Title: THE ACTION OF QUATERNARY AMMONIUM COMPOUNDS ON MICROBES by

M. N. Meysel' and V. P. Umanskaya (USSR)

Source: Microbiologiya, Vol XVIII, No 1, Jan-Feb 1949, pp 11-19, (Russian bimonthly periodical)

CONFIDENTIAL

THE ACTION OF QUATERNARY AMMONIUM COMPOUNDS ON MICROBES

M. N. Meysel', V. P. Umanskaya
(Inst of Microbiol, Acad of Sci USSR, and All-Union Sci-Res Chemico-Pharmaceutical Inst imeni S. Ordzhonikidze, Moscow)

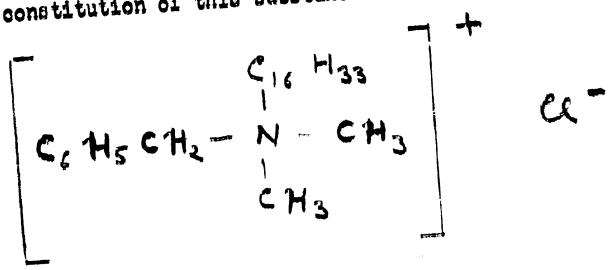
The structural organization of the cell, as we know, provides for the coordinated and harmonic flow of enzymatic processes which are the basis of cellular activity. Variations in cellular functions, and their inhibition and repression usually develop under the influence of effects greatly differing in intensity from those which are sufficient for the inhibition and repression of the activity of corresponding enzymes secreted by the cell. This is explained in the first place by the fact that the cell reacts to external stimuli not as a combination of enzymes but as a complicated structural system, the different structural elements of which exhibit either a greater sensitivity or a greater degree of resistance than the enzymes which make up their composition. Therefore the study of the action of any external factor can not be restricted to an analysis of the effect of this factor on an isolated enzymatic system, but must be extended to a functional-morphological study of the reaction of the cellular protoplast as a whole.

These studies can be conducted more efficiently on more differentiated cells exhibiting pronounced cellular structure. These objects of study can serve as models; by studying their reactions we are able in a series of cases to discover the character of the processes which occur in less dif-

ferentiated organisms. An obligatory condition for this type of study must be an identical response to the reactions by both the model organisms and those to which the results of the study are extended. In a comparative study of the effect of a series of bacteriostatic and bactericidal substances the essential similarity of their effects on bacteria, fungi and algae was clarified. This is the case, for instance, with sulfamide preparations (Brian (11), Maysel' and Zavarzin (5)). Thus the mechanism of the effect of sulfamides is more efficiently studied on larger and more differentiated organisms. The situation is the same with regard to bactericidal agents such as gramicidin. According to our data the effect of gramicidin on yeasts within general limits is similar to its effect on bacteria. We therefore consider it essential for the comprehension of the nature of bacteriostatic, bactericidal, and bacteriolytic effects to study these phenomena on larger and more differentiated model organisms, with due regard, naturally, for the specificity and the special features which are determined by the degree of organization and differentiation of the cell organism.

This investigation is devoted to the bactericidal and bacteriolytic effect of an unusual class of disinfectant soap-like substances with low surface tension in solution, the so-called quaternary ammonium compounds or detergents. These substances are used to some extent at present as good disinfectants which are harmless to man and animals. There is data on their being used successfully for disinfecting dishes and equipment in the food industry, and for the treatment of skin and instruments in surgical practice (Huyck (13), Valko (18)). Among the Russian authors Braz worked on the synthesis of bactericidal quaternary ammonium compounds. There was at our disposal a very active substance of this class, a cation type substance - cetyl zepherol (more accurately cetyl-diamethyl-benzyl-ammonium-chloride), which was the first of them studied in detail with regard to their bacteriostatic and bacteriocidal effect (Umanskaya (9)).

The chemical constitution of this substance is as follows:



It is known that quaternary ammonium compounds greatly inhibit the metabolism of bacteria (Baker, Harrison, and Miller (10)) and yeasts (Sevag and Ross (15)), and that their reduction of the surface tension can not in itself explain their bactericidal action. An ~~the~~ essential significance must be attached to the molecular weight of the compound, and in particular, to the length of the chain of the alkyl group of homologous substances; high bactericidal activity is possessed by those compounds whose alkyl groups contain 9 to 16 atoms of carbon (Shelton, van Campen and Nisonger (16)). Of special interest are their effects on proteins and lipoids (phospholipoids); quaternary ammonium compounds have the ability to combine with these substances and to denature them. Proteins and lipoids, existing in a medium significantly reduce the effectiveness of these antiseptics by adsorbing them. However, the effect of the detergents can not be explained by direct denaturation of the proteins of the cellular protoplasm, for bacteria are destroyed in concentrations of these substances many times lower than those which denature protein in vitro. Consequently there must be some more sensitive components or structures within the living bacteria cell which are affected by the quaternary ammonium compounds. On theoretical considerations there is an attempt to explain in the following manner the cytolytic effect observed during the use of these compounds. The surface-active ions of the antiseptic become attached to the oppositely charged surface of the

cellular membrane and accumulate on it, causing irreversible changes in the membrane which effect its permeability, so that what results is elimination of nitrogen and phosphorus compounds from the cell, in other words, a process suggesting the hemolysis of erythrocytes. This results in death of the cell without actual disruption of its structural organization, after which autolysis starts, continuing with various speeds depending on the species of microbe, (Hotchkiss (12)). Direct evidence of this mechanism does not exist, however. There is an attempt to treat the different parts of the cellular reaction to the quaternary ammonium compounds as the result of the inactivation of specific enzymes, especially oxidation enzymes. However, the whole question is still vague as to what degree the inactivation represents a primary effect and/is responsible for the resulting phenomena.

Thus the action of this unusual and interesting group of antiseptic agents, which leads to the death and lysis of microbes, can not be considered explained. Meanwhile the nature of the reaction of quaternary ammonium compounds with components of cellular protoplast is of fundamental value both for the explanation of the point of application of this class of substances and for increasing the knowledge of the interrelationship of cellular structures and functions.

For solution of these problems we resorted to a model test employing a yeast-like organism Endomyses Magnusii, to which we had previously devoted considerable study. In this article the data is primarily on cytological investigations.

The methods of cytological research were described in detail in our previous reports, and therefore we limit ourselves to a brief summary of the methods used. Together with the observation of the changes in the living cell under the effect of cetyl-zephèrol we made use of the staining of the living cell with Janus green and Neutral Red to show chondriosomes and volutin (Meysel' (4)), treatment of the living cell with fluorescent

substances for subsequent fluorescence microscopic study of the early stages of the changes in the protoplast (Meysel', and Zavarzina (6)), and fixation and staining by Heydenhain's method according to a modification made by one of the authors of the present article (Meysel' (4)) for producing permanent cytological preparations. The absorption of oxygen and the giving off of carbon dioxide by the cultures were determined by the usual methods using Warburg's apparatus and Odintsova's microfermentation equipment.

General Characteristics of Cellular Changes

We have already noted that yeast organisms showed approximately the same sensitivity to quaternary ammonium compounds as bacteria did. In our tests the bacteriostatic effect was already observed in dilutions of 1:120,000 for Endomces Magnusii and 1:250,000 for Torulopsis latvica. In cultivation of yeast organisms on an artificial sugar-inorganic Rider medium the resistance of the culture with regard to cetyl-zephèrol increased in those cases when the medium was rich in vitamins.

Cetyl-zephérol in concentrations of 1:10,000---1:20,000 quickly destroyed the yeast organisms even in the presence of 10% of blood serum. The effective functioning of this disinfecting agent produced 100% destruction of microbes and the absence of any cells resistant to it; all of the culture was completely destroyed.

We studied the effect of various concentrations of cetyl-zephèrol on individual cells of Endomyses Magnusii directly under the microscope. For these tests two-day cultures grown in wort or wort-agar mediums were taken from the mediums washed with sterile tap water and a suspension of cells from this culture (Figure 1,a) was combined directly on the object glass with a solution of cetyl-zephérol. The range of the concentrations of this substance was fixed from 1:10,000, to 1:300,000. The first of these concentrations caused abrupt destruction of the cells in the course of one to

two minutes. The details of the process were difficult to discern at this speed. Therefore we also used concentrations of 1:40,000,---1:100,000, and in these cases the process occurred more slowly and we were able to follow step by step the basic cellular changes. The first thing which was noticed with an ordinary microscope was the sudden intensification of vacuolization of the cell under the influence of cetyl-zepherol. The protoplasm became full of large alveoles so that it seemed to have frothed, the wall of the cell contracted, gelatinized, and began to refract light more strongly. Later ensues a stage of contraction of the protoplast and plasma so that the latter is filled with small aveoles. The contraction of the protoplast increased so that it seemed that the droplets of lipoids and nuclear structures disappeared and were replaced by small grains. The contraction of the protoplast was so great that it separated from the cellular membrane, giving a picture suggesting acute plasmolysis. (Figure 1 b). However, this is already an irreversible state. Staining of the living cell with Janus Green and Neutral Red makes apparent further details of the cellular changes. First the chondriosome structure is affected. In the early stages of the cellular reaction vacuoles and lipoids (fat phanerosis) develop in the chondriosomes. Then these organoids expand, assume bulb or spherical shapes and the greater part of them loses the ability to be stained by Janus Green and Netural Red, which are then retained only by the peripheral structures. Later the chondriosomes break up, disperse in small granules, and dissolve in the protoplasm. The latter begins to increase its adsorption of the dye and becomes diffusely colored in blue by the Janus Green and in red by the Neutral Red. At the time that the protoplasm and its organoids were undergoing such considerable changes, the contents of the vacuolar structure for a long time remained unchanged. The volutin which forms a coacervate with the Neutral Red and usually quickly dissolves during various affections of

the cell in this case remains for sometime in the form of red granules and only later decolorizes.

Even finer changes were observed by fluorescence-microscopic examination under blue rays. For this study the cells washed from the suspension medium were treated while living with fluorescent substances: acridine orange, [illegible] berberine sulfate, and coriphosphine. We observed briefly the results obtained with each of these substances.

The Acridine orange in very weak solutions (1:100,000--1:200,000) created in the Endomyces Magnusii a deep-green fluorescence of the protoplasm and a light-green fluorescence of the nucleus. The vacuoles which accumulated a great deal of the dye were colored a fiery red. The early changes, not observed under the microscope in white light, were expressed in a sharply increased brightness of the cytoplasm and nucleus. The protoplasm began to fluoresce a bright green and the nucleus a yellow green. This is caused by an increased adsorptive power of the protoplast. The accumulation of Acridine orange in the cell in time increased and the fluorescence of the protoplasm acquired a stronger yellow color, became orange, and finally red. The orange and red fluorescence of the protoplasm coincides with its irreversible damage to it through gelatinization and coagulation. These changes in the color of the fluorescence which are connected with denaturation of the proteins of the cell were first observed in plant cells by Struger (17). He noted that any disturbance of the cell which led to its death was accompanied by the change of the fluorescent green color of adsorbed acridine to red. In checking these observations ourselves, we found that this change of the fluorescent color occurs only for a specific type of degeneration of the protoplast and does not invariably accompany the death of the cell. (Meysel' and Zavarzina (6)). The type of denaturation of the protein of the protoplasm under the effect of the cetyl-zephirolis expressly that characteristic for unusual necrotic processes leading to particularly strong binding of Acridine Orange by the protoplast.

The fluorescent substance berberine sulfate penetrating into the living cell accumulates in the first place on the chondriosome, leading to it an intense gold-yellow fluorescence. Early changes under the influence of the cetyl-zephirol were shown in the increased fluorescence of the protoplasm and nucleus. Then agglutination and granular decomposition of the chondriosome followed and as this progressed, fluorescence and alveolar frothing of the protoplasm increased. In the final stages the differential brightness of the separate cellular structures disappeared and the general fluorescence of the protoplast gradually faded.

The fluorescent substance coriphosphine gives to the protoplasm a bright green fluorescence, to the fatty inclusions an azure one, to the chondriosomes a yellow, and the precipitates in the vacuoles a red fluorescence. Due to the selective brightness of the fat droplets we are able to observe clearly the fat phanerosis in the protoplasm under the influence of cetyl-zephirol. The chondriosomes expand and become drop-shaped. The fluorescence of the protoplasm becomes increasingly yellow.

Summing up the changes in the living cells under the effect of cetyl-zephirol which were observed by ordinary and fluorescence microscopy, we can align them somewhat schematically in the following manner:

1. First the adsorptive capacity of the protoplast is increased. This in particular is clearly noticeable due to the accumulation of fluorescent substances in the protoplasm and nuclei and indicates the physico-chemical changes of the protein basis of the protoplasm.

2. Then changes in the structure and nature of the chondriosomes are observed. After the latter are vacuolized, part of the lipoproteins decompose with liberation of the lipoids (fat phanerosis). Judging by the fluctuation in colorability of the chondriosomes, the nucleoprotein framework of these organoids is essentially changed.

3. Changes in the protoplasm progress; it is strongly vacuolized, and at the same time it contracted, as if congealing, and then gelatinized. The lipoproteins of the protoplasm partially disintegrated with the appearance of free fat droplets.

4. The aggregate state of the nucleoproteins of the nucleus changes; they gelatinize and strongly adsorb the dye.

5. The protoplast progressively undergoes necrotic changes; small alveolar vacuolization and small granular coagulation. It shrinks, contracts, and recedes from the cell wall. The chondriosomes break up and dissolve. The nucleus acquires a coarse structure. These changes are irreversible and lead to the death of the cell. At this stage and possibly somewhat earlier autolysis of the cell begins, progresses swiftly and leads to nearly complete dissolving of the protoplast (Figure 1 c). Consequently, the death and lysis of the cell as a result of the activity of quaternary ammonium compounds (of the cation type) is produced by the disruption of the cell's structural organization, and primarily of the lipoprotein and nucleoprotein structures. The disruption of the structure increases the hydrolyzing activity of the enzymes (Oparin - 7, 8; Kursanov - 2), which leads to autolysis of the protoplast.

The specimens fixed at various stages of the cellular changes make it possible after treatment by cytological methods to define more accurately the character of cellular degeneration. In the cells of Endomyces Magnusii from the control cultures, the chondriosome parts show up clearly as consisting of delicate fine threads equally distributed in the protoplasm; in the round oidium cells, the chondriosomes are rod-shaped. The nucleus in normal cells is large and round with clearly differentiated karyosomes (Figure 2 a). The initial stages of the changes (in the case of a 1:150,000 dilution of cetyl-zephirol, acting over a period of 1 hour) are characterized by alveolar

structures of the protoplasm, and the swelling and joining together of the chondriosomes. Somewhat higher concentrations of cetyl-zephirol (1:100,000) cause further processes of degeneration in the protoplast and its structure. Vacuolization is relatively more strongly expressed. The expanding chondriosomes separate into more darkly and lightly colored parts, and it is possible that this indicates the separation of the lipoids from the nucleoproteins. The contours of the chondriosomes become indistinct, which indicates the partial dissolving of these structures. The nucleus also undergoes acute degenerative changes of the pyknosis or Karyorrhexis variety (Figure 2b). Very high concentrations of the antiseptic (1:10,000) causes, as was already noted, a rapidly occuring contraction of the cell and separation of the protoplast from the cellular membrane. The protoplasm is poorly differentiated and is indistinct and clotted. The chondriosomes in the cells are either not apparent at all, are found in a state of disintegration into small, powdery grains, or are distinguished by ~~indistinct~~ blurred contours which are characteristic for dissolving structures. The nucleus is shriveled, small and in a state of breaking up (Figure 2 c).

Functional Changes of the Cell

As was to be expected, ~~the~~ cetyl-zephirol powerfully disrupts the essential structures of the cell and particularly sharply affects the oxidation enzyme system. The indirect inhibition of the respiratory function of bacteria by the effect of detergents had been noted by Baker and his coworkers (10). Our model, Endomyces Magnusii, also reacted to cetyl-zephirol by a rapid reduction in the amount of oxygen absorption. With concentrations of 1:10,000 this process is suppressed completely in the course of 2 minutes. When concentrations of 1:40,000 are used, respiration of the cell stops after several minutes. Even with concentrations of 1:100,000 the absorption of oxygen by a suspension of the culture is reduced by around 50 percent (Table 1).

We investigated some systems of enzymatic oxidation activity both in normal cultures of Endomyces Magnusii and in those cultures which had been treated with various concentrations of cetyl-zephirol. A detailed report of the data produced will form the topic for a special report. Here we are concerned only with the dehydrogenase activity and the condition of the cytochromic system.

The activity of the glucose dehydrogenases was determined using Thunberg's method and basing the determination on the time required to decolorize methylene blue. It was noted that although a suspension of Endomyces Magnusii cells decolorized the dye after 12 to 15 minutes, the same suspension treated with cetyl-zephirol (1:10,000) did not decolorize methylene blue after 48 hours. Treating the cells with a solution of this detergent in a concentration of 1:80,000 sharply retards the decolorization so that it takes place only after 30 to 35 minutes. Thus the cetyl-zephirol retards the activity of the dehydragenase and in higher concentrations completely suppresses it.

The cytochromic system of Endomyces Magnusii was studied by the Kriss method with the aid of a microspectroscope. A column of ~~determined~~ definite height was prepared from a washed and drained culture, and scanned in a microscope equipped with a spectroscopic eye_piece. Normal cultures of our Endomyces exhibited four absorption bands which we identified as cytochromic bands; a(610-598 mμ), b(556-560 mμ), c(552-548 mμ), and d(528-520mμ). Under the effect of cetyl-zephirol there was noted the disappearance of band d, the fusion of bands b and c, and the loss of distinct boundries in these bands, The most stable was shown to be the absorption band a.

Although the oxidation systems we investigated reacted strongly to the cetyl-zephirol, the zymase complex which regulates the alcohol fermentation is considerably more resistant. The comparative resistance of the fermentative functions of yeast organisms to cetyl-zephirol has already been noted by Loginova (3).

When the detergent is introduced directly into Warburg beakers before the determination of oxygen absorption by suspensions of Endomyces Magnusii, it was possible to note a more or less distinct effect only for concentrations of cetyl-zephirol of not less than 1:20,000. Greater dilutions did not produce any substantial depression (Table 2).

The effect of the cetyl-zephirol on the fermentation is somewhat increased, if the detergent is placed in contact with the suspension for 1 to 2 hours before determining the intensity of fermentation. However, in that case the effect produced is considerably less severe in comparison to the reaction of oxidation systems (Table 3).

Miscroscopic examination of the cells of Endomyces from this suspension which was conducted at the same time clearly indicated that they were damaged by the cetyl-zephirol. However, the process of fermentation is clearly not inhibited proportionally to the degree of damage to the cell. These observations once more stress the fact that the coordinated oxidation processes in cells depend on the condition of the cells' structural organization a great deal more than the processes connected with the anaerobic cleavage of carbohydrates.

Conclusions

1. Quaternary ammonium compounds of the cation type such as cetyl-zephirol have the same general effect on yeast and yeast-like organisms as on bacterial cells. Consequently, these yeast organisms can be used as convenient models for the study of the mechanism of the effect on microbic cells of substances of a similar type.

2. The destruction of a series of essential cellular structures, first of all of lipoproteins and nucleoproteins, forms the basis for the effect of cetyl-zephirol on the microbe cells.

3. Cytoplasm and cytoplasmic organoids, the chondriosomes, are quickly changed by cetyl-zephirol. These changes are expressed first in the growth of the adsorptive powers of the protoplast, and then in the fission of lipoproteins with liberation of free lipoids (fat phanerosis). After this lysis of the propoplast occurs, which is of secondary origin. It depends on increased activity of the proteolytic ferments of the cell, occurring after the primary damage to the cellular structure.

4. The nucleus also undergoes wide changes. It starts swelling, and then breaks up and partially dissolves.

5. One of the characteristic features of the effect of cetyl-zephirol on the cell is the sharply expressed contraction of the protoplast, and the separation of it from the cellular membrane, resembling plasmolysis. This phenomena is irrevisible.

6. The cetyl-zephirol strongly inhibits the respiratory function of the cell and in a considerably less degree the fermentative function.

Bibliography

1. Braz, G., "Synthesis of the Bactericidal Compounds of the Series of Quaternary Ammonium Compounds", Protokol. Uchen. soveta Vsecoyuznogo Nauchno-Issledovatel'skogo Khimiko-Farmatsevticheskogo Instituta, 4 August 1944.

2. Kursanov, A., "Reversibility of the Action of Enzymes in Living Plant Cells", Izdatel'stvo Akademii Nauk SSSR, 1940.

3. Loginova, L., "Investigation of New Types of Antiseptics for Alcohol Production", Otchet Vsesoyuznogo Instituta Spiritovoy Promyshlennosti, 1943.

4. Meysel', M., "Reorganization of the Protoplast of Yeast Organisms in the Fermentation Process", Mikrobiologiya, Vol VIII, pp 381, 1939.

5. Meysel', M., and Zavarzina, N., "Functional Morphological Analysis of the Effect of Sulfonamides on the Cell", Referaty Rabot Akademii Nauk SSSR za 1945, Otdeleniye Biologicheskikh Nauk, pp 139.

6. Meysel', M., and Zavarzina, N., "Fluorescent Microscopic Investigation on the Living Cells of Microorganisms", Mikrobiologiya, XVI, pp 394, 1947.

7. Oparin, A., "The Activity of Enzymes in the Living Cell", Uspekhi Khimii, Vol 3, pp 200, 1934.

8. Oparin, A., "The Direction of Enzymatic Activity in the Living Cell", Trudy Moskva Doma Uchenykh i Instituta Biokhimiya Akademii Nauk SSSR, No 4, pp 5, 1940.

9. Umanskaya, V., "Quaternary Ammonium Compounds as Bactericidal Substances", Protokol Uchenogo Soveta Vsesoyuznogo Nauchno-Issledovatel'skogo Khimiko-Farmatsevticheskogo Instituta, 4 August 1944.

10. Baker, Z., Harrison, R., and Miller B., "The Bactericidal Action of Synthetic Detergents", Journal of Experimental Medicine, Vol 74, pp 611, 1941; "Inhibition by Phospholipids of the Action of Synthetic Detergents on Bacteria", Journal Experimental Medicine, Vol 74, pp 621, 1941.

11. Brian, P., "Effect of p-Amino-Benzoic Acid on the Toxicity of p-Amino-Benzene-Sulfonamide to the Higher Plants and Fungi", Nature, Vol 153, pp 83, 1944.

12. Hotchkiss, R., "The Nature of the Bactericidal Action of Surface Active Agents", Annuals of the New York Academy of Sciences, Vol 46, article 6, pp 479, 1946.

13. Huyck, S., "The Effect of Cetylpyridinium Chloride on the Bacterial Growth in the Oral Cavity", Journal American Pharmaceutical Association Scientific Edition, Vol 34, pp 5, 1945.

14. Rahn, O. and van Eseltine W., "Quaternary Ammonium Compounds", Annual Review of Microbiology, Vol 1, pp 173, 1947.

15. Sevag, M., and Ross, O., "Studies on the Mechanism of the Inhibitory Action of Zephiran on Yeast Cells", Journal of Bacteriology, Vol 48, pp 677, 1944.

16. Shelton, R., van Campen, M., and Nisonger, L., "Correlation of Structure and Germicidal Activity of Certain Acyclic Quaternary Ammonium Salts", Boston Meeting of the American Chemical, 1939.

17. Strugger, S., "Fluorescent Microscopic Investigation of the Absorption and Storage of Acidine Orange by Living and Dead Plants Cells", Jenaische Z. Naturwiss, Vol 65, pp 97, 1940.

18. Valko, E., "Surface Active Agents in Biology and Medicine", Annuals of the New York Academy Sciences, Vol 46, article 6, pp 451, 1946.

[Figures referred to are available in the original document in CIA; tables follow:]

Table 1

Intensity of the Absorption of Oxygen by a Culture of Endomyces Magusii as Influenced by Various Concentrations of Cetyl-Zephirol.

Concentration of Cetyl-zephirol	Absorption of O_2 in cubic millimeters per hour by 1 milligram of dry material(Q_{O_2})	Concentration of Cetyl-zephirol	Absorption of O_2 in cubic millimeters per hour by 1 milligram of dry material(Q_{O_2})
Control	69	1:100,000	28
1:300,000	68	1: 40,000	0
1:150,000	58	1: 30,000	0

Table 2

Intensity of Fermentation in Endomyces Magnusii in the Presence of Cetyl-zephirol.

Concentration of Cetyl-zephirol	Evolution of CO_2 in cubic millimeters per hour by 1 milligram of dry material (Q_{CO_2})
Control	41
1:150,000	40
1:80,000	39.5
1:20,000	26
1:10,000	20

Table 3

Intensity of Fermentation produced by Endomyces Magnusii After the Latter has been Acted Upon by Cetyl-zephirol.

Concentration of Cetyl-zephirol	Evolution of CO_2 in cubic millimeters per hour by 1 milligram of dry material (Q_{CO_2})
Control	58
1:100,000	35
1:40,000	22