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SUBJECT OF INVESTIGATION

STUDY
ON
THE INTRACELLULAR CALCIFICATION
BY
ORAL LEPTOTHICHIA

RESPONSIBLE INVESTIGATOR

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Abstract

The intracellular calcification of oral filamentous micro-organisms which were identified Bacterionema matruchotii from the bacteriological examinations in this study was analysed chemically in relation to its mechanism.

The intracellular calcium and phosphorus were found to increase following the in vitro calcification. In addition, the ratio of calcium and phosphorus approximates to 2.12 which is the ratio of themselves in hydroxyapatite. These findings was observed in both living and killed cells, therefore, it can be said the intracellular calcification are independent from the metabolic process of the microorganisms.

Heating or magnesium ion which possessed the specific inhibition against the in vitro calcification of rachitic cartilage were found to affect the intracellular calcification as well. From the experiment on the other inhibitory factors, formalin if it exist in the calcification throughout, was found most effective.

These findings suggest that the intracellular calcification consisted of three phases: crystal nucleation, crystal growth and crystal limitation as the phenomenon observed in another organic tissues. And also the existence of intracellular organic substance responsible to the calcification as like collagen as found in bone tissues was assumed.

Extraction of the responsible substanee from the cells was repeated, but the extracts were not stable in their calcifiability except some instance in which hydroxyapatite was demonstrated by electron diffraction method. Study is going on the way to get the stable extract.

STUDY

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C o n t e n t s

the calcifying solution concerned, the influence of co-existed ions was negligible as is shown in Fig. 1.

Other special methods are indicated in each section.

III. Experimentals and Comments

1. The identification of the microorganisms.*

The plaque samples of seventy male adults were supplied for the isolation source.

Plaque samples taken by platinum roop were diluted by saline solution in approximately 1,000 to 10,000 times and 0.1 ml of each suspension was inoculated on the isolation media. Media used consisted of BHI : 3.7 %, Yeast Extract : 0.2 % and Agar (powder): 1 %. Inoculated plates were incubated at 37°C in the moistured top for 7 days. Colonies were examined under the low magnified microscope taking aim at the hairy structure of objective colony. Isolates were purified by three passages on the same plate.

Biochemical characteristics including nitrate reduction, indole production, gelatine liquefaction, Voges-Proskauer reaction, esculin hydrolysis, hippurate hydrolysis, urease production, carbohydrates fermentation, phosphatase production and lipase production were examined.

The employed method followed to those described in the "Manual of Microbiological Method"[5]. Carbohydrates fermentation was tested on glucose, maltose, sucrose, lactose, galactose, dextrin, inulin, raffinose and salicin. The medium consisted of Trypticase (BBL): 2%, Yeast Extract (Difco) : 0.2% and 0.2% BTB solution : 1.2% was used for the basal medium. The medium which contained test carbohydrate in 1% was autoclaved except the case for glucose, lactose, maltose, dextrin and galactose. These carbohydrates were as aqueous-20% solution respectively sterilized by filtration (Millipore Filter, HA) and aseptically distributed to autoclaved basal medium at a final concentration of 1 %.

For the phosphatase detection, Yeast Extract added BHI agar was supplemented by phenolphthalein phosphate which were synthetized in our laboratory or obtained commercially (Corp. for Biochemical Research, Los Angeles).

For the lipase detection, the medium consisted of Peptone: 1%, Beef Extract (Difco) : 0.3%, Yeast Extract : 0.2%, Agar (powder) : 1%, Sorbitan monooleate : 0.1% and $CaCl_2$: 0.1% was used. The appearance of crystal around the colony was examined every day.

The biochemical and fermentative characteristics of forty four cultures isolated are summerized in table 3 and 4 . Looked at various carbohydrates, (Table 3), whereas most strains fermented glucose, sucrose and maltose, no strain fermented inulin. Raffinose was fermented by about half number of the isolates, and lactose and galactose were fermented by 10% numbers of the isolates.

Hydrolysis of both esculin and hippurate was observed in all isolates. Indole production and gaseous carbon dioxide were not observed by all strains. Acetyl-methyl-carbinol was produced from glucose by all isolates except one strain. Dichotomous branching was observed, in general, and aerobic conditions was found to be more preferably for the growth.

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* This part will be published soon in the Bulletin of Tokyo Dental College.

Both phosphatase and lipase activity were not detected, whereas weak urease activity was found in some strains.

The present study is confined to strains starting from the colony which possessed hairy structure. Accordingly, the isolation ratio (frequency of occurence) was not so high. Compared the present findings with those described by many authors (Table 5,6), it was found that the isolates were quite common to those cultures reported by Davis and Baird-Parker (Type 2)[1], Richardson and Schmidt[10], and Gilmour and Beck[11] both in morphology and physiology. Because each biochemical characteristic of isolates showed relatively homogenously, it is agreeable to classify this group of microorganisms in one genus as Gilmour et al. have proposed. It should be pointed out that the study upon the nutritional requirements of the microorganisms and upon establishment of the selective medium are urgent matter for further investigation upon this group of microorganism.

Special character which appeared to be correlated to the intracellular calcification was not noted except the negativeness of phosphatase and lipase production which are regarded some significant enzyme during the general calcification in the Robinow's theory[12].

2. Analysis of intracellular calcium and phosphorus during the calcification, and inhibition study.

1) Mineral increasing in the cells.

At first, the calcification experiment was carried out using calcifying solution in which test microorganism containing cellulose tube (Visking Co. 20/32) was hung. Calcifying solution was kept in flask and cellulose tube was stopped at the neck of the flask with cotton plug. The flask was incubated at 37°C for 3 weeks and 4 ml of calcifying solution outside of cellulose tube was taken every 4 days for the determination of calcium and phosphorus.

The values gained were summerized in Fig. 2. Cytochemically, after 3 weeks treatment, cells were strongly positive against Alizarin red S method. It was clarified that calcium and phosphorus in the solution outside of cellulose tube decreased during the full test period. But the values of calcium and phosphorus assumed to be taken intracellulary were very limited.

It is likely that calcium and phosphorus in the calcifying solution precipitated weakly when it is kept under the condition tested, therefore, the actual intracellular calcium and phosphorus should be determined directly with changing calcifying solution every one or two days.

7 day cultured cells were applied for calcification in the flask with glass stopper at 37°C. The calcifying solution was changed every one day and this procedure was performed for 23 dyas. At intervals, calcified cell samples were taken, washed and used for the determination of intracellular calcium and phosphorus.

The intracellular calcium and phosphorus following the growth in nutrient broth were also determined as control. Both values showed some increasing tendency as is shown in Fig. 3. It is interesting that the ratio of calcium and phosphorus readily is nearly 2.

The values of calcium and phosphorus per mg dry weight of the calcified cells in the calcification experiment increased following to the treatment period as is shown in Fig. 4. It was noticed that the cells contained nearly the same quantity of calcium and phosphorus at the first stage i.e. after five day

treatment. Then the difference between both values became significantly, but the ratio of calcium and phosphorus were calculated between 1.96 and 2.16 (Table 7). This finding theoretically supported the previous finding by Takazoe[3] who demonstrated the intracellular crystals as hydroxyapatite.

2) Some parameters which influence upon the intracellular calcification.

Influence of culture age of the test microorganisms upon the intracellular calcification was examined.

Cells were harvested at different culture period as 3 dyas, 7 days, 10 days, and 12 days. Every samples were treated by the method described above and applied for calcification experiment. At the three day cultured cells, the ratio of calcium and phosphorus was 1.87 indicating that the intracellular crystal component -- if existed -- was a little different from hydroxyapatite. It was shown that more than 7 day cultured cells have the calcifiable ability fitting to our purposes (Table 8).

Influence of the viability of the starting cells upon the calcification was examined. 0.5% formalin treatment at 37°C for 2 hours was used for killing. After this treatment, cells were compltely washed and used along with living cells. After the treatment in calcifying solution for 10 days, it was recognized that the viability of the cells did not affect the calcification (Table 9). However, when the period of formalin treatment was prolonged, for example, when formalin was added in the calcifying solution throughout the experiment at the concentration of 0.5%, the effect was quite significant as inhibitory action as was shown in Fig. 4. The curve of phosphorus in this case was almost horizontal but the curve for calcium showed weak increasing tendency for which mechanism is unknown.

It is possible, at any rate, that the significant inhibitory action of formalin against calcification might be due to its close coupling ability with active free amino acid of some calcifiable organic substance in the cell.

Influence of ether-acetone treatment of the cells upon the calcification was also examined.

Cells were treated by mixture of ether and acetone (1 : 1) at 4°C for 24 hours. Cells were centrifuged and dried in air. Thus, the sample became dried powder in nature.

This treated cells still possessed the stainability against Gram reaction with relatively higher tendency to be negative. The picture of the cell disruption was hardly found.

After the calcification, materials were decalcified by 2.5% EDTA solution (pH 7.0) at room temperature for 10 days period which was found to be sufficient for the decalcification of this materials as detected for calcium in the supernatant.

Ether-acetone treated cells and once decalcified samples were applied for the calcification test again for 14 days.

The results were summerized in Table 10. Control showed the almost same terms as the finding mentioned above. In the column for the ether-acetone treated cells, every samples showed low values than control in calcium and phosphorus keeping with the common ratio. The results on the calcification ability of once decalcified samples showed the evidence that the material possess the calcifying ability at least per mg dry weight under the condition treated. In addition, the absolute terms determined were higher than the ether-acetone treated control. The reason for this fact remained unsettled.

3) Inhibition study in relation to the crystal nucleation.

Further, as an exploration of mechanism for the intracellular calcification, the existence of nucleation in this system was examined by the inhibition method used by Sobel[13]. Heating at 76°C for 10 minutes and the treatment by 3 mM iodoacetate solution with 1 mM / 1 magnesium at 37°C, 2 hours were used for the inhibitry method against the crystal nucleation. 200 mg wet weight of washed cells were distributed into 70 ml-centrifuging tubes and each tubes were treated respectively according to the scheme shown in Table 11. After respective treatment, final calcification were performed for 10 days, changing calcifying solution every day.

Examined treatments were proved as inhibitory on the intracellular calcification as well. The determined values of the intracellular calcium and phosphorus are summerized in Table 12. Inhibition against calcification on each treatment were calculated as summerized in Table 13. On the non-precalcified cells, heating method is more effective on living cells than formalin-killed cells, to the contrast, treatment by iodoacetate solution with magnesium was more effective on formalin-killed cells. This tendency was also recognized in the case of precalcified cells.

Above all, precalcified cells were less inhibited by following inhibition treatment. The difference of the inhibition degree by the examined method between precalcified cells and non precalcified cells are precisely recognized, especially on formalin-killed cells. The inhibitory mechanism in these findings are yet unknown. It is possible to assume, however, that heating effect is possible extraction of responsible substance for calcification or simple inactivation. The results concerning iodoacetate with magnesium suggest that the metabolic processes of the microorganisms are not essential for the mineralization. The fact that the complete inhibition was not recognized in this experiment suggest that the original wahsed cells possessed readily the crystal nucleus, or inhibition methods was unsatisfatory in the concentration or in the treatment period.

3. Preliminary study on the extraction of intracellular calcifiable substance.

The responsible fraction[14] which is supposed to exist intracellulary from the findings above obtained was studied to extract.

Glacial acetic acid and citric acid[15] were tested for extractability of calcifiable substance. Results showed glacial acetic acid-treated cell residue lost their calcifiability (Table 14). Then the extraction was done following the method by Ennever[16] as follows.

About 15 litres of BHI broth supplemented by 0.2% Yeast Extract were inoculated with 3 day cultured test microorganism and incubated at 37°C for 7 - 10 days aerobically. After the cultivation, harvesting of cells and washing by a large quantity of deionized water were done by means of centrifugation (8,000RPM) at 4°C. Washed cells were suspended in 500 ml glacial acetic acid for 8 - 18 hours. Whole materials were repeatedly centrifuged to seperate the residue cells. 250 ml of saturated sodium chloride solution was added to the cell free solution obtained and dialysed in cellulose tube (Visking Co. 36/32) against running deionized water for 48 hours. Precipitates were centrifuged (10,000 RPM) at 4°C and kept in refrigerator at 4°C.

The recoveries of the extract were variable ranging from 10 to 20% by wet weights.

Extracts obtained several times were examined for the calcifiability in the calcifying solution.

Though the samples showed positive against alizarin red S method even in a few days, the determined values of calcium and phosphorus do not approximate the steady state for possible assuming of the appearance of hydroxyapatite. The results were summarized in Table 15, 16. It was noted that some instance as experiment No.2 in table 16 which was carried out at room temperature resulted adequate calcification. In this case, the electron diffraction of the material showed the pattern of hydroxyapatite as indicated and culculated in Fig. 5 and Table 17.

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Table 1. Calcifying solution

0.7 M	NaCl	)
0.05M	KCl	} ..... Stock Solution
0.02M	$NaHCO_3$	)

Stock solution is ten-fold diluted and K_2HPO_4 is added as 12mg per cent phoaphorus. Then pH is adjusted to 6.0 using carbon dioxide gas and then $CaCl_2$ is dissolued as 4mg per cent calcium. Finally, pH is adjusted to 7.4 by airation. Kept with tighly sealing.

Table 2. Preliminary experiment on Calcium recoveries from the test solution with phosphate in various concentration.

Test tube No.	1	2	3	4	5	6	7
Ca-sol.(10 γ/ml)	1.0 ml	1.0	1.0	1.0	1.0	1.0	1.0
P-sol. (0.04M-K_2HPO_4)	.	1.0 ml	1.0	1.0	2.0	2.0	2.0
Determined Ca	10.02 γ	10.02	10.02	10.02	10.02	10.22	9.82

Test tube No.	1	2	3	4	5	6	7	8
Ca-sol.(10 γ/ml)	5.0 ml	5.0	5.0	5.0	5.0	5.0	5.0	5.0
P-sol. (0.04M-K_2HPO_4)	.	.	1.0 ml	1.0	1.0	2.0	2.0	2.0
Determined Ca	49.89 γ	50.30	49.89	50.10	50.30	50.30	49.89	49.69

Calcium standard solution tested was mixed with 0.04M-K_2HPO_4 (P: 1.24 mg/ml), diluted with deionized water, pH adjusted, and were passed through the column with Amberlite IR-4B under the speed of 10 ml/20 minute. Column was washed twice with 10 ml deionized water. Whole filtnates were determined by EDTA chelating Method.

Fig. 1. The influence of co-existed ions in the calcifying solution upon the phosphorus content (470 $m\mu$)

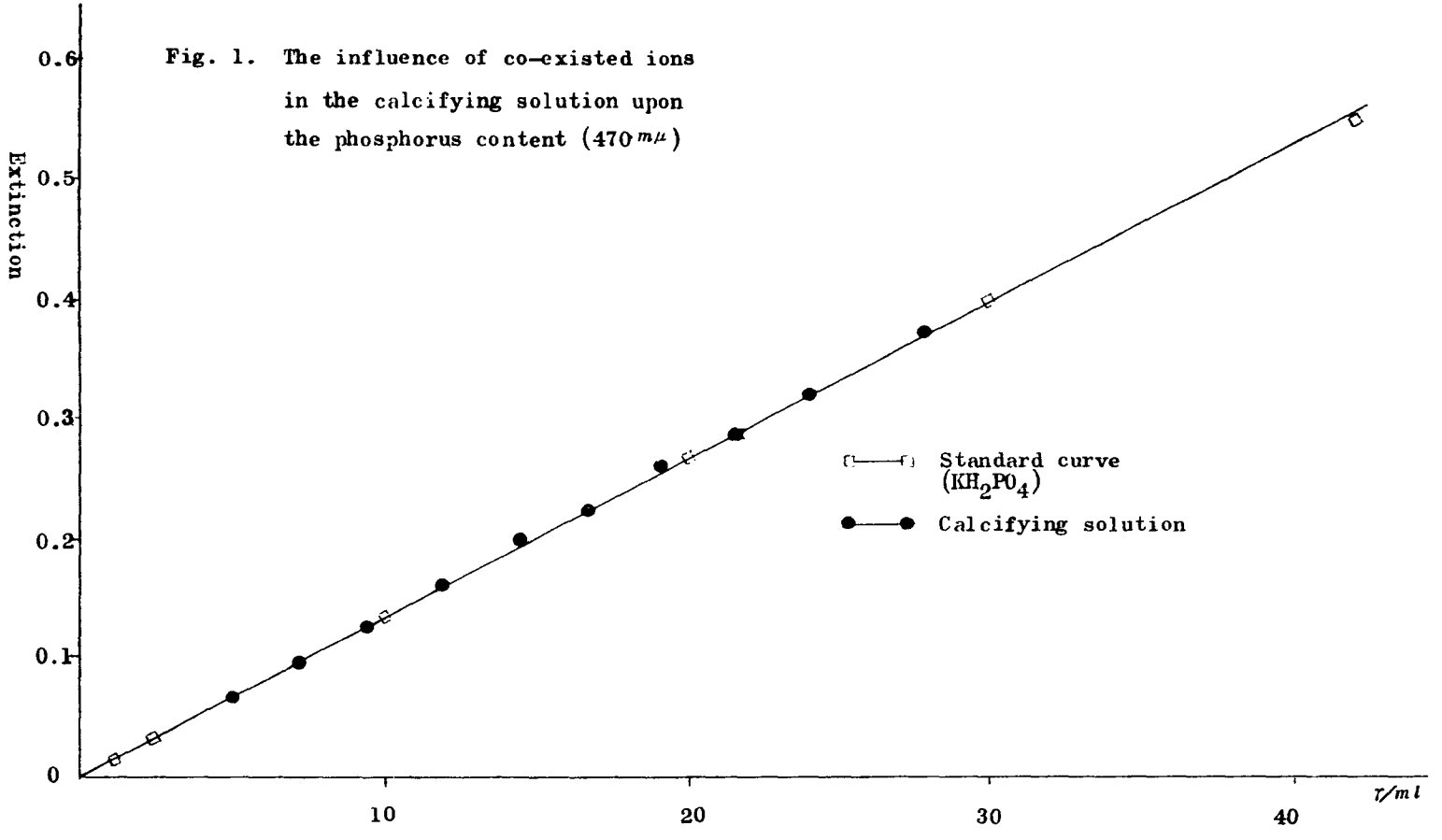


Table 3. Fermentative characteristics of isolated organisms

	Glucose	Sucrose	Maltose	Raffinose	Lactose	Galactose	Inulin	Dextrin
L2	++	++	-	-	-	-	-	-
L4	++	++	++	-	-	-	-	++
2KI	++	++	++	-	++	++	-	+
ICI	++	++	++	+	-	-	-	+
IC2	++	++	++	+	++	++	-	+
IC3	++	+	+	+	+	-	-	+
NSI	++	++	++	-	-	-	-	+
TUI	++	++	++	+	++	++	-	+
# 2	++	+	++	+	-	-	-	±
# 4	++	++	++	+	-	-	-	++
# 5	++	++	++	-	-	-	-	++
# 8	++	++	++	-	-	-	-	+
# 10	++	++	++	-	-	-	-	++
# 11	++	++	++	++	-	-	-	++
# 12	++	++	++	-	-	-	-	++
# 13	++	++	++	-	-	-	-	++
# 14	++	++	++	-	-	-	-	++
# 16	++	++	++	-	+	-	-	-
# 17	++	++	++	++	-	±	-	++
# 18	++	++	++	++	-	+	-	++
# 19	++	+	+	-	-	-	-	+
# 20	++	+	+	++	-	-	-	+

	Glucose	Sucrose	Maltose	Raffinose	Lactose	Galactose	Inulin	Dextrin
# 22	+	+	++	+	-	-	-	+
# 23	++	++	++	+	-	-	-	++
# 25	+	++	++	-	-	-	-	+
# 76	++	++	++	-	-	-	-	+
# 79	.	.	.	.	.	.	.	.
# 80	++	+	++	-	-	-	-	++
#84	++	++	++	++	-	±	-	+
#85	++	++	++	++	-	-	-	++
#86	++	++	++	++	-	-	-	+
#92	++	++	++	++	-	-	-	+
# 97	+	+	++	+	-	-	-	+
# 98	++	++	++	++	-	-	-	+
#106	++	++	++	++	-	+	-	++
#112	++	++	++	-	-	+	-	+
#116	+	+	-	++	-	-	-	±
#117	+	+	±	-	-	-	-	±
#127	++	++	++	-	-	-	-	+
#128	++	++	++	-	-	-	-	+
#131	++	++	++	++	-	-	-	++
#132	++	++	++	++	-	-	-	+
#133	.	.	.	.	.	.	.	.
#137	++	++	++	++	-	-	-	++

Table 4. Biochemical characteristics of isolated strains

	Esculin	Hippurate	Urease	Indole	CO_2	Acetoin	NO_3——NO_2	Gelatinase	Lipase	Phosphatase	Catalase	Gaseous charact.	Branching
L2	w	+	+	−	−	+	+	−	−	−	+	A	+
L4	+	−	+	−	−	+	+	−	−	−	+	A	+
2KI	+	+	+	−	−	+	+	−	−	−	+	A	+
ICI	+	+	−	−	−	+	+	−	−	−	+	A	+
IC2	+	−	+	−	−	−	+	−	−	−	+	A	+
IC3	+	+	+	−	−	+	+	−	−	−	+	A	+
NSI	−	+	−	−	−	+	+	−	−	−	+	A	+
TUI	+	+	+	−	−	w	+	−	−	−	+	A	+
# 2	+	+	+	−	−	+	+	−	−	−	+	A	+
# 4	+	+	−	−	−	+	+	−	−	−	+	A	+
# 5	+	+	−	−	−	+	+	−	−	−	+	A	+
# 8	+	+	+	−	−	+	+	−	−	−	+	A	+
# 10	+	+	+	−	−	+	+	−	−	−	+	A	+
# 11	+	+	+	−	−	+	+	−	−	−	+	A	+
# 12	+	+	+	−	−	+	+	−	−	−	+	A	+
# 13	+	+	−	−	−	+	+	−	−	−	+	A	+
# 14	+	+	−	−	−	+	+	−	−	−	+	A	+
# 16	+	+	−	−	−	+	+	−	−	−	+	A	+
# 17	+	+	+	−	−	+	+	−	−	−	+	A	+
# 18	+	+	−	−	−	+	+	−	−	−	+	A	+
# 19	+	+	+	−	−	+	+	−	−	−	+	A	+
# 20	+	+	+	−	−	+	+	−	−	−	+	A	+
# 22	+	+	+	−	−	+	+	−	−	−	+	A	+
# 23	+	+	+	−	−	+	+	−	−	−	+	A	+
# 25	+	+	−	−	−	+	+	−	−	−	+	A	+
# 76	+	+	−	−	−	+	+	−	−	−	+	A	+
# 79	+	+	+	−	−	+	+	−	−	−	+	A	+
# 80	+	+	−	−	−	+	+	−	−	−	+	A	+
# 84	+	+	+	−	−	+	+	−	−	−	+	A	+
# 85	+	+	+	−	−	+	+	−	−	−	+	A	+
# 86	+	+	−	−	−	+	+	−	−	−	+	A	+
# 92	−	+	−	−	−	+	+	−	−	−	+	A	+
# 97	+	+	+	−	−	+	+	−	−	−	+	A	+
# 98	+	+	+	−	−	+	+	−	−	−	+	A	+
#106	+	+	+	−	−	+	+	−	−	−	+	A	+
#112	+	w	+	−	−	+	+	−	−	−	+	A	+
#116	+	+	−	−	−	+	+	−	−	−	+	A	+
#117	+	−	−	−	−	+	+	−	−	−	+	A	+
#127	+	+	+	−	−	+	+	−	−	−	+	A	+
#128	−	+	−	−	−	+	+	−	−	−	+	A	+
#131	+	+	+	−	−	+	+	−	−	−	+	A	+
#132	+	w	−	−	−	w	+	−	−	−	+	A	+
#133	+	+	−	−	−	+	+	−	−	−	+	A	+
#137	+	+	−	−	−	+	+	−	−	−	+	A	+

w: weak positive A: aerobe

Table 5. Fermentative characteristics of organisms morphologically similar to Bacterionema matruchotii, reported by several authors

	Kligler	Mendel	Bulleid	Bibby	Bartels	Morris Type 1	Morris Type 2	Morris Type 3	Morris Type 4	Davis and Baird-Parker	Richardson and Schmidt	Gilmour and Beck	Our strains
Glucose	+	+	slow	+	+	–	slow	+	+	+	+	+	100%
Sucrose	+	+	–	98%	+	–	slow	+	+	+	+	+	100%
Maltose	+	.	slow	88%	+	–	–	+	+	+	+	+	93%
Raffinose	.	.	.	+	–	–	–	–	–	+,–	+,–	*19/55	55%
Lactose	–	+	–	–	–	–	–	+	+	–	–	–	12%
Galactose	.	.	slow	–	.	.	.	.	.	+,–	–	–	14%
Inulin	.	.	–	–	.	–	–	–	+	.	–	–	0%
Dextrin	.	.	–	.	+	–	–	–	–	+	+	.	90%

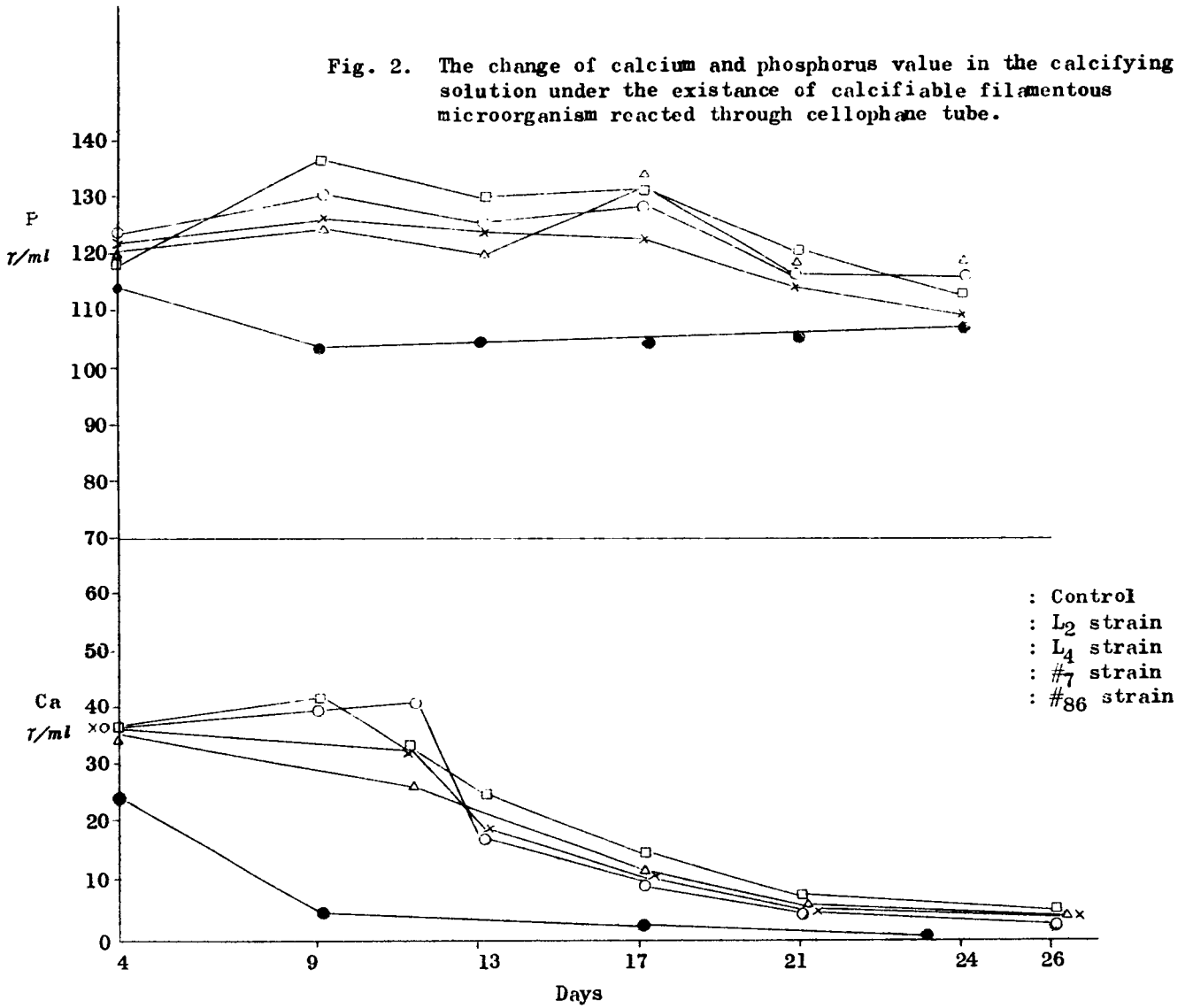
* : aerobically tested
% : express positive

Table 6. Biochemical characteristics of organisms morphologically similar to Bacterionema matruchotii, reported by several authors

	Kligler	Mendel	Bulleid	Bibby	Bartels	Morris Type 1	Morris Type 2	Morris Type 3	Morris Type 4	Davis and Baird-Parker	Richardson and Schmidt	Gilmour and Beck	Our strains
Esculin	.	.	.	.	.	.	.	.	.	+	.	48/55	93%
Hippurate ...	.	.	.	.	.	.	.	.	.	+	.	55/55	93%
NH_3product ..	–	.	.	.	.	.	.	.	.	–	.	.	.
Urease	.	.	.	.	.	.	.	.	.	.	.	.	57%
Indole	–	.	.	–	.	.	.	.	.	–	–	–	–
CO_2	–*	–*	.	–*	.	.	.	.	.	–	–*	25/55	–*
Acetoin	.	.	.	–**	.	–	.	–	+	+	+	+	98%
NO_3---NO_2 ...	.	.	.	96%	.	–	.	+	+	+	+	55/55	100%
Gelatin	–	–	.	–	.	.	.	.	.	–	.	–	–
Catalase	.	.	.	.	.	–	.	–	–	+	+	+	100%
Gaseous charact....	An, F, A,	A	F, A	Pref. An	An to F	Strict An, some strains become O_2 tolerant				Pref. A	A	Pref. A	A
Branching ...	–	+	+	Rare	+	Rare				+	+	+	100%

* : By Durham tube test.
An: anerobe, F: facult ative, A; aerobe.
**: By original Voges-Proskauer.
% : Express positive

Fig. 2. The change of calcium and phosphorus value in the calcifying solution under the existance of calcifiable filamentous microorganism reacted through cellophane tube.



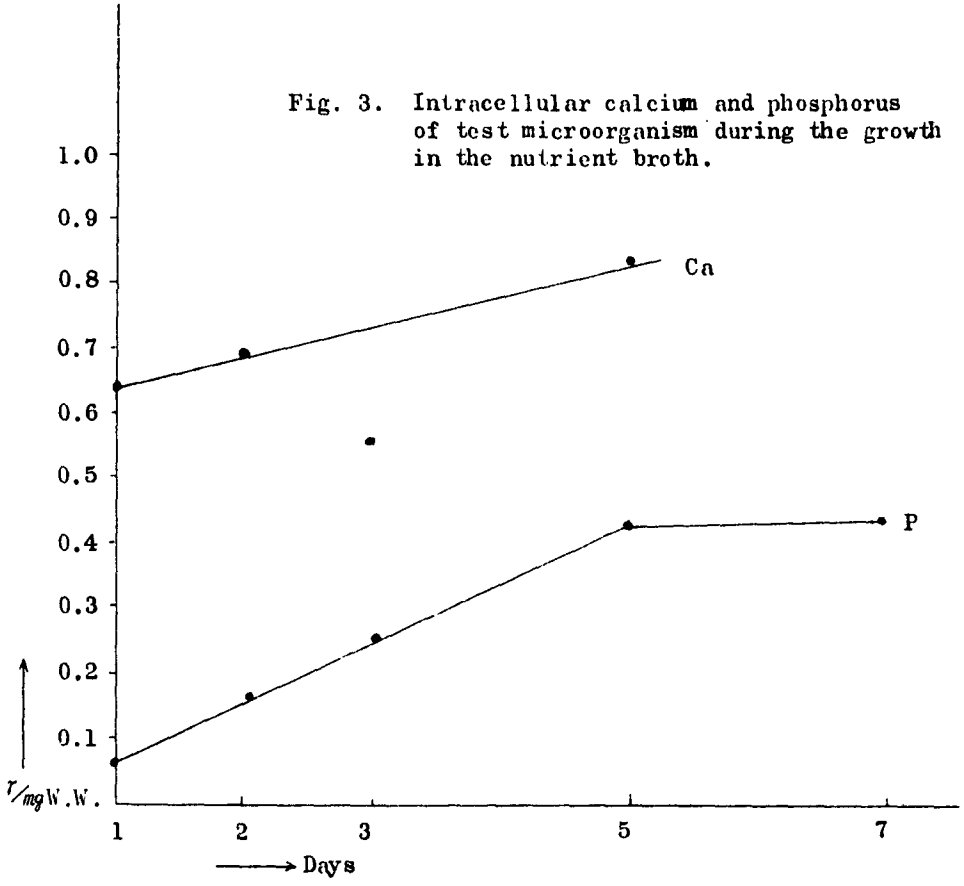


Fig. 3. Intracellular calcium and phosphorus of test microorganism during the growth in the nutrient broth.

Fig. 4. Intracellular calcium and phosphorus during the intracellular calcification.

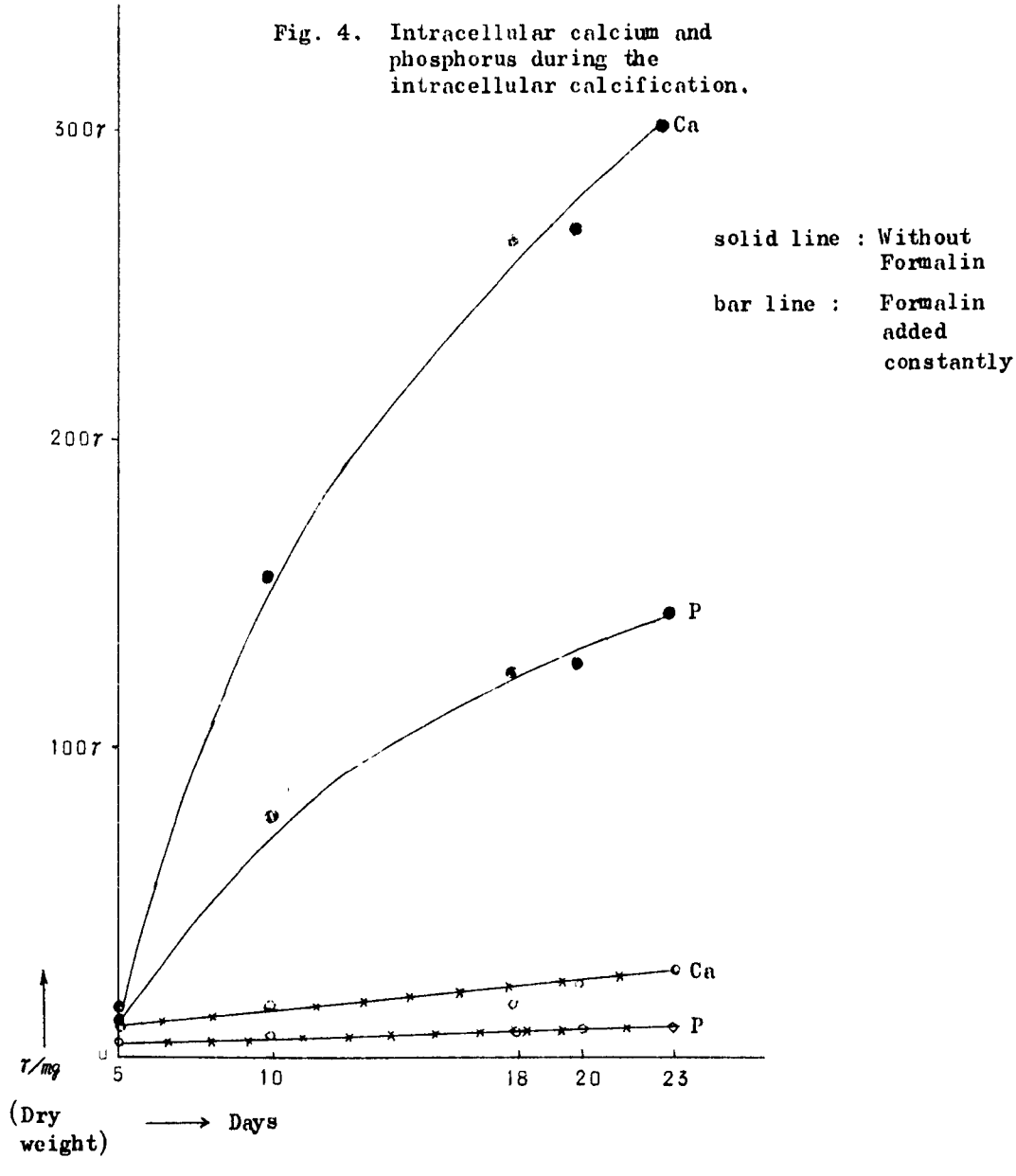


Table 7. Intracellular calcification in vitro

Period (days)	Ca (γ/mg)	P (γ/mg)	Ca/P
I (5)	13.02	11.65	1.12
II (10)	156.70	79.92	1.96
III (18)	265.84	123.24	2.16
IV (20)	265.51	125.39	2.11
V (23)	298.16	142.20	2.10

Table 8. The effect of culture period on the following calcification.

Period	Ca (γ/mg)	P (γ/mg)	Ca/P
3 days	101.4	54.02	1.87
7 days	212.6	100.31	2.11
10 days	134.8	65.10	2.07
12 days	181.2	84.73	2.14

Table 9. The effect of viability of the cell on the following calcification.

	Ca (γ/mg)	P (γ/mg)	Ca/P
Living cell	246.07	111.29	2.21
Formalin killed cell	249.11	116.50	2.14

Table 10. The effect of ether-acetone treatment on the following following calcification and recalcifiability of the cells.

	Ether-Acetone treated cell						Non-treated cell		
	Non-treated			Calcified with calcifying sol. and then decalcified by E D T A sol.					
	Exp.1.	Exp.2.	Exp.3.	Exp.1.	Exp.2.	Exp.3.	Exp.1.	Exp.2.	Exp.3.
Ca γ/mg	48.19	77.77	137.23	151.52	192.65	195.48	237.60	263.53	170.25
P γ/mg	25.79	40.34	69.22	74.16	91.70	92.59	120.42	128.38	83.83
Ca/P	1.87	1.93	1.98	2.04	2.10	2.11	1.97	2.05	2.03

Table 11. Experimental scheme of inhibition against crystal nucleation in the microorganism

Pre-calcifying 37°C, 2h)	Mg+monoiodo acetate treating (37°C, 2h)	Heating (76°C, 10 min)	Non inhibition
Treated living cell	No. 1	No. 2	.
Treated formalin-killed cell	No. 6	No. 7	.
Non treated living cell	No. 3	No. 4	No. 5
Non treated formalin-killed cell	No. 8	No. 9	No. 10

Table 12. Intracellular calcium and phosphorus under inhibitory conditions.

Tube. No.	Ca (γ/mg)	P (γ/mg)	Ca/P
No. 1	196.16	93.55	2.10
No. 2	183.44	83.16	2.21
No. 3	197.79	90.09	2.20
No. 4	122.00	55.63	2.19
No. 5	246.07	111.29	2.21
No. 6	166.44	79.49	2.09
No. 7	247.16	112.61	2.19
No. 8	90.20	41.13	2.19
No. 9	168.23	76.74	2.19
No.10	249.11	116.50	2.14

Table 13. Inhibited quantity of calcium and phosphorus by some anti-nucleation procedures. (γ/mg DW)

Treating	Pre-calcification				Non pre-calcification			
	Heating (76°C, 10 min)		Mg+monoiodo acetate (37°C, 2h)		Heating (76°C, 10 min)		Mg+monoiodo acetate (37°C, 2h)	
	Ca	P	Ca	P	Ca	P	Ca	P
Living cell	62.63	28.13	49.91	17.73	124.07	56.65	48.28	21.20
Formalin-killed cell	1.95	3.88	82.67	37.00	80.88	39.75	158.91	75.37

Table 14. The effect of organic acids on the calcifying activity.

	Acetic acid		Citrate* (pH 4.0)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Ca (γ/mg)	48.84	11.55	243.93	191.16
P (γ/mg)	24.95	5.39	116.30	107.65
Ca/P	1.96	2.14	2.10	1.76

* McIlbain Buffer solution

Table 15. The tide of calcium and phosphorus combined with extract of the test microorganism.

	37°C			Room temp.		
Period	Ca (γ/mg)	P (γ/mg)	Ca/P	Ca(γ/mg)	P (γ/mg)	Ca/P
4 hrs.	3.60	1.87	1.93	2.40	1.54	1.56
8 hrs.	3.61	1.62	2.23	1.79	1.21	1.48
1 day	3.24	1.09	2.97	0.84	1.42	0.59
2 days	3.26	2.13	1.53	2.61	2.27	1.15
3 days	2.13	2.50	0.85	0.03	2.65	0.01
5 days	5.69	1.05	5.42	0.08	1.04	0.08
10 days	3.61	4.16	0.87	0.11	1.88	0.06

Table 16. The calcifiability of the extracts under the various conditions.

Exp.	Condition	Extract			Residue		
		Ca γ/mg	P γ/mg	Ca/P	Ca γ/mg	P γ/mg	Ca/P
1	Room-temp.	9.23	3.23	2.86	2.70	2.87	0.94
	37°C	10.81	5.30	2.04	98.18	46.86	2.10
	37°C Thymol add.	7.46	4.31	1.73	40.52	22.21	1.82
2	Room-temp.	168.66	75.70	2.23	1.86	4.24	0.44
	37°C	12.31	1.68	7.32	35.39	17.40	2.02
3	Room-temp. 1	253.37	150.60	1.68	712.66	132.88	5.36
	Room-temp. 2	260.64	107.1[illegible]	2.43	739.02	123.12	6.00

Period for calcification: Exp.1: 16 days.
Exp.2: 16 days.
Exp.3: 14 days.

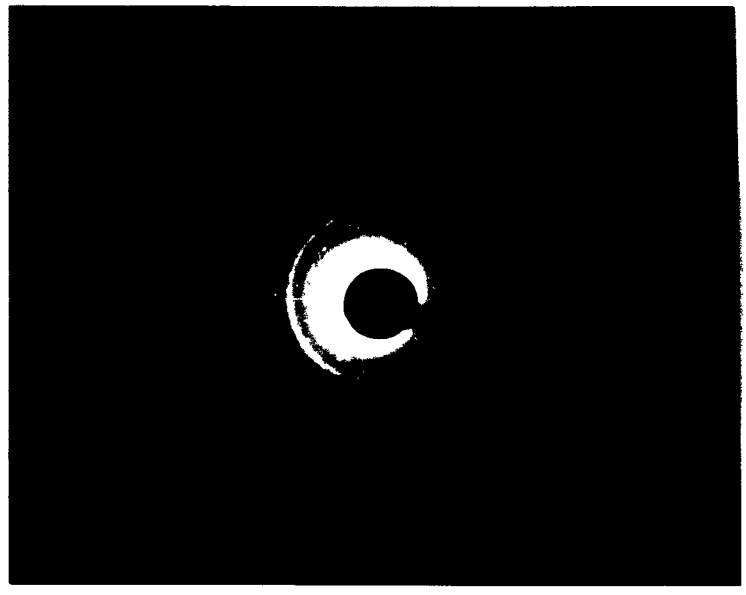


Fig. 5. The electron diffraction pattern of calcified extract from the test microorganism.

Table 17. The identification of electron diffraction pattern obtained from the calcified extract.

Measured lattice spacings		Hydroxyapatite* $Ca_{10}(OH)_2(PO_4)_6$	
d (Å)	I	d (Å)	I/I_o
3.40	s	3.44	40
		3.11	8
2.80	vs	2.79	100
		2.62	8
2.26	w	2.27	16
		2.13	4
		2.06	4
1.93	w	1.94	20
1.85	w	1.84	20
1.72	w	1.71	16
		1.45	8
		1.31	8
		1.24	4
		1.11	8

d: spacings
I: intensities
s: strong
vs: very strong
w: weak
I/I_o: relative intensities

*ASTM data (1-1008)

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25 p. incl. illus, tables, 16 refs.
(Contract DA92-557-FEC-35975) Unclassified report

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The intracellular calcium and phosphorus were found to increase following the in vitro calcification. In addition, the ratio of calcium and phosphorus approximates 2.12 which is the ratio in hydroxyapatite. These findings were observed in both living and dead cells, therefore, it can be said the intracellular calcification is independent from the metabolic process of the microorganisms.

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These findings suggest that the intracellular calcification consisted of three phases: crystal nucleation, crystal growth, and crystal limitation the same phenomenon observed in other organic tissues. Also the existence of intracellular organic substance responsible for calcification similar to collagen as found in bone tissues was assumed.

Extraction of the responsible substance from the cells was repeated, but the extracts were not stable in their calcifiability except some instance in which hydroxyapatite was demonstrated by electron diffraction method. Studies are now in progress to obtain the stable extract. (Author)

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DESCRIPTORS

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