MINIMUM MEDIUM FOR *CORYNEBACTERIUM RENALE* AND FILAMENTOUS GROWTH DUE TO DEFICIENCY OR EXCESS OF INORGANIC IONS

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In their previous report⁷⁾, HIRAI and YANAGAWA demonstrated that it was possible to obtain the luxuriant growth of the 3 types¹⁷⁾ of *Corynebacterium renale* in a chemically defined medium, and that the amino acid and growth factor requirements of *C. renale* differed among the 3 types.

It is necessary for basic studies on metabolism, biosynthesis, and genetic analysis in bacteria to establish a chemically defined minimum medium. The 3 types of this organism were, therefore, investigated for nutritional requirements to formulate such a minimum medium.

Evidence is presented in this paper to show that the organisms can be cultured in a medium composed of mineral salts, glucose as energy source, ammonium sulfate, simple amino acids as nitrogen sources, sodium bicarbonate, and simple vitamins as growth factors. It is another purpose of this paper to mention such filamentous growth as caused by deficiency or excess of inorganic ions in the minimum medium.

MATERIALS AND METHODS

Strains: The fifteen strains of *C. renale* described previously⁷⁾ were used. Of them, Nos. 8, 45, and 42 were selected as the representative strains of types I, II, and III, respectively.

Preparation of media: A minimum medium was prepared in the same manner as described previously⁷⁾, except that 1.5% special agar noble (Difco) was added when a solid medium was required.

Distilled water used: Demineralized water was made free from any inorganic ion, or trace metal by autoclaving with 2% (w/v) alumina¹⁶⁾ (Toyo Kagaku product of chromatography grade).

Inorganic ions used: The following inorganic ions were tested for effect on growth, including filamentous growth, in the minimum medium: FeSO_4 , FeCl_2 , $\text{Fe}_2(\text{SO}_4)_3$, FeCl_3 , MnSO_4 , MnCl_2 , MnHPO_4 , MgSO_4 , and CuSO_4 .

Growth measurement: Growth in the liquid medium was estimated by turbidimetry using a Tokyo Koden type 7 A photoelectric colorimeter equipped with a 570 m μ filter. Growth on the solid medium was estimated by calculating the average of the colony size.

Microscopic observation: Bacterial cells stained (Gram stain) were photographed under the light microscope. Size of the bacterial cell was measured on the photograph. The length of the bacterial cell was expressed in each preparation as the average of 30 cells randomly selected.

RESULTS

A. Minimum medium

1. Establishment of minimum defined medium for each type of *C. renale*

An attempt was made first to formulate a synthetic medium which would support growth of all the 3 types of *C. renale*. The composition of such medium is given in Table 1. From this synthetic medium, a minimum medium was devised for each type. Amino acid and growth factor requirements were determined for each type by making various changes in the composition of the synthetic medium.

The composition of the minimum medium thus prepared for each type of *C. renale* is shown in Table 3. Type I (strain No. 8) grew well in a medium containing glutamic acid and valine as amino acids, and biotin, thiamine, pantothenic acid, and inositol as growth factors. The minimum medium for type II (strain No. 45) was composed of only glutamic acid as amino acid and biotin, nicotinic acid, and P-aminobenzoic acid as growth factors. Growth of type III (strain No. 42) was the most exact. The amino acids required for this type included glutamic acid, valine, isoleucine, methionine, and cystine. This type also required biotin, nicotinic acid, pyridoxine, thiamine, and glucosamine essentially. Without even one of these growth factors, there was no growth of this type.

2. Growth of 5 strains of each type in the minimum media

It was suggested in the experiments mentioned above that the minimum medium of *C. renale* might differ among the 3 types of strains. In order to confirm this, 5 strains were indiscriminately selected from each type. Growth of these strains in the minimum media was compared among the types. The results are shown in Table 3. The strains of type I, without exception, showed good growth in the minimum medium

Table 1. Composition of Minimum Medium Used

Component	Concentration	Component	Concentration
Basal		Growth factor supplement	
Salt A:		Biotin	5 μ g/10 ml
NaCl	62 mg/10 ml	Thiamine-Hcl	100
NaHPO ₄	28	Ca-Pantothenic acid	100
KH ₂ PO ₄	6.8	Nicotinic acid	100
KCl	4	P-Aminobenzoic acid	100
Salt B:		Pyridoxine-HCl	100
FeSO ₄	100 μ g/10 ml	Inositol	5 mg/10 ml
MgSO ₄	100	Glucosamine	5
MnSO ₄	5		
CuSO ₄	1	Amino acid supplement	
Salt C:		L-Glutamic acid	100
CaCl ₂	0.5 mg/10 ml	L-Valine	12
Glucose	100	L-Isoleucine	5
(NH ₄) ₂ SO ₄	20	L-Cystine	0.5
NaHCO ₃	5	L-Methionine	3

Table 2. Amino Acid and Growth Factor Constituents of Minimum Media for 3 Types of *C. renale**

Minimum medium for type I (No. 8)	Type II (No. 45)	Type III (No. 42)
Amino acid supplement		
L-Glutamic acid100 mg/10 ml**	L-Glutamic acid ..100 mg/10 ml	L-Glutamic acid ..100 mg/10 ml
L-Valine 12		L-Valine 12
		L-Isoleucine 5
		L-Methionine 3
		L-Cystine 0.5
Growth factor supplement		
Biotin 5 μ g/10 ml	Biotin 5 μ g/10 ml	Biotin 5 μ g/10 ml
Thiamine-HCl100	Nicotinic acid100	Nicotinic acid100
Ca-Pantothenic acid100	P-Aminobenzoic acid100	Pyridoxine-HCl100
Isositol 5 mg/10 ml		Thiamine-HCl100
		Glucosamine 5 mg/10 ml

* The basal component indicated in Table 1 was used in common to the 3 types.

** The concentrations of each amino acid and growth factor are the same as shown in Table 1.

Table 3. Growth of Strains of Each Type in Minimum Medium*

Minimum medium for:	Type I strains					Type II strains					Type III strains				
	8**	15	24	34	65	18	20	35	45	75	42	43	44	47	48
Type I	+++***	+++	+++	++	+	+	+	+	+	—	—	—	—	—	—
Type II	+	+	+	+	—	+++	+++	+++	+++	—	—	—	—	—	—
Type III	+	+	++	++	—	+++	+++	++	++	+++	+++	+++	+	+++	+++

* The medium used for each type is indicated in Table 2.

** Strain No.

*** Results expressed by the optical density measured at 48 hours of incubation at 37°C.

+++ O.D. more than 0.4 + O.D. 0.11~0.2

++ " 0.2~0.4 ± " 0.06~0.1

— " 0 ~0.05

for type I. The minimum media for types II and III were inferior to the minimum medium for type I in supporting profuse growth of type I.

The strains of type II exhibited their best growth in the minimum media for types II and III, except one. The minimum medium for type I did not support good growth of the strains of type II. Strain No. 75 did not grow in the minimum medium for type II, but showed good growth only in the minimum medium for type III. Cystine contained in the minimum medium for type III was necessary for growth of this strain.

All the strains of type III grew only in the minimum medium for type III. The amino acids and growth factors contained in the minimum medium for type III were found essential for all the strains of type III.

3. Growth curve in the minimum media

Chart 1 gives the growth curves of the representative strains of the 3 types in each minimum medium. The growth curve of strain No. 8 of type I in nutrient broth is shown as a control. The maximum bacterial concentration of each strain reached 10^8 /ml in every minimum medium. The time necessary for a strain to reach the

Chart 1. Growth of the 3 Types of *C. renale* in Minimum Media*

* The media used are indicated in Table 2.

- Strain No. 8 (type I) in nutrient broth
- No. 8 (type I) in minimum medium
- △—△ No. 45 (type II) in minimum medium
- ×—× No. 42 (type III) in minimum medium

maximum concentration was not the same. It was 48 hours for types I and II, and 73 hours for type III. The generation time in the exponential phase of growth was 90 minutes for type I (strain No. 8) and type II (strain No. 45), and 180 minutes for type III (strain No. 42).

4. Colony size in the minimum media

The colony sizes of the 3 strains of each type were compared between each minimum medium and nutrient agar (Figs. 1~6). The minimum medium was not at all inferior to nutrient agar in the size and number of colonies of these strains grown after incubation at 37°C for 48 or 72 hours. Colonies of strain No. 42 grew on the minimum medium in 72 hours and on nutrient agar in 48 hours.

No pronounced change was noticed in the antigenic characteristic when the strains were examined by the precipitin reaction in gel after 5 serial subcultures on the minimum medium.

B. Filamentous growth due to deficiency or excess of inorganic ions

In the investigation of the minimum medium, it was noted that *C. renale* formed long filaments. The filaments were approximately 4 times as long as the normal cell. The cell showed no remarkable change in width. Subsequent investigation indicated that the deficiency or excess of such inorganic ions as supplemented in the form of trace element was responsible for this phenomenon. Experiments were carried out using the minimum media.

1. Fe⁺⁺

The effect of Fe⁺⁺ (FeSO₄) on the growth of *C. renale* is summarized in Table 4. No or poor growth of each strain was observed in media without added Fe⁺⁺.

Type I (strain No. 8) grew abundantly in a medium with an addition of more than 1 µg/ml of Fe⁺⁺. Cells were normal in size in the medium with an addition of 0.5~100 µg/ml of Fe⁺⁺.

Type II (strain No. 45) required more than 5 µg/ml of Fe⁺⁺ for good growth. Cells of this type grown in the presence of 0.5 and 1 µg/ml of Fe⁺⁺ were 3 to 4 times

Table 4. Growth and Elongation Effect of Various Concentrations of Iron Ion on the 3 Types of *C. renale**

Fe ⁺⁺ Conc'n (FeSO ₄ : μ g/ml)	Type I (strain No. 8)		Type II (No. 45)		Type III (No. 42)	
	Growth	Elongation	Growth	Elongation	Growth	Elongation
0	±**	Normal~1.5 X***	—	—	—	—
0.5	++	Normal~1.5 X	+	3~4 X	—	—
1	+++	Normal~1.5 X	++	3~4 X	—	—
5	+++	Normal	+++	Normal~1.5 X	+	3~4 X
7.5	+++	Normal	+++	Normal	+++	Normal~1.5 X
10	+++	Normal	+++	Normal	+++	Normal
25	+++	Normal	+++	Normal~1.5 X	+++	Normal
50	+++	Normal	+++	Coccoid	+++	Normal~1.5 X
100	+++	Normal	+++	Coccoid	+++	Coccoid

* The minimum medium for each type is indicated in Table 2.

** Growth was graded as follows: —, no growth; ±, very slight growth; +, slight growth; ++, growth inferior to +++; +++, sufficient growth.

*** The length of the filament was compared with that of the normal cell. "1.5 X" means 1.5 times the length of the normal cell.

Table 5. Growth and Elongation Effect of Various Concentrations of Manganese Ion on the 3 Types of *C. renale**

Mn ⁺⁺ Conc'n (MnSO ₄ : μ g/ml)	Type I (strain No. 8)		Type II (No. 45)		Type III (No. 42)	
	Growth	Elongation	Growth	Elongation	Growth	Elongation
0	+++**	Normal	+++	4~5 X	+++	4~5 X
0.2	+++	Normal	+++	2~3 X	+++	3~4 X
0.4	+++	Normal	+++	1.5 X	+++	2~3 X
0.5	+++	Normal	+++	Normal	+++	Normal
0.6	+++	Normal	+++	Normal	+++	Normal
0.8	+++	Normal	+++	1.5 X	+++	Normal
1	+++	Normal	+++	2~3 X	+++	1.5 X

* and ** See the footnote of Table 4.

as long as the regular cell (Figs. 7 and 8). On the other hand, cells were short, taking a coccoid form, in a medium with an addition of 50 and 100 μ g/ml of Fe⁺⁺ (Fig. 10). Cells of regular size of this strain were found only when 7.5~10 μ g/ml of Fe⁺⁺ had been added to the medium (Fig. 9).

Type III (strain No. 42) required more than 7.5 μ g/ml of Fe⁺⁺ for good growth. In a medium with an addition of less than 1 μ g/ml of Fe⁺⁺, no growth was detected. In the presence of 5 μ g/ml of Fe⁺⁺, cells were elongated and became 3 to 4 times as long as the regular cell (Fig. 12). In the presence of an excess amount of Fe⁺⁺, such as 100 μ g/ml, cells took a coccoid form (Fig. 14). Cells of regular size were found in a medium with an addition of such amount of Fe⁺⁺ as ranging from 7.5 to 50 μ g/ml (Fig. 13).

Other Fe⁺⁺ and Fe⁺⁺⁺ compounds, such as FeCl₂, Fe₂(SO₄)₃, and FeCl₃, were also used. Similar results were obtained from media to which varying amounts of these compounds had been added.

2. Mn⁺⁺

No growth was affected by the amount of Mn⁺⁺ (MnSO₄) added (Table 5).

Filamentous growth appeared in a medium which was deficient in or excess of Mn^{++} .

Type I (strain No. 8) showed no filamentous growth. In the case of type II or III (strain No. 45 or No. 42), filamentous growth appeared in a medium with an addition of not more than $0.4 \mu\text{g/ml}$ of Mn^{++} . With no addition of Mn^{++} , cells were elongated and became 4 to 5 times as long as the regular cell. With an increase in the amount of Mn^{++} , the elongation diminished. Cells of regular size appeared after an addition of $0.5\sim 0.6 \mu\text{g/ml}$ of Mn^{++} (type II) or $0.5\sim 0.8 \mu\text{g/ml}$ of Mn^{++} (type III). Filamentous growth again appeared in a medium containing an excessive amount of Mn^{++} , or more than $0.8\sim 1.0 \mu\text{g/ml}$ of Mn^{++} , but the filaments were not so long as those seen in the case of Mn^{++} deficiency. Figs. 15 to 22 show the typical cells of type II or III (strain No. 45 or No. 42) in media with addition of varying amounts of Mn^{++} . Occasionally, some filaments 10 to 20 times as long as the regular cells were observed in a medium containing no Mn^{++} (Fig. 20). It was noticed that elongated cells of type III grown in a medium deficient in Mn^{++} possessed constrictions which were located at regular intervals, each interval corresponding roughly to the length of the regular cell (Figs. 20 and 21).

Other Mn^{++} compounds, such as $MnCl_2$ and $MnHPO_4$, were also tested. Filamentous growth was induced by deficiency or excess of these compounds in the same manner as in the experiment with $MnSO_4$.

3. Mg^{++} and Cu^{++}

Mg^{++} ($MgSO_4$) (Table 6) and Cu^{++} ($CuSO_4$) (Table 7) gave similar results when added to the minimum medium in varying amounts.

Table 6. Growth and Elongation Effect of Various Concentrations of Magnesium Ion on the 3 Types of *C. renale**

Mg^{++} Conc'n ($MgSO_4$: $\mu\text{g/ml}$)	Type I (strain No. 8)		Type II (No. 45)		Type III (No. 42)	
	Growth	Elongation	Growth	Elongation	Growth	Elongation
0	+++**	Normal	+++	1.5~2 X	++	2~3 X
1	+++	Normal	+++	1.5~2 X	+++	1.5~2 X
5	+++	Normal	+++	Normal	+++	1.5~2 X
10	+++	Normal	+++	Normal	+++	Normal
25	+++	Normal	+++	1.5~2 X	+++	Normal
50	+++	Normal	+++	2~3 X	+++	1.5~2 X
100	+++	Normal	+++	3~4 X	+++	1.5~2 X

* and ** See the footnote of Table 4.

Table 7. Growth and Elongation Effect of Various Concentrations of Copper Ion on the 3 Types of *C. renale**

Cu^{++} Conc'n ($CuSO_4$: $\mu\text{g/ml}$)	Type I (strain No. 8)		Type II (No. 45)		Type III (No. 45)	
	Growth	Elongation	Growth	Elongation	Growth	Elongation
0	+++**	Normal	+++	2~3 X	+++	2~3 X
0.1	+++	Normal	+++	1.5 X	+++	1.5 X
0.25	+++	Normal	+++	Normal	+++	Normal
0.5	+++	Normal	+++	Normal	+++	Normal
0.75	+++	Normal	+++	1.5 X	+++	Normal
1	+++	Normal	+++	2~3 X	+++	2~3 X

* and ** See the footnote of Table 4.

Excess or deficiency of these ions caused elongation of cell form in types II and III, but not in type I. Cells of regular size were found in a medium with an addition of 5~10 $\mu\text{g}/\text{ml}$ of Mg^{++} (type II) or 10~25 $\mu\text{g}/\text{ml}$ of Mg^{++} (type III), and 0.2~0.5 $\mu\text{g}/\text{ml}$ of Cu^{++} (type II) or 0.25~0.75 $\mu\text{g}/\text{ml}$ of Cu^{++} (type III).

4. Colonial morphology and antigenicity of filamentous culture

The colony of filamentous cells was a little larger, and more dried and granular than that of regular cells. When determined by the precipitin reaction in gels, no antigenic differences were found in the 3 types of *C. renale* between the culture of filamentous cells and that of regular cells.

DISCUSSION

The previous investigation showed that a minimum medium could be formulated for each of the 3 types of *C. renale* classified by YANAGAWA et al.¹⁷⁾, and that the minimum medium differed in composition among the 3 types of *C. renale*.

Type I grew profusely in a minimum medium containing the following amino acids and growth factors: glutamic acid, valine, biotin, thiamine, pantothenic acid, and inositol. The minimum medium for type II contained glutamic acid, biotin, nicotinic acid, and P-aminobenzoic acid. Type III required glutamic acid, valine, isoleucine, methionine, and cystine as essential amino acids, and biotin, nicotinic acid, thiamine, pyridoxine, and glucosamine as essential growth factors. Thus, the types are arranged in the decreasing order of complexity of both amino acids and growth factors required for the minimum medium as follows: types III, I, and II.

It should be emphasized here that inositol markedly stimulated the growth of type I of *C. renale*. It is said that inositol is required by many yeasts and molds as one of the growth factors. No investigators, however, have ever mentioned about the requirements by any bacterial species, except MACLACHLAN and THATCHER⁸⁾ who reported that inositol was stimulatory to growth of *Corynebacterium sepedonicum*. No metabolic role of inositol has been known as yet. Generally, since inositol occurs almost exclusively in the phospholipid fraction, its primary role may be as structural component of the essential phospholipids. The characteristic need of inositol by *C. renale* and *C. sepedonicum* will be an interesting subject for research in future.

Another point of interest was that glucosamine stimulated the growth of type III of *C. renale* markedly. GAUKE et al.³⁾, GYORGY et al.⁶⁾, and TOMARELLI et al.¹⁴⁾ reported that a factor which contained a group of N-acetyl-D-glucosamine oligosaccharides and polysaccharides, and which stimulated the growth of *Lactobacillus bifidus* had been found in human milk and named bifidus factor. It is well known that N-acetylglucosamine serves as precursors for synthesis of bacterial cell walls. The need of the glucosamine by type III of *C. renale* will be another interesting problem to be solved.

The authors established a minimum defined medium for *C. renale*. The isolation of auxotrophic mutants, which is in progress, will be of value in performing genetic analysis and studying metabolism and biosynthesis in *C. renale*.

During the course of this study it was noted that filamentous growth appeared due to the deficiency or excess of some inorganic ions, such as Fe^{++} , Mn^{++} , Mg^{++} , and Cu^{++} . The filamentous form was observed in types II and III, but not in type I.

Filamentous growth of bacteria induced by a variety of environmental conditions has been observed by many investigators. Generally, the agents which cause filamentous growth can be classified as follows: (i) poisons and metabolic inhibitors^{1,4,10,13)}, (ii) nutritional deficiencies, including those of metals^{2,11,15,16)}, and (iii) physical conditions,

such as radiation and pressure^{9,12,19}).

As for the deficiency of inorganic ions, WEBB¹⁵) reported that bacteria varied in their requirements for magnesium ion, and that *E. coli* ML 35 was apparently more sensitive to magnesium-ion deficiency than *Aerobacter aerogenes* was. Similarly, BROCK²) mentioned that magnesium-ion deficiency caused a marked filamentation in growing cells of *E. coli* ML 35. WINDER and O'HARA¹⁶) reported biochemical consequences of filamentous growth produced by iron deficiency in *Mycobacterium smegmatis*. According to them, the protein and RNA contents of such elongated cells of *M. smegmatis* were nearly equal to those of normal cells, but the DNA content of the elongated cells was much reduced. They suggested that a lack of DNA might repress replication of bacterial cells and inhibition of cell division, thereby resulting in filament formation.

In the case of manganese deficiency dealt with in this report, constriction was observed in the filament at such regular intervals as corresponding roughly to the length of a normal cell (Figs. 20 and 21).

The reason why no long filament could be divided into cells of regular size may be considered in 2 ways: (1) Septum formation may have stopped in the early or middle stage, or (2) septum formation may have almost finished, but no cells can separate from one another. In the former case, manganese deficiency may interrupt the completion of septum formation. Cytoplasmic-membrane synthesis, which is the first step of septum formation, may have been stopped by manganese deficiency. In the latter case, cell division may have almost finished, but the resultant cells are connected with one another and with the outermost layer, which is present characteristically in this microorganism¹⁸).

Detailed information on filamentous growth will be obtained by electronmicroscopic analysis and biochemical procedures, such as estimation of DNA or RNA.

SUMMARY

Minimum media were developed for the 3 types of *Corynebacterium renale*. The results obtained are summarized as follows.

1) Minimum medium for type I was one containing glutamic acid and valine as amino acids, and biotin, thiamine, pantothenic acid, and inositol as growth factors.

2) Type II grew well in a medium containing glutamic acid, valine, biotin, nicotinic acid, and P-aminobenzoic acid.

3) Type III required glutamic acid, valine, isoleucine, methionine, and cystine as essential amino acids, and biotin, thiamine, nicotinic acid, pantothenic acid, and glucosamine as essential growth factors. Thus, the 3 types were arranged from the complexity of both amino acids and growth factors required for the minimum medium in the following decreasing order: types III, I, and II.

4) Maximum growth of each type in each minimum medium reached 10^8 /ml. The time necessary for one type to reach the maximum growth was not the same; it was 48 hours for types I and II, and 72 hours for type III. The colonies of the 3 types grown on the solidified minimum medium were not at all smaller in size than those grown on nutrient agar.

5) In type I strains, no or poor growth was observed in a minimum medium with no addition of Fe^{++} or Fe^{+++} . No addition of Mn^{++} , Mg^{++} , or Cu^{++} to this medium affected growth of these strains.

6) Filamentous growth was characteristic of types II and III in a minimum

medium deficient in, or excess of, these inorganic ions. The optimal concentrations of these ions for minimum medium were 10 $\mu\text{g/ml}$ for Fe^{++} , 0.5 $\mu\text{g/ml}$ for Mn^{++} , 10 $\mu\text{g/ml}$ for Mg^{++} , and 0.5 $\mu\text{g/ml}$ for Cu^{++} . In type I, however, filamentous growth was never induced, regardless of the concentration of any of these inorganic ions.

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Corynebacterium renale の minimum medium について,
ならびに無機イオン欠乏および過剰による
filamentous growth について

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Corynebacterium renale の3つの型では, アミノ酸および発育素要求性に差異があることをさきに述べた. この報告では, 各型の minimum medium を確立し, なお filamentous growth について記述する.

各型 *C. renale* の minimum medium の基礎培地の組成は全く同じで, $1/50$ M 磷酸緩衝液 pH 7.4, 塩化ナトリウム, 塩化カリウム, 硫酸アンモニウム, 重炭酸ナトリウムおよびブドウ糖を含む. アミノ酸および発育素に関して以下に述べる.

I 型菌の minimum medium は, glutamic acid および valine の2種アミノ酸に, biotin, pantothenic acid, thiamine および inositol の4種発育素を含む. II 型菌のそれは, glutamic acid に biotin, nicotinic acid および P-aminobenzoic acid を必要とする. III 型菌の minimum medium は, glutamic acid, valine, cystine, isoleucine

および methionine の5種アミノ酸のほかに, pyridoxine, thiamine, biotin, nicotinic acid および glucosamine を含み, I および II 型に比較して, より複雑な成分を必要とした.

minimum medium に発育した3つの型の菌は, 集落の大きさおよび形態は, 普通寒天に発育した集落のそれと変わりなく, また抗原性にも変化がなかった.

上記の実験中に, 無機イオンの欠乏および過剰による本菌の filamentous growth が観察された. Fe^{++} は3つの型の菌に共通に, 必須的に要求される. II および III 型菌は, Fe^{++} 低濃度では, 通常の菌形の4~5倍の長さを持つ filamentous form を示し, Fe^{++} 高濃度では, 球菌状になった. Mn^{++} , Mg^{++} および Cu^{++} も, 最適濃度範囲外の濃度では, filamentous form をもたすが, 特に II および III 型に著明であった.

EXPLANATION OF PLATES

PLATE I

Colonies of representative strains of the 3 types of *C. renale* growing on the respective minimum media. 48-hour cultures.

Fig. 1. Colony of type I (strain No. 8)	} on nutrient agar plate.
Fig. 2. Colony of type II (strain No. 45)	
Fig. 3. Colony of type III (strain No. 42)	
Fig. 4. Colony of type I (strain No. 8)	} on the minimum medium.
Fig. 5. Colony of type II (strain No. 45)	
Fig. 6. Colony of type III (strain No. 42)	

PLATE II

Filamentous forms of type II (strain No. 45) and type III (strain No. 42) of *C. renale* induced by addition of various concentrations of Fe^{++} to the minimum medium. Figs. 7 to 10 show such forms of type II (strain No. 45) and Figs. 11 to 14 those of type III (strain No. 42). 48-hour cultures. $\times 1,000$.

- Fig. 7. Cells of type II grown on nutrient agar.
- Fig. 8. Cells of type II grown on the minimum medium with addition of $5 \mu\text{g/ml}$ of Fe^{++} .
- Fig. 9. Cells of type II grown on the minimum medium with addition of $10 \mu\text{g/ml}$ of Fe^{++} .
- Fig. 10. Cells of type II grown on the minimum medium with addition of $100 \mu\text{g/ml}$ of Fe^{++} .
- Fig. 11. Cells of type III grown on nutrient agar.
- Fig. 12. Cells of type III grown on the minimum medium with addition of $5 \mu\text{g/ml}$ of Fe^{++} .
- Fig. 13. Cells of type III grown on the minimum medium with addition of $10 \mu\text{g/ml}$ of Fe^{++} .
- Fig. 14. Cells of type III grown on the minimum medium with addition of $100 \mu\text{g/ml}$ of Fe^{++} .

PLATE III

Filamentous forms of type II (strain No. 45) and type III (strain No. 42) of *C. renale* induced by addition of various concentrations of Mn^{++} to the minimum medium. Figs. 15 to 18 show such forms of type II (strain No. 45) and Figs. 19 to 22 those of type III (strain No. 42). 48-hour cultures. $\times 1,000$.

- Fig. 15. Cells of type II grown on nutrient agar.
- Fig. 16. Cells of type II grown on nutrient agar with no addition of Mn^{++} .
- Fig. 17. Cells of type II grown on the minimum medium with addition of $0.2 \mu\text{g/ml}$ of Mn^{++} .
- Fig. 18. Cells of type II grown on the minimum medium with addition of $0.4 \mu\text{g/ml}$ of Mn^{++} .
- Fig. 19. Cells of type III grown on nutrient agar.
- Fig. 20. Cells of type III grown on the minimum medium with no addition of Mn^{++} .
- Fig. 21. Cells of type III grown on the minimum medium with addition of $0.2 \mu\text{g/ml}$ of Mn^{++} .
- Fig. 22. Cells of type III grown on the minimum medium with addition of $0.4 \mu\text{g/ml}$ of Mn^{++} .