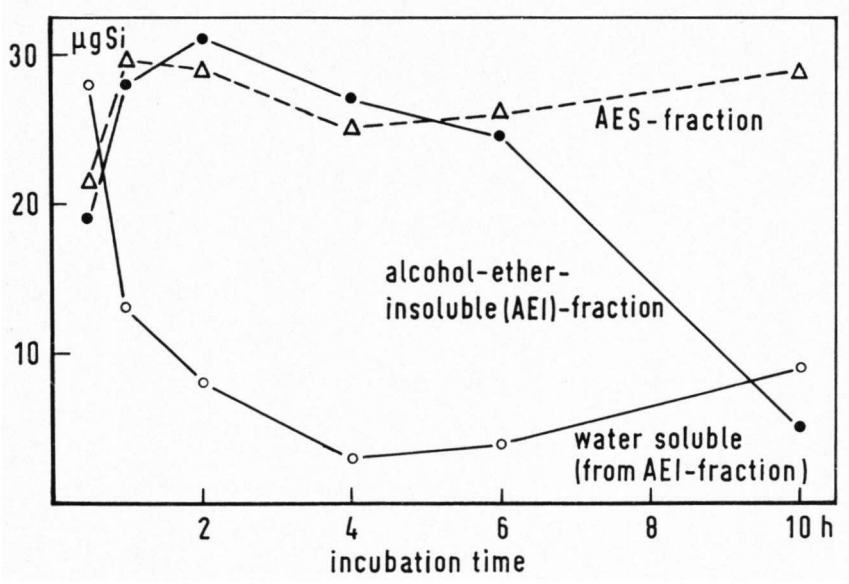

Fig. 2. Distribution of silica within three fractions of *Pr. mirabilis* cell-free extracts, depending on the incubation time. Samples were taken at successive intervals from the alcohol-ether-soluble (AES) fraction, the alcohol-ether-insoluble (AEI) fraction, and the water-soluble fraction derived from the latter by a 30 min extraction at 50 °C.

followed by a slow but steady increase thereafter. The AEI-fraction from which the soluble Si is derived, gains silicate during the first two hours, but starts loosing Si quite rapidly during the further incubation. The Si-concentration of the supernatant (AES-fraction) on the other hand increases during the first 60 min, then decreases but starts to increase again after 3 hours.

It is obvious from these data that the total amount of Si decreases during incubation, which could partly be due to polymerization. However, when the total Si-concentration of an aerated cell-free extract was followed without fractionation, it was found that the Si-content really decreases and is lost from the medium. It should be added here, that while fusing the samples the typical odor of kakodyl was noticed, which indicates the presence of AsH_3, SbH_3 and other hydrides. Since neither As nor Sb was present in our samples we concluded that the reaction must be due to the presence of silicon hydrides or oxyhydrides. The formation of volatile (oxy-)-hydrides would then account for the loss of Si during the incubation, in accordance with earlier observations that extracts of Si-incubated bacteria show infrared bands for Si-H- and alcyl-Si-compounds (Heinen 1965b). An analogy to the formation of silicon (oxy-)-hydrides is given by the processes that lead to volatilization of incorporated selenium by hydride formation, as reported by SHRIFT (1958, 1961), GANTHER & BAUMAN (1962a and b) and other investigators (BREMER & NATORI 1960, COWIE & COHEN 1957).

3.4. Solubility of bound silicate

The water-solubility of a part of the Si-compounds present in the AEI-fraction pointed to low-molecular substances. So did the observation that about 40% of this fraction's Si disappears on dialysis of the alcohol-ether-insoluble precipitate against bidestilled water for 18 hours. Therefore it was not surprising, and in accordance with the results obtained with intact cells, that free silicic acid was released from the AEI-fraction upon acid hydrolysis. As shown in *fig. 3*, alterations occur with increasing incubation time: After 2 hours the substances are rapidly hydrolysed, and the amount of silicic acid released does not show any substantial change after 5 min hydrolysis. If samples of the AEI-fraction after 4 hours of incubation are treated the same way, release of silicic acid increases with prolonged hydrolysation time and yields more free silicate than after 2 hours. On the contrary, the total amount of free silicic acid after 8 hours' incubation is very low, and slowly released during the entire period of hydrolysation.

A decrease of hydrolysable substances was also found in the AES-fraction. When the amount of silicic acid released after 30 min of hydrolysis was determined at various intervals during the incubation, the data given in *fig. 4* were obtained. This shows that the compounds are formed within the first hour of incubation and that the amount then gradually decreases, remaining on the low level reached, for at least two hours.

4. DISCUSSION

The experiments reported show that cell-free extracts of non-Si-adapted *Pr. mirabilis* cells are capable to accumulate silicate, thereby changing the properties of the incorporated material. According to the literature on bacterial cell-free extracts obtained by sonication (FUJITA *c.s.* 1965; ARIMA & OKA 1965; REPASKE & LIZOTTE 1965; NAIK & NICOLAS 1966; ASANO & BRODIE

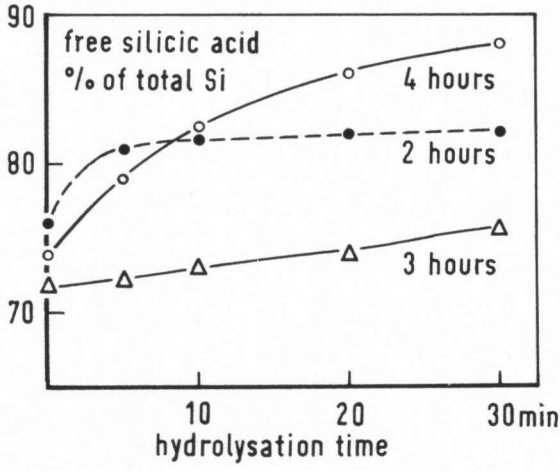

Fig. 3.
Liberation of free silicic acid (molybdate-active silica) by acid hydrolysis of the AEI-fraction, and alterations of the amount released after different incubation times (2, 4 and 8 hours).

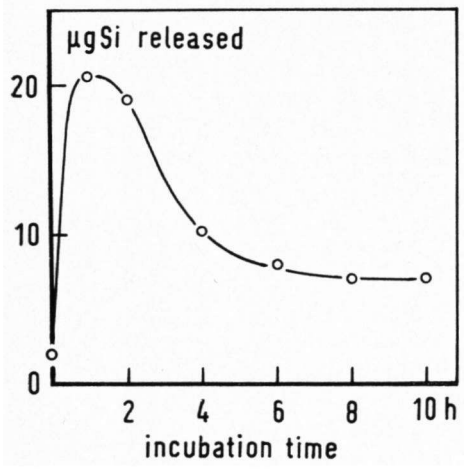

Fig. 4.
Release of molybdate-active Si from the AES-fraction after 30 min of acid hydrolysis, varying with increased incubation time.

1965, 1966) these extracts contain fragmented cytoplasmic membrane systems, which *in vivo* are responsible for substrate-dependent ion uptake, as could be demonstrated cytochemically for *Proteus* by VAN ITERSON & LEENE (1964) and LEENE & VAN ITERSON (1965). Since these fragments are precipitated upon treatment with organic solvents or salt (ASANO & BRODIE 1965a, b), we may assume that the alcohol-ether-insoluble fraction obtained from our cell-free extracts will at least to a certain extent contain the particulate membrane fragments. Taking these aspects into consideration, the time- and temperature-dependent release of soluble Si-compounds from the AEI-fraction on prolonged water extraction obviously represents the discharge of the previously accumulated silicate from fragments with a limited binding capacity, similar to the leakage of ions from "loaded mitochondria" (CARAFOLI 1965; ROSSI *c.s.* 1966). The alterations of Si-concentrations observed in the three fractions analysed in subsequent intervals of incubation *(fig. 2)* confirm this view: Most of the silicate is primarily incorporated into the membrane-containing AEI-fraction, followed by transfer reactions that lead to secondary compounds, which are found in the two other fractions. A variety of Si-containing products will be formed during these secondary reactions, as seen from the time-dependent formation of acid-labile compounds in both the AEI- and AES-fraction (*figs. 3* and *4*). These products may partly be present as C-O-Si ester- or ether-type components, or as weak Si-Si bonds like silico-oxalic acid and related compounds (GMELIN 1959). Formation of this latter type of Si-compounds implies a reduction of the incorporated silicate. Both the decrease of the total Si-concentration reported here, and the formation of volatile reducing substances previously observed (HEINEN 1965c) show that bacterial extracts can handle the accumulated silicate in different ways, including the reductive formation of Si-Si- and Si-C-compounds. The distribution of Si-compounds therefore depends on the type of compound formed and will thus be altered with the time

of incubation. After incubations not exceeding 2 hours, the Si is quite evenly distributed and the compounds formed will be relatively uniform, so that short incubations will be well suited for the determination of the primary reactions occuring with silicate in cell-free extracts. The high concentration of water-extractable Si-compounds after 30 min of incubation and their decline during the following hour, which is accompanied by an increase of the total Si-content of the AEI-fraction, on the other hand, shows that within this period a stronger binding of the accumulated silicate occurs. The amount of compounds with various properties will increase rapidly once the accumulation of silicate has started.

REFERENCES

ARIMA, K. & T. OKA (1965): A new combination of cytochromes occurring in Pseudomonas riboflavina. *J. Biochem.* **58**: 320.

ASANO, A. & A. F. BRODIE (1965): The properties of the non-phosphorylative electron transport bypass enzymes of Mycobacterium phlei. *Biochem. Biophys. Res. Comm.* **19**: 121.

— & — (1965): Oxidative phosphorylation in fractionated bacterial systems. XVIII. Phosphorylation coupled to different segments of the respiratory chains of Mycobacterium phlei. *J. Biol. Chem.* **240**: 4002.

BAILY, P. (1960): The biochemistry of lungs in relation to silicosis. 1. The amino acid content of normal guinea pig lung tissue. *Arch. Biochem. Biophys.* **87**: 144.

BAUMANN, H. (1960): Bestimmung der Kieselsäure in biologischem Material. *Z. physiol. Chem.* **319**: 38.

BREMER, J. & Y. NATORI (1960): Behavior of some selenium compounds in transmethylation. *Biochim. Biophys. Acta* **44**: 367.

CARAFOLI, E. (1965): Active accumulation of Sr^{++} by rat-liver mitochondria. II. Competition between Ca^{++} and Sr^{++}. *Biochim. Biophys. Acta* **97**: 99.

COWIE, D. B. & G. N. COHEN (1957): Biosynthesis by E. coli of active altered proteins containing selenium instead of sulfur. *Biochim. Biophys. Acta* **26**: 252.

DEUEL, H. (1957): Organische Derivate von Tonmineralien. *Agrochimica* **1**: 248.

— (1959) Reaktionen von Silikaten mit organischen Verbindungen. *Makromolek. Chem.* **34**: 206.

FUJITA, M., S. ISHIKAWA & N. SHIMAZONO (1966): Respiratory chain and phosphorylation site of the sonicated membrane fragments of Micrococcus lysodeikticus. *J. Biochem.* **59**: 104.

GANTHER, H. E. & C. A. BAUMANN (1962): Se metabolism. I. Effects of diet, arsenic and cadmium. *J. Nutr.* **77**: 210.

— & — (1962): Se metabolism. II. Modifying effects of sulfate. *J. Nutr.* **77**: 408.

GMELINS *Handbuch der Anorganischen Chemie.* 8. Auflage **15**, Teil B (1959), p. 582. Verlag Chemie, Weinheim/Bergstraße.

HEINEN, W. (1960): Silicium-Stoffwechsel bei Mikroorganismen. I. Aufnahme von Silicium durch Bakterien. *Arch. Mikrobiol.* **37**: 199.

— (1963a): Silicium-Stoffwechsel bei Mikroorganismen. IV. Die Wirkung organischer Verbindungen, insbesondere Glucose, auf den Silicium-Stoffwechsel bei Bakterien. *Arch. Mikrobiol.* **45**: 162.

— (1963b): Silicium-Stoffwechsel bei Mikroorganismen. V. Untersuchungen zur Mobilität der inkorporierten Kieselsäure. *Arch. Mikrobiol.* **45**: 172.

— (1965a): Silicium-Stoffwechsel bei Mikroorganismen. VII. Verteilung der Kieselsäure in Zell-Fraktionen von Pr. mirabilis und der Nachweis von Kohlenhydrat-Kieselsäure-Estern. *Arch. Mikrobiol.* **52**: 69.

— (1965b): Time-Dependent distribution of silicon in intact cells and cell-free extracts of

Proteus mirabilis as a model of bacterial silicon transport. *Arch. Biochem. Biophys.* **110**: 137.

— (1965c): A "Three Gases Technique" as a method for the determination of volatile hydrides and related compounds in a modified Warburg assay. *Arch. Biochem. Biophys.* **111**: 236.

— (1967): Ion accumulation in bacterial systems. II. Properties of silicate-metabolizing cell-free extracts and particulate fractions from Proteus mirabilis. *Arch. Biochem. Biophys.* **120**: 93.

HOLZAPFEL, L. & W. ENGEL (1954): Der Einfluss organischer Kieselsäure-Verbindungen auf das Wachstum von Aspergillus niger und Triticum. *Z. Naturforsch.* **9b**: 602.

ITERSON, W. VAN & W. LEENE (1964): A cytochemical localization of reductive sites in a gram-negative bacterium. *J. Cell Biol.* **20**: 377.

LEENE, W. & W. VAN ITERSON (1965): Tetranitro-blue tetrazolium reduction in Proteus vulgaris. *J. Cell Biol.* **27**: 241.

LEWIN, J. C., R. E. REIMANN, W. F. BUSSY & B. E. VOLCANI (1966): Silica shell formation in synchronously dividing diatoms. *Cell Synchrony* **9**: 169.

NAIK, M. S. & D. J. D. NICHOLAS (1966): Phosphorylation associated with nitrate and nitrite reduction in Micrococcus denitrificans and Pseudomonas denitrificans. - *Biochim. Biophys. Acta* **113**: 940.

OKUDA, A. & E. TAKAHASHI (1964): The role of silicon. - *Proc. Symp. at the Intern. Rice Res. Inst.* 123.

POLICARD, A., A. COLLET, H. DANIEL. MOUSSARD & S. PREGERMAIN (1960): Etude au microscope élèctronique des dépots de silice expérimentalement produits dans le rein. *Bull. de microsc. appl.* **10**: 72.

REPASKE, R. & C. L. LIZOTTE (1965): The electron transport system of Hydrogenomonas eutropha. II. Reduced nicotinamide adenine dinucleotide-menadione reductase. *J. Biol. Chem.* **240**: 4774.

ROSSI, C. S., J. BIELAWSKI, E. CARAFOLI & A. L. LEHNINGER (1966): The relationship of the Ca^{++} efflux rate to the "superstoichiometry" of respiration-linked Ca^{++} accumulation by mitochondria. *Biochem. Biophys. Res. Comm.* **22**: 206.

SAUER, F., D. H. LAUGHLAND, & W. M. DAVIDSON (1959): Silica metabolism in guinea pigs. *Can. J. Biochem. Physiol.* **37**: 183.

SHRIFT, A. (1958): Biological activities of Se-compounds. *Bot. Rev.* **24**: 550.

— (1961): Biochemical interrelations between selenium and sulfur in plants and microorganisms. *Fed. Proc.* **20**: 695.

WEBLEY, D. M., R. B. DUFF & E. W. A. MITCHELL (1960): A plate method for studying the breakdown of synthetic and natural silicates by soil bacteria. *Nature* (Lond.) **188**: 766.

YOSHIDA, S., Y. OHNISHI & K. KITAGISHI, (1962): Chemical forms, mobility and deposition of silicon in rice plant. *Soil Sci. Plant Nutr.* **8**: no. 3, 15.