Studies on Glass Mould VI. On the Problem of Osmotic Control

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In preceding papers, studies on isolation of the fungi which grow on glass surface^{1,2)}, on cultural characteristics³⁾, on growth in concentrated media, on measurements of osmotic pressures of the mycelial cells⁴⁾, on aerial germination⁵⁾ and on identification of the species⁶⁾, were published in sequence.

Next problem to be solved is how and why the fungus cells keep their obligate tonophily.

When tonophilic fungi were transferred into the media containing salts or sugars in higher concentration, they showed higher osmotic pressure of cells. Consequently, the cell was able to keep its osmotic pressure always higher than that of external solution. By such a way, the fungus exhibits its osmoregulative function and adapts itself to a new environmental condition and meets the need of water uptake.

For the elevation of the osmotic pressure of cells, an increase in osmoactive substances of cells must take place. On this concern, some experiments were carried out, of which results will be reported here.

Tonophily of fungus was already investigated by Eschenhagen (1889)⁷⁾. He confirmed that the osmotic pressure of *Asp. niger* cells was raised from 8.5% to 32% (expressed in NaNO_3 -value) by transferring the mycelia from 1% to 34% glucose-containing media. Mayenburg (1901) has also studied the osmotic control of the fungus cell and reached a conclusion that it would be carried out by formation of new osmoactive substances in the cell⁸⁾. According to his experiments, cells of *Asp. niger* were plasmolysed when immersed in solutions of high concentrations of NaNO_3 , Na_2SO_4 , NaCl , NH_4Cl , and glucose, but were not deplasmolysed. Further, mineral substances were measured chemically only in diminutive quantity, even when cells were immersed in high concentrations of mineral solution, such as NaCl , Na_2SO_4 , and NH_4Cl .

Pantanelli (1904) has observed microscopically in detail. He pointed out that even a single hypha should have cells of different ages, e. g. old cells found near the base of a hypha showed high permeability, which was noted by rapid deplasmolysis⁹⁾. On the contrary, newly formed younger cells near the tip of a hypha were rarely deplasmolysed after long incubation in bathing solution, which indicated low permeability. Ethyl alcohol, butyl alcohol given in a little quantity, acted as developing stimulus (auslösender Reiz) on pressure within the cell. Analogously, he believed that the high osmotic pressure of cell would be induced by high concentration of media. The problem of osmotic control is, however, still open to further criticism, even on the physiology of usual fungi.

From the results above mentioned the present author has taken following points in special consideration in the experiments.

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1. Age of cells should be distinguished under a microscope in order to choose young and active cells as experimental materials.
2. Permeability of cells may be tested by the way of deplasmolysis.
3. Permeability of cells may be tested by the way of chemical analysis, too.
4. In order to notify the characteristic properties of tonophilic fungi, usual fungi should be used as controls.

Materials and Methods

Fungus strains: *Aspergillus vitricolae* was cultivated on malt agar slants for several months, from which inocula were harvested. This strain did never produce ascospores, but produced only conidiospores, which were served for inocula. The strain used was isolated from glass and has the property of obligate tonophily, i.e. it grows only on concentrated media. *Asp. oryzae* and *Asp. niger* grow best on usual media, but also are able to tolerate concentrated media. Two strains of *Asp. niger* which produced conidiospores only were used as controls.

Culture methods: Basal medium A (natural medium). Malt extract was prepared freshly each time before cultivation. Ten grams of powdered malt were immersed in 500 ml of water, saccharified overnight at 60°, and filtered. This extract showed reducing power corresponding ca. 0.8% maltose. This medium was found to be the most favorable one for growth and it served well, when other media did not permit enough fungus growth.

Basal medium B (synthetic medium). This liquid medium was prepared as follows.

NH ₄ Cl	2.75 g	sucrose	15 g
MgSO ₄	0.25 g	FeSO ₄	trace
KH ₂ PO ₄	0.25 g	water	500 ml

Salt medium. Sodium chloride was added to each basal medium in a requisite concentration. Obligately tonophilic fungi should be cultured in this medium.

Experiment 1. Plasmolysis of fungus cells.

Thirty milliliters of each liquid medium in an Erlenmeyer flask of 100 ml volume were seeded with inocula after autoclaving for 15 min. at 120°, and incubated at 30°. As soon as small mycelial patch appeared, hyphae were picked up and transferred to a slide glass to be observed under a microscope. It took 2-5 days for tonophilic fungi, 1-2 days for usual fungi after inoculation. During continuous observations, evaporation of the medium should be prevented on the slide glass, since this might result the change in concentration. For the purpose, peripherous border of the area was closed with vaseline. Cells of tonophilic fungi, even when young, were so fragile that plasmolysis experiment was confronted by difficulties. But cells of usual fungi were treated with much facility, on account of easy growth and less fragile cell structure.

Results are shown in Table 1.

It was extremely difficult to measure the osmotic pressure of hypha cells, which had grown on the glass itself. Although every attempt on tonophilic fungi failed, it succeeded in single case of *Asp. oryzae*. Hyphae of *Asp. oryzae* which was formed aerielly on cleansed glass showed that 3.4 M (20%) NaCl was isotonic by the method of plasmolysis. By analogy with this usual fungi, *Asp. vitricolae* probably has a higher osmotic value than 20% NaCl solution.

Table 1. Measurements of osmotic pressure by plasmolytic method.
Three strains of *Aspergillus*.

<i>Asp. oryzae</i>				
NaCl content, added to malt medium	0	4.76%	9.1%	13%
Osmotic pressure, expressed by isotonic NaCl-solution	0.69 M (41%)	1.9 M (11%)	2.9 M (17%)	3.2 M (18.7%)
<i>Asp. niger</i>				
NaCl content, added to malt medium	0	5%	10.8%	
Osmotic pressure, expressed by isotonic NaCl-solution	0.69 M (4%)	1.7 M (10%)	2.75 M (16%)	
<i>Asp. vitricolae</i>				
NaCl content, added to malt medium				15%
Osmotic pressure, expressed by isotonic NaCl-solution				3.4 M (19.7%)

Experiment 2. Deplasmolysis experiments.

Experiments were designed to ascertain two points. One point is to know the viability of cells. When plasmolysed cells were brought into water, they should attain original shape (deplasmolysis), if they remained viable. If they were not deplasmolysed, they must be concluded to be dead. This was very important, because when protoplasmic contents of dead cells were salted out by high concentration of salt solution, the cell gave an appearance of plasmolysis. Because, a salt is considered to have penetrated in a cell, when deplasmolysis occurred following on plasmolysis in hypertonic salt solution.

A) Even when cells of usual fungi were used, typical plasmolysis-pattern just like that of higher plants were not expected. For example, every cell of a hypha does not necessarily exercise the regular plasmolysis. Old cells were already dead or became so inactive that they could not behave normally. Several new cells near the tip of the hypha should be used for plasmolysis.

Second, third or fourth cell from the tip of a hypha was chosen as the material in the experiments. As Pantanelli pointed, old, delicate but yet living cells took the shape similar to cells plasmolysed in hypertonic solution, but did not recover their original shape when transferred them in water. Protoplasts of such cells might be injured and die during operation. In this case, it was not the true plasmolysis. Choice of proper cells is, therefore, essential for such experiments.

Several examples are to be described in the following.

Asp. oryzae: White colored mycelia of 2 day culture, in which spores scarcely formed, were immersed in 10%, 15%, 20% NaCl solution respectively. Within 15 min. cells were plasmolysed, and then deplasmolysed by replacing the solution by water. The type of plasmolysis was not "convex" form of *Rhodo discolor* but rather "concave" form of onion scale and usually took heavily irregular shapes, in extremely high

concentration of salt solution, almost all cell sap was lost and the protoplasm was separated into several small bodies. Such a phenomenon was initiated often by rapid plasmolysis, occurred especially in old cells and in most cases did not recover again.

In hypotonic solution, inward osmosis of water took place and the swelling of cells was observed. Similar results were obtained when basal medium B was used, and KNO_3 was applied in place of NaCl (Fig. 1A).

Asp. niger: Mycelia obtained by 2 day culture in basal medium A were immersed in 5% solution of NaCl . Mycelial cells were plasmolysed in 15 min. Deplasmolysis progressed more slowly than that of *Asp. oryzae* and observations were a little easier than the latter. Plasmolysis type and others were almost similar to those of *Asp. oryzae*. (Fig. 1C).

Asp. vitricolae: Mycelial cells of 5 day culture in the basal medium A added with

Fig. 1. A. Plasmolysis, *Asp. oryzae* was cultured for 2 days in malt medium, hyphae immersed in 20% NaCl solution.
 B. Plasmolysis, *Asp. vitricolae* was cultured for 4 days in malt medium, containing 10% NaCl , hyphae immersed in 20% NaCl solution.
 C. Plasmolysis, *Asp. niger* was cultured for 2 days in malt medium.
 a) hyphae immersed in 4% NaCl solution.
 b) hyphae immersed in 12% NaCl solution. No deplasmolysis.
 c) hyphae in 12% NaCl solution. Plasmolysed within 20 min.
 d) the same as c), almost deplasmolysed after 50 min. incubation.
 D. Silver reaction of NaCl . Hyphae cells of *Asp. vitricolae*, cultured in 12% NaCl malt medium for 4 days.
 a) silver reaction after 3 washings with isotonic KNO_3 solution.
 b) the same as a), after 5 washings.
 c) the same as a), after 7 washings.

15% NaCl, were plasmolysed when immersed in the solution of 20% NaCl, were deplasmolysed when transferred in water. But this process could be observed in rare cases, because cells were extremely fragile and readily injured by the operations (Fig. 1B).

B) In regard to permeability experiments, the whole process was carried out more easily with success in the case of *Asp. niger*. Hypha cells of 2 day culture in the basal medium A were plasmolysed in 15 min., when immersed in 5% NaCl solution, then deplasmolysed within 30 min. The same cells were plasmolysed again in 17% NaCl solution, but followed by no deplasmolysis even after 30 min., probably because they were damaged by repeated plasmolysis.

Another example. Hypha cells were plasmolysed in 5% NaCl solution, then deplasmolysed, and further plasmolysed once more in 23% NaCl solution, but followed by no deplasmolysis in the same solution.

Similar results were obtained when KNO_3 was used as plasmolyticum. No plasmolysis was observed in sucrose solution.

Asp. oryzae was plasmolysed in hypertonic solution, but time of deplasmolysis was delayed, so that observations were made with some difficulties.

Experiment 3. Measurements of NaCl endosmosis by the way of the silver reaction.

Usual fungus strains, *Asp. niger*, *Asp. oryzae*, and two strains of obligately tonophilic fungus, *Asp. vitricolae* were cultured in basal media and salt media, respectively. Active cells near the tip of hypha were chosen for experiments.

Younger cells taken on a slide glass were immersed in the silver reagent to be exposed to direct sun light. If chloride ion is present, change of color might take place. Under a microscope black metal granules attached around the periphery of hypha were recognized. When chloride ion was removed by washing, it was able to see whether the black granules inside cell be present or not. As following experiments showed, it was verified that NaCl was present in an appreciable amount in the cell, too, when cultured in salt solution.

A) Silver reaction of cells cultured in salt media.

Asp. niger and *Asp. vitricolae* were cultured for 2-4 days in basal media added with 12% NaCl. Mycelia were picked up by a forceps, rinsed out attached NaCl with isotonic NaNO_3 solution. Removing excess water with filter-paper, small mycelium was immersed in a drop of reagent. A number of silver particles were noticed. From 1st till 4th washing, there was no decrease in silver particles but from 7th till 10th washing, number of them in cells decreased markedly (Fig. 1D).

B) Mycelial cells produced in basal medium A were transferred to NaCl solution.

Asp. oryzae and *Asp. niger* were cultured in basal media A and cells were immersed in 16% NaCl solution for one and half hours. When tested directly, silver particles were observed everywhere, not only the periphery but also inside the cell. On the contrary, cell inside only was filled with silver particles when tested after 3 times washings in 10 min. with isotonic solution. These results seemed to prove the existence of endosmosis of NaCl with certainty.

C) Mycelial cells cultured in high concentrations of Na_2SO_4 solution were transferred into NaCl solution.

Both tonophilic and usual fungi were cultured in Richards' solution added with 17% Na_2SO_4 . Silver particles observed were markedly less than those in the experiment B on usual fungi. But silver particles appeared even after 10 min. and much increased after 1 hour incubation in cases of tonophilic fungi. Further was remarked

that NaCl once endosmosed was rapidly exosmosed when washed with isotonic KNO₃ solution (24%).

D) Measurement of permeability by estimation of salt content.

Asp. oryzae was cultured in Richards' solution added with 9.4% NaCl, incubated for 8 days at 30°. The medium solution was decanted, NaCl attached to hyphae was removed by washing with 50 ml of isotonic KNO₃ solution (20%). Last trace of Cl reaction in wash water disappeared after 3-4 washings, which needed about 1 hour. Fresh weight of mycelia from culture in an Erlenmeyer flask amounted to 4.37 g, of which 0.5 g gave 0.17 g of dry weight (66% water content).

Fresh mycelia were boiled for 30 min. and ground in a mortar, filtered and washed. Filtrate together with wash water was estimated of NaCl content. NaCl 0.6%, ashes 20%. Organic substances: inorganic substances 4:1.

E) Wash method.

The time required for accomplishment of a single washing could be reduced to one minute. When such an operation was repeated 10 times, NaCl disappeared completely in wash solution. NaCl content of mycelia, 1.1-1.5%. In this method it amounted to about twice as much as that by former method. Water content of mycelia 60%, ashes 24%. Consequently, organic substances: inorganic substances 3:1.

Comparing these results with those of experiment D, it may be deduced that NaCl exosmosed out of the cell by washing.

F) Permeability of the cell to Na-sulfate.

Asp. oryzae was cultured in Richards' solution added with 17% Na₂SO₄. After 8 day incubation, mycelia were washed with isotonic 14% NaCl solution until sulfate ion disappeared. 2.043% Na₂SO₄*, 50% water.

These results seem to indicate that Na₂SO₄ was permeable, inspite of less permeability than chloride.

Using tonophilic strains, similar experiments were performed. It needed more

Table 2. Results of cell sap analysis in connection with cell permeability to salt.

mycelia fresh weight	mycelia dry weight	water content	cell sap				
			NaCl	dry weight	ashes	org. : inorg.	red. as glucose
<i>Asp. oryzae</i> cultured in NaCl-media							
4.37 g	34%	66%	0.305%	28%	6.04%	4 : 1	0.47 g
2.5 g	40%	60%	1.1-1.5	32%	8.0%	3 : 1	—
<i>Asp. vitricolea</i> cultured in NaCl-media							
0.83 g	38%	62%	+	29.3%	7.8%	2.7 : 1	—
<i>Asp. oryzae</i> cultured in Na ₂ SO ₄ -media							
6.8 g	44%	56%	Na ₂ SO ₄ 2.04%	28.3%	6.3%	3.5 : 1	—
<i>Asp. vitricolae</i> cultured in Na ₂ SO ₄ -media							
4.73 g	42%	58%	3.2%	45%	16%	1.1 : 1	—

* Sulfate was estimated by the method of Andrew¹²⁾.

days for cultivation. Less mycelial yields were obtained.

Results were listed altogether in Table 2.

Discussion

On the problem of the osmoregulation, Pfeffer¹³⁾ stated that initiation or increase of solutes in cells must be of utmost importance, because he thought that mineral salts of external solution could not penetrate into the cell through the cell membrane. Eschenhagen, Mayenburg, and Pantanelli, who have studied tonophily of fungi, supported Pfeffer's concept. According to their experiments, *Asp. niger* etc. could accommodate themselves virtually to hypertonic environments by increasing osmotic pressures within cells. They believed that high concentration of media might stimulate the cell to cause the accumulation of solutes in hyphae cell. Anatonosis was suggested as a possible origin of smaller molecular weight compounds. For example, polysaccharides could be converted into sugars, sugars into organic acids. In other words, they thought that cells couldn't absorb sufficient osmoactive substances such as minerals from outside for maintenance of pressure gradient.

Based on results mentioned in this paper, the present author would conclude that mineral substances could diffuse inward across fungus cell membrane in the both cases of organisms, i. e., usual fungi and tonophilic fungi. One of these discrepancies lying between two opposing concepts is thought to be depending on different wash methods. For preparation of cell sap, Eschenhagen washed the mycelia with isotonic potassium nitrate solution (corresponding to 11% NaCl-media) until last trace of chloride reaction in wash solution disappeared. It needed longer time, e. g., more than one hour for complete removal of NaCl. Only 0.004 g (0.1%) NaCl were measured in mycelia, fresh weight 4 g of *Asp. niger*. The content was much less than that of culture media. According to my opinion, NaCl inside the cell must have been removed by drastic washing. That Eschenhagen concluded the absence of permeability to NaCl, is therefore, insignificant. Mayenburg and Kinoshita have chosen also the same wash method, which led to an error in conclusion.

In an earlier paper⁴⁾, effect of washing on NaCl content of cells has been tested. The results are given here again. *Asp. vitricolae* was cultured in NaCl media.

Table 3. Relation between time and number of washing of mycelia and NaCl contents, *Asp. vitricolae*.

Time and number of washing	medium	incubation	depression		NaCl-content		sugar content	
			medium	cell sap	medium	cell sap	medium	cell sap
10 min. 3 washings	koji-media	12 days	-11	-3.84	16%	2.8%	—	0.8%
30 min. 3 washings	"	"	-11	-2.5	—	—	—	—
60 min. 5 washings	"	"	-11	-1.3	—	—	—	—
5 min. 2 washings	"	23 days	-17.3	-14.9	22%	15.9%	3.6%	3.6%
without washing	"	"	-17.3	-18.5	22%	18.5%	3.6%	3.6%

Viewed from various results mentioned, permeability of *Asp. vitricolae* cells to salt seemed largest, that of *Asp. niger* cells smallest and that of *Asp. oryzae* cells lying between two. These facts tell us the reason why experimental procedures of plasmolysis and deplasmolysis met with difficulties in the case of *Asp. vitricolae*, while more easily in cases of *Asp. niger* and *Asp. oryzae*.

In a previous paper, the author measured depressions of the cell sap. Thereby, an appreciable salt content was estimated in cell saps, although NaCl in cell sap was always smaller than that of medium. NaCl content of cell sap was raised, as NaCl increased in the culture medium. This fact also indicated the inward movement of NaCl into cell.

There is one more point to be noticed. Concentration of medium or bathing solution may be responsible for the permeability of cell membrane, in such a manner that the higher is the concentration, the larger is the permeability of cells. From these facts, it may be acceptable that the highest tonophily of *Asp. vitricolae* was accompanied by the most rapid salt penetration.

In this connection, much interesting are Thatcher's observations, which were carried out using parasitic fungi; *Uromyces*, *Sclerotinia* and *Botrytis*¹⁴⁾. In every case, he measured markedly greater osmotic pressure as well as permeability of fungi than those of host plant cells and concurrently he stated that host cells in the vicinity of parasitic fungi had particularly higher grade of permeability to water and nutrients.

As regards viability, remarkable difference appeared under a microscope between usual fungi and glass moulds, when cultured in liquid media. The latter is delicate and much more readily injured. The less viability of glass moulds in solution may be rather natural, because they grow normally only by absorbing water vapor from air, without uptake of water.

Because of its fragility and minuteness, operations were brought about with much difficulties and fair results were not always expected. But it is not too much to say that glass mould cells are permeable to salts, its rate considerably depending on kind and concentration of each salt. Osmotic pressure may be maintained for the most part by salts absorbed, with only a limited amount due to other substances formed in the cell.

Summary

In regard to adaptation of glass moulds and other fungi to high concentration of media, permeability of fungi cells to salts was studied.

1. Besides strains of *Asp. vitricolae*, usual fungi strains, *Asp. oryzae*, *Asp. niger* were chosen as test organisms.

2. For the purpose to observe plasmolysis of fungi cells, younger hyphal cells were chosen.

3. Cells of *Asp. vitricolae* were so delicate that they were readily injured during operations, which caused some difficulty for experimentation. On the contrary, *Asp. oryzae* and *Asp. niger* were much stable and rich in mycelial yield, which allowed more smooth procedures.

4. Osmotic pressure of the cells measured by the method of plasmolysis resulted always in higher values than that of external solutions.

5. Cells once plasmolysed in certain salt solution recovered the original shape, when they were transferred into water. Older cells often took an appearance like

plasmolysis in salt solution, but they were scarcely deplasmolysed, when transferred into water. Plasmaproteins of the same cell seems to be salted out, perhaps because they were dead during operation.

6. Permeability to salt was tested by deplasmolysis experiments. Deplasmolysis occurred gradually after plasmolysis in the same salt solution. The rate of permeation of salts was larger in the case of *Asp. vitricolae* than in cases of *Asp. oryzae* and *Asp. niger*.

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摘 要

大槻虎男： ガラス菌の研究，第6報．浸透調整の問題について

ガラス菌その他が高浸透圧培地に培養された場合に，菌糸細胞はその浸透圧を高くして，これに適應することは，前に数回にわたって報告された．このとき浸透調整がいかになおされるかを知るために塩類の透過性に関して諸実験をおこなった．

1. 1本の菌糸にも基部には古い細胞があり，活力とぼしく，あるものはすでに死んでいる．先端に近いところに若い活力に富む細胞がある．実験は先端に近い細胞についておこなわれた．

2. 原形質分離型は不規則であるが，凹面型が主として観察された．ひどく分離したとき凸面型をとり，このときは死を招くことが多い．

3. 分離細胞を水にもどすと，原形質分離回復が起こる．これによって実験に供した菌糸細胞の生死を確かめた．

4. ガラス菌を高張溶液に浸し，分離を起こさせると，しばらくして回復が起こってくる．これは塩類が細胞内に浸入したことを示す．コウジカビ，クロカビは回復に長い時間を必要とする．また硫酸塩は食塩より長い時間を要した．

5. 食塩の透過については銀反応を利用してその存在を顕微化学的に証明した．

6. 過度の洗滌は菌糸細胞内の塩の外浸透を起こし，不正確な結果を招くことを，塩の定量をすることで明らかにした．他の著者らとの結果のちがいがここに原因することを論議した．（お茶の水女子大学理学部植物学教室）